Success in Academic Surgery
Series Editors: Lillian Kao · Herbert Chen

Catherine J. Hunter
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Success in Academic Surgery: Basic Science

Third Edition

Springer

Success in Academic Surgery

Series Editors

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All of the intended volume editors are highly successful academic surgeons with expertise in the respective fields of basic science, clinical trials, health services research, and surgical education research. They are all also leaders within the Association for Academic Surgery (AAS). The previous AAS book, Success in Academic Surgery: Part I provided an overview with regards to the different types of surgical research, beginning one's academic career, and balancing work and life commitments. The aims and scopes of this series of books will be to provide specifics with regards to becoming successful academic surgeons with focuses on the different types of research and academic careers (basic science, clinical trials, health services research, and surgical education). These books will provide information beyond that in the introductory book and even beyond that provided in the Fall and International Courses. The target audience would be medical students, surgical residents, and young surgical faculty. We would promote bulk sales at the Association for Academic Surgery (AAS) Fall Courses (www.aasurg.org) which take place prior to the American College of Surgeons meeting in October, as well as the AAS International Courses which take place year-round in Australasia, Colombia, West Africa, and France. Courses are also planned for India, Italy, and Germany and potentially in the United Kingdom and Saudi Arabia. As the AAS expands the course into other parts of the world, there is a greater need for an accompanying series of textbooks. The AAS has already received requests for translation of the book into Italian. These books would be closely linked with the course content and be sold as part of the registration. In 2011, there were 270 participants in the Fall Courses. In addition, we would anticipate several hundred participants combined per year at all of the international courses.

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Editors

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Contents

How to Establish, Staff, and Fund Your Basic Science or Translational Research Laboratory

1

Priya Dedhia and J. Joshua Smith

Abstract

Establishing a basic or translational research laboratory is a significant undertaking that requires rigorous commitment, supportive mentorship, and meticulous planning. This chapter will review the interview process for a faculty position and resources that are typically required, including space, time, personnel, equipment, and mentorship. Various options in fulfilling such needs will be reviewed. Finally, we will briefly consider how to maintain the laboratory once it has been established and how to respond to unexpected challenges.

Keywords

Basic science research · Translational research · Laboratory · Faculty interview Set up · Staff · Space · Equipment · Regulations

Introduction

After engaging in prior research and committing to a career as a basic or translational surgeon-scientist, understanding the resources and strategies to conduct research is critical. Defined broadly, resources include protected time, research space, funding resources, equipment and supplies, and key technical personnel,

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along with supportive collaborators and senior mentors [1]. The resources and strategies required vary based on investigative needs and personal preferences. This chapter will discuss considerations necessary for a successful and fulfilling career as an independent basic or translational surgeon-scientist.

Faculty Interview

The key to a successful start to a basic and translational science career for a surgeon-scientist is to secure support and resources that allow the lab to flourish. After a productive research training period before or during residency, the optimal timeframe to identify and acquire these assets is during the interview and negotiation process (Fig. 1.1). The interview process for the basic and translational surgeon-scientist starts with interview solicitation, which typically occurs through unsolicited cover letters, personal meetings, and response to job listings. Cover letters, whether unsolicited or solicited, should include the following key components: (1) general information about your training, major accomplishments, and research and career goals, (2) how the institution aligns with your research and career goals – this section should include potential mentors, general support for research, and aspects of the environment or culture that are attractive to you, and (3) the unique aspects you will bring that will grow the department or institution. *The cover letter should be unique to each institution and demonstrate deep understanding of the culture and goals of the target institution.*

After successfully obtaining an interview, preparation is crucial not only to excel in the interview, but also to map out the early career pathway. Important interview documents for the basic and translational surgeon-scientist include the research prospectus and the budget. The research prospectus should include long-term career goals, the aims of 1–2 planned research projects, relevant supporting publications by the candidate, grant submission plans over the next 3–5 years, and a bibliography. The budget should include all resources you would need to start and run the lab. A budget template can usually be obtained from your research mentor or peer mentor.

Commonly, in the first interview, you will garner interest from the institution, whereas in the second interview you will determine if the institution can deliver the support you need. Once a second interview is offered, you should request to meet with potential mentors and collaborators, clinical partners, and clinical support staff. You should also tour lab facilities to ensure they meet your needs and are close to mentors. Meeting with early career research faculty can also identify barriers to

Fig. 1.1 Overview of the faculty interview process

success and additional institution-specific resources that may be necessary for running a lab. Finally, giving a research talk during the second interview can gauge departmental engagement and support for research (and can also be included on one's CV).

Once offered, your contract should be reviewed with a research mentor, a clinical mentor, and a peer mentor. Important aspects of the contract include protected time for research, laboratory space, expectations regarding RVU generation, OR block time assignment, administrative support for clinical activity, research activities, patient management, and grant submissions, and whether the surgeon-scientist will be embedded in a laboratory or start their laboratory as a standalone entity. These needs are reviewed in detail below.

Protected Time

During the surgeon-scientist's early career, protected research time refers to hours financed by the department and institution to carry out research and participate in scientific events. Even when funded by governmental grants, the surgeon-scientist's salary is often covered in-part by the department and institution due to funding agency salary caps. Specifically, in 2024 the NIH salary cap is $221,900 so if an investigator is at 10% on an R01 grant (1.2 calendar months) then the department can expect a contribution of $28,891 (salary + fringe) per year for the 5-year duration of the grant. Consequently, understanding that supporting the career of a surgeon-scientist results in a financial loss to a department can help you navigate conversations with your department. To run a lab, you must develop a vision to drive the science and culture in your lab. Developing this vision requires you to physically spend time in the lab and carry out experiments, but also read papers, work on manuscripts, and write grants without the distraction of clinical or administrative responsibilities. Carving out this time can be challenging without adequate support because clinical teams may not understand the value of your research responsibilities. As such, the backing of your chairperson, your division chief, and your clinical partners is essential to protecting your time. *Protecting 50–75% of your time for research during the first 3–5 years is ideal for the basic and translational surgeon-scientist.* This model translates to ~0.5 to 1 clinic day and 1 OR day per week. An alternate strategy in some surgical specialties is to limit service responsibilities to 1 week per month. Institutional support for funded academic research and a chairperson and / or division chief with basic or translational science experience are more likely to result in 50–75% protected time. In addition, demonstrating a high likelihood success through publications and a detailed research prospectus can improve your ability to secure protected time.

In the event that the optimal amount of protected time cannot be achieved or to maximize protected time, additional strategies can increase your effective time for research. First, ensure that your relative value unit (RVU) target reflects your optimal research protected time for your specialty. Even if your contract indicates 50% protected time, you will not have this time if your RVU target is too high. An

appropriate RVU target should be discussed with your clinical mentor, because this target will be specific to your specialty and case mix. Second, you should aim to obtain one OR block day per week. For elective specialties, block time prevents disruption to your research schedule, and allows you to better organize your time. Third, your clinical partners should be committed to your research efforts. Their commitment will be necessary to limit your new patient visits, avoid clinical obligations at the time of your grant submissions, and to safeguard your protected time. Other approaches to maximizing your time for research include physically separating your lab and clinical spaces to reduce interruptions and to group clinic and OR time on adjacent days in order to leave the rest of the week free for research pursuits. Another model for success is to limit your practice to a narrowly defined niche, particularly one that does not generate many emergencies. Overlapping your clinical and research programs will also improve productivity in both endeavors. Sharing your research vision with your clinical and administrative support team by bringing them to your lab and conveying your research goals at a social event can also get your team to protect your schedule. Ultimately, having the support of your chair, division chief, clinical partners, and support staff can offset inadequate protected time to some degree.

Mentorship Team

Early mentorship is critical to succeed as a surgeon-scientist. We recommend mentorship and support in the following areas: clinical, research, career development, and sponsorship. Occasionally, a senior surgeon can provide support in all of these areas, but more commonly, multiple mentors are necessary. Importantly, not all mentors or sponsors need to be in the Department of Surgery. Also, your career development mentor and sponsor do not have to be at your institution. *Ideally, these mentors should be identified prior to or immediately after starting your faculty position, and you should collectively meet with your mentorship team quarterly or biannually to review progress and discuss challenges.*

A clinical mentor will introduce you as an expert to referring physicians and new colleagues and can offer guidance and technical support with difficult clinical scenarios or operative challenges. Your clinical mentor can also help with institution-specific workflows and appropriate coding and documentation. Especially at RVU-based institutions, accurate coding can maximize research productivity by increasing RVUs generated for the same clinical volume.

A research mentor will guide development of your research program by helping you formulate salient research questions, identify collaborators, and write grants and manuscripts. Relying on your research mentor to help develop a budget, hire and manage personnel, and navigate institutional research policies and protocols can help you minimize delays and mistakes. Furthermore, asking your mentor to direct high caliber postdoctoral applicants to your lab may improve the quality of candidates that you may be able to initially attract on your own. You and your research mentor do not need similar or overlapping research programs. In fact, a

mentor with a unique technical skillset or background in a different disease can complement your research and help you develop an independent research program. Important considerations in identifying a research mentor for early career basic and translational surgeon-scientists are: (1) a track record of funding, (2) prior experience mentoring a surgeon-scientist or a clear understanding of the unique needs of a surgeon-scientist, (3) successful mentees with publications and independent funding, and (4) adequate time to meet regularly and review your grants and manuscripts.

Planning your career trajectory is an important component of your growth as a basic and translational surgeon-scientist. Embarking on leadership and administrative roles too early in your career can jeopardize your research program. However, refusing key opportunities can stifle your development. A mentor focused on your career development and future career goals can help you effectively balance growth of your research program with professional opportunities. Although clinical or research mentors can sometimes provide guidance in this realm, their career experiences may not reflect your goals. As such, your career development mentor should help you navigate promotion and tenure, conference attendance, participation in institutional committees and national societies, and other service obligations such as manuscript and grant reviews. Regular discussion about these topics as well as your long-term career goals with your career development mentor will help you achieve your goals with minimal sacrifice.

In addition to the aforementioned mentors, a sponsor can accelerate the growth of your national reputation – an essential element of promotion and tenure. Your sponsor should be a prominent surgeon and / or scientist who has led prominent societies in your field and given national and international talks. Because of her or his renowned status, your sponsor can recommend you for invited talks, leadership positions, and conference participation as a moderator or keynote speaker. For appropriate sponsorship, you must discuss your career goals and research accomplishments with your sponsor.

You may not be able to identify all of these mentors and sponsors right away; however, selecting a clinical and research mentor at the start of your faculty career or earlier is important. Identifying a career development mentor or sponsor can be challenging, but participation in conferences or meeting with a visiting professor can provide such opportunities. Most basic and translational surgeon-scientists in leadership positions will support young surgeon-scientists by meeting regularly and providing guidance. When introducing yourself to a potential mentor or sponsor, succinctly indicate the purpose of your meetings and how frequently you would like to meet. Demonstrate your appreciation of their time by providing an agenda for each meeting and regularly indicating your gratitude.

If you are unable to identify a particular mentor or encounter challenges that your mentorship team is unable to support, a professional coach can supplement your mentorship team. A coach can help you crystallize your short-term and long-term career goals, navigate the complexities of your environment, and develop self-awareness. By providing guidance and accountability, a coach can help you achieve impact while maintaining authenticity.

Research Space: Independent vs Embedded

Space requirements will vary depending on your scientific endeavors. The options for housing your lab space include having your own dedicated lab space, embedding your lab in a mentor's lab space, or sharing space with multiple faculty members with common research interests. Establishing an independent lab space requires a significant investment of time and resources. These undertakings can be particularly challenging to the early career surgeon-scientist who must simultaneously balance developing a clinical practice, cultivating a research program, hiring personnel, and writing grants and manuscripts. Furthermore, setting up your own independent lab space will require a budget to outfit the lab with equipment you will use regularly and the recurring cost of the space. However, this model has the noteworthy advantage of protecting your lab space from a mentor or shared space investigator leaving and altering either available equipment or space.

Because of the complexities associated in an independent lab space, many early career faculty opt to either embed their lab in a mentor's lab or inhabit a shared lab space with other faculty. These models have the advantages of shared equipment, shared personnel, and ready access to senior principal investigators (PIs) and their established group of investigative staff. A shared space with a mentor or senior PIs can not only facilitate feedback on research pursuits and grants, but also strengthen grant applications by demonstrating proximity to mentors. In addition, your research staff will be supported by members of the other lab(s) for technical advice and camaraderie. These additional connections for members in your lab are critical when you will frequently be unavailable due to clinical duties and your lab is small. In an embedded space, relying on a mentor's lab manager for ordering and protocol adherence can also provide you and your lab members critical time and resources for experiments and grant writing. Despite the numerous benefits of an embedded or shared lab space, problems can arise when a mentor or PI leaves the space. In this event, having discussed a predetermined plan for your lab space and shared equipment with your chair, the PI in which your lab will be embedded, and institutional leadership can prevent future challenges that can halt your lab's progress.

Equipment and Supplies

After identifying a lab space, you will need to equip it. Obtaining an equipment list from your prior research mentor is an excellent starting point. Prior to purchasing your own equipment, you should determine if your institution provides shared equipment that can be easily accessed in a timely manner. Large, expensive equipment can often be accessed through core facilities. If shared equipment has a long wait time for usage, you can consider an alternative such as using a mentor or collaborator's equipment or purchasing your own. Occasionally equipment can be obtained from institutional storage. For example, if your institution has a research facilities manager, you should ask for appropriate generic equipment (such as shakers, water baths, etc.) that have been retained from labs that have retired or moved. As expected, you will have easy access to equipment that you own, but these items

can be costly. On the other hand, shared equipment will be less expensive but also may be less accessible. When sharing equipment with a mentor or collaborator, you must clearly delineate usage and consider paying a portion of maintenance fees if you will frequently use the equipment.

When purchasing your own equipment, obtaining new investigator or institutional discounts will significantly reduce costs. Comparing multiple bids from equipment companies can also provide cost-savings. Important considerations for major purchases are functionality and upkeep. Discussing performance and maintenance of equipment you are interested in purchasing with a PI who owns the same equipment can provide insight into equipment value.

Supplies are necessary reagents to execute your experiments. You may be able to negotiate a discount on initial supplies purchase as part of a new investigator discount. Alternatively, purchasing frequently used supplies in bulk quantities on your own or with another PI can substantially reduce costs. For reagents with expiration dates that you will use in small quantities, sharing with another PI or your mentor can save funds and prevent wastage.

Laboratory Personnel and Culture

Motivated lab personnel are essential to your success as a basic and translational surgeon-scientist. There are multiple options for the number and type of lab personnel to include in your lab, and selection depends on your interests and funds as well as quality of the candidate. Often, an intellectually curious, motivated, and resourceful candidate at any level will be more valuable than more senior staff, but may require considerable initial investment of your time. Potential lab personnel include trainees (undergraduate and medical students, residents, and postdoctoral fellows) and staff (research technicians and scientists), Table 1.1.

Although trainees will usually require more one-on-one mentorship to develop their skills, they can apply for fellowships – which can substantially decrease personnel cost. Furthermore, successful trainees will naturally attract future trainees to your lab. When recruiting trainees to your lab, ensuring their academic goals align with your research goals and budget and regularly discussing expectations can minimize discord. Frequently, trainees, especially surgical residents, will be motivated to work for longer periods in the lab and be motivated to publish in a timely fashion in order to establish her or his future career.

Research staff can provide continuity in your lab but can be more costly than trainees and may not be as motivated. However, maintenance of equipment, animal colonies or cell lines as well as administrative responsibilities such as ordering can be delegated to research staff depending on their capabilities. More experienced research staff, such as research scientists, can provide valuable technical expertise and can even assist with grant or manuscript writing. This level of proficiency is scarce and can be very costly. In contrast, an inexperienced technician may be less expensive but still effective for tasks that require less expertise.

Recruiting personnel to fill research needs is not sufficient to maintain a healthy culture in your lab. Understanding your own needs and interaction style and selecting

Table 1.1 Advantages and disadvantages of laboratory personnel

Personnel type	Cost	Advantages	Disadvantages
Research technician	$$	Can be motivated	Inexperienced Requires significant time from other lab members Often leaves in 1–2 years
Research scientist	$$$$	Can provide continuity between other labs members Experience in complex lab techniques	May be rigid in approach May not be open to learning new techniques
Postdoctoral fellow	$$$	Can be intellectually curious May have experience in novel technique Can be supported by fellowships	Challenging to hire exceptional post-doctoral fellows
Research resident	$$	Can be intellectually curious Can be supported by fellowships A successful experience can result in a steady influx of residents	May have limited experience Requires significant time investment Often leaves in 1–2 years
Graduate student	$$	Can be intellectually curious Can be motivated Can be supported by fellowships	May have limited experience Requires significant time investment
Undergraduate student	$	Can be motivated Can be supported by fellowships	Inexperienced Requires significant time from other lab members Often leaves in 1–2 years

candidates that complement your characteristics can transform your lab's potential for success. For example, if you prefer specific work hours and regimented meetings, recruiting an exceptional postdoctoral fellow who is less formal and works odd hours may cause friction. You must also ensure your team works well together by having your lab members meet with and weigh in on any new candidates. Similarly, addressing conflict between lab members quickly and professionally by highlighting common goals can prevent stagnation of research. Highlighting accomplishments of all members of the lab is equally important and can improve cohesion and trust in your lab. Importantly, avoiding harsh criticism when your lab members make mistakes and allowing them to learn from their mistakes can not only enrich their development, but also prevent falsification of data due to pressure for results. Ultimately, working with your mentorship team and understanding your personal and lab needs will help you identify and maintain the right cohort for your lab.

Collaborators

Collaborations are critical to the growth of most PIs, especially the basic or translational surgeon-scientist. Collaborators differ from a mentor in that they do usually do not provide career guidance, but still amplify your research program by providing specialized technology, equipment, personnel, etc. When first starting your lab, identifying potential collaborators can be challenging. Your mentors can provide

initial suggestions for collaborators, but often the most successful collaborations are unexpected. Attending seminar series outside of your expertise or talks that may not appear to be immediately relevant to your research can result in the most innovative collaborations. Also, conferences designed to foster convergent science can yield unexpected collaborations.

When embarking on a collaboration, starting with a narrow scope can offer insight into the relationship between you and your collaborator. Before initiating a larger project, determine if you and your collaborator contribute expected amounts of effort and meet mutually agreed upon deadlines. Also, consider discussing effort for grants and authorship for publications prior to finalizing a collaboration to avoid future disagreements. As an early career surgeon-scientist, ensuring that the relationship is beneficial to your collaborator with authorship, effort, or access to clinical expertise and unique tissues can strengthen your interactions. Certainly, a synergistic collaboration can develop into a long-term relationship that can introduce new avenues of research for all PIs.

Responding to Failure

Understanding that "failure is a steppingstone to success," is key to longevity as a basic or translational surgeon scientist. Developing a strategy for grant submissions can mitigate the challenge of most grants not being funded. Many scientists apply for 5–10 grants for every funded grant. In addition, map out the grants you plan to submit with your research mentor. Grants with the highest likelihood of success should be prioritized. When examining the summary statement for a grant, review the critiques with your mentors, collaborators, and other funded colleagues to devise a strategy to address the critique. Their input can help you determine if you should revise and resubmit the grant, redirect to another funding agency, refocus the concept and aims, or abandon the concept. Similarly, experimental failures and manuscript rejections are common (and expected). As you tackle these challenges with your mentor, also take time to discuss your plans with your lab to instill confidence and reinvigorate their efforts. At times, these setbacks are opportunities to reimagine your science, recalibrate your priorities, or move in a new direction based on critical feedback and input from your mentorship team.

A critical aspect of running a basic and translational lab is keeping close tabs of your research budget. If you recognize early that your funding is limiting your research, you can adjust your grant submission strategy accordingly. In addition, your research successes such as publications, awards, and/or media recognition can convince your chair and institution to provide bridge funding. Alternative strategies to fully exploit your resources include having trainees apply for internal and external fellowships, sharing research staff or resources with another PI, obtaining patient donations for your research, and applying for institutional pilot awards or core facilities vouchers. If you have not received appropriate funding 1 year prior to the end of your protected time, you should meet with your mentorship team to review your priorities, identify any barriers, and devise a strategy to extend your protected time,

if appropriate. After reflection, you may realize your priorities lie elsewhere, or you may intensify your efforts to advance your research program. Developing a prospectus similar to your faculty interview can be a tool to request additional protected time or resources. This document should outline your clinical and research accomplishments, highlights of your research program, notable barriers that you have encountered, and your request to the department. These requests, which result in significant financial loss to your department, must be offset by your accomplishments and contributions to the department. Work with your mentors early to develop a contingency plan if your request to extend protected time is not met. Refocus your energy and address your motivation for basic or translational research. The way you respond to failure will define your successes.

Summary

Establishing a basic or translational research laboratory is a significant undertaking that requires rigorous personal commitment and initiative, meticulous planning, passionate and supportive mentorship, and committed departmental and institutional leadership. Other necessary aspects for a successful scientific mission include protected time, space, personnel, and equipment. Table 1.2 reviews the key points establishing and managing your lab.

Table 1.2 Key points for establishing and managing a successful laboratory

Protected time	Mentorship team	Research space and equipment	Personnel and culture	Collaborators
Negotiate protected time in your contract RVUs and OR block time should reflect protected time Limit practice to narrow clinical niche Overlap clinical and research programs Separate lab from clinical workspace Supportive partners and leadership	Clinical mentor: provides operative support, education on workflows, coding Research mentor: provides scientific expertise, support for writing grants and manuscripts Career development mentor: recommends academic pursuits based on your career goals, promotion and tenure Sponsor: provides opportunities to develop your national reputation Coach: provides guidance and accountability to navigate challenges	Space options 1) Independent Requires substantial time, funds Provides stability 2) Embedded/ Shared Provides access to mentor/other PIs, personnel, equipment Can be substantially affected by mentor/other PI leaving New investigator discount can provide cost savings for equipment and supplies	Trainees Require increased oversight Can apply for fellowships May be more motivated Staff More costly Variable in experience Can delegate administrative responsibilities Healthy lab culture relies on positive interactions, psychological safety to make mistakes, and celebration of accomplishments	Identify collaborators through mentors, seminar series, and talks Start with smaller projects and pilot awards Discuss expectations such as effort and authorship for large projects in advance

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How to Fund Your Basic Science or Translational Research Laboratory

2

Morgan F. Pettigrew and Sam C. Wang

Abstract

Due to the increasing pressure for clinical productivity and the time-consuming nature of high-quality research, becoming an independently funded surgeon-scientist is a daunting challenge. This chapter will cover strategies to secure extramural funding to support your research endeavors. We will focus on obtaining National Institutes of Health (NIH) K- and R-level awards since NIH funding is the most widely accepted marker of research success in a surgeon-scientist's career.

Keywords

Basic science research · Translational research · Funding · Grants · K08 · R01

Introduction

The importance of obtaining funding is two-fold. The most obvious is that financial resources are necessary to support the functions of a lab, including hiring personnel and purchasing reagents. Table 2.1 shows the costs that a small wet bench laboratory may accrue at our institution. As you can see, just the total personnel costs for a research scientist lab manager, one postdoctoral fellow, and one research technician are over $200,000 per year. The cost of disposable materials such as culture media, gloves, and pipette tips, adds up quickly over the course of a year. There are also annual service contracts for your specialized machinery and other regular overhead costs for your institution, all of which may run from thousands to tens of

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Table 2.1 Examples of laboratory expenses

Type of expense	Cost
Personnel (salary + fringe)	
Lab manager (research scientist)	$90,000/year
Postdoctoral fellow	$90,000/year
Research technician	$50,000/year
Animals	
50 cages of mice at $0.94/cage/day	$17,115
Materials	
1 case of DMEM cell culture media	$536
1 case of nitrile gloves	$110
1 case of culture plates	$85
1 case of pipette tips	$91
1 case of centrifuge tubes	$86
1 antibody	$350
1 hour of flow cytometry core use	$90

Table 2.2 Work RVUs and average Medicare reimbursement for common surgical oncology procedures [3, 4]

Procedure	Work RVU	Average Medicare reimbursement
Whipple	53.8	$3203.19
Distal pancreatectomy	26.3	$1612.06
Total gastrectomy with Roux-en-Y reconstruction	39.5	$2340.28
Partial hepatic lobectomy	39.0	$2407.62

thousands of dollars. You must also account for one-time equipment purchases that may be for mundane items (e.g., pipettes, water baths, thermocyclers) or highly specialized (and expensive) pieces (e.g., confocal microscopes, single cell sequencers) that are essential for your research focus. Finally, you also need to account for the cost of doing large one-time experiments that provide a sizeable batch of data such as CyTOF or spatial transcriptomics.

Notably, we did not include the principal investigator's salary in our cost estimate. The average department of surgery assistant professor salary ranges widely based on specialty. Using our subspecialty for illustrative purposes, a junior surgical oncologist's annual salary is approximately $300,000 [1]. When one considers fringe costs, the total personnel cost for the surgeon-scientist may be up to $375,000–$400,000 per year.

Table 2.2 shows the work relative value units (RVUs) and average Medicare reimbursement for some major surgical oncology procedures. In order to generate enough RVUs to cover the surgeon-scientist's salary, it would be necessary to perform an average of 12 major procedures per week [2]. This would leave little time to perform research. While we acknowledge that procedure reimbursement and salary calculations are highly complex and vary widely across institutions, our rough back-of-napkin estimates demonstrate that surgeon-scientists are often considered "money-losers" by their institutions. These investigators will require significant

institutional investment in the form of money and space; there is also the opportunity cost of performing experiments at the bench rather than generating clinical revenue in the operating room.

From a purely financial standpoint, the source of funds you use to cover your research lab, whether they are from your startup package, philanthropy, or the National Institutes of Health (NIH), is irrelevant. However, you must consistently achieve benchmarks of research success to validate your department and institutional leadership's decision to support your research-based career in lieu of a clinical practice. One such benchmark is competing successfully for extramural awards (further detailed below). Competitive extramural funding is the most prestigious award type as it requires that your grant application be judged against applicants from other institutions. Obtaining these types of awards, whether they are from foundations, societies, or the NIH, is important for your career trajectory, not only to obtain resources to support your research lab but also to burnish your academic bona fides. Obtaining NIH R-level grants is generally considered an essential marker of success for a surgeon-scientist.

As will be discussed in the next section, there are numerous funding sources available to junior investigators to offset the costs of running a lab. The most realistic extramural awards that young faculty members may obtain early in their careers are pilot or foundation-type awards that provide only tens of thousands of dollars over 1 or 2 years. These are not sufficient to fully support the function of most translational and basic science laboratories. Only R-level grants from the NIH that provide many hundreds of thousands of dollars for annual direct costs over 3 to 5 years can support a lab independent of significant institutional funding. Thus, R-level grants and their equivalents, such as the VA Merit awards, are necessary to become an independent investigator. Because of the importance of obtaining R-level grants in a surgeon-scientist's career, we have focused our chapter on advice for achieving that goal. We consider smaller funding mechanisms, such as institutional pilot grants, foundation awards, and career development awards, as helpful milestones. However, these smaller awards should be considered stepping stones on the way to your ultimate goal of becoming an independent investigator (i.e., obtaining R-level funding) rather than the final destination.

Funding Sources

General Considerations

There are multiple potential funding sources to consider, especially early in your career. You should seek out multiple and diverse funding mechanisms to maximize your chance for success. Funds available to support your lab can be classified in multiple ways. First, there are *internal* opportunities, which are from your institution, and *extramural* opportunities, which are obtained from sources outside of your institution. Another category is *non-competitive* or *competitive*. Non-competitive awards are those that are bequeathed to you, such as a startup package or philanthropic

funds, while competitive grants are awards that involve numerous applicants vying for a limited pool of money. Industry partners are another potential source of funds. Be aware that certain grants can only be obtained early in your career, which is variably defined. Some mechanisms define eligibility based how long you have been out of training or how long you have been a faculty member. Other awards may seek only assistant professors or only associate professors and above. It is essential to read the eligibility instructions before embarking on your grant writing journey.

Intramural Sources

Your startup package will provide a significant source of funding to launch your lab. You need to establish a vision for your research program and negotiate for adequate funds to support that vision before taking a position. Ideally, the institution's financial commitments to you are documented in your letter of intent or final offer contract. Your startup package may consist of a total sum allocated upon beginning your faculty position, some amount to be allocated annually, or a front-loaded amount for the first year to get started and buy equipment and then lower amounts for succeeding years.

Thoughtful discussions during negotiation of your startup package with your chairperson, service chief, and scientific mentor are critical as you estimate realistic numbers and set expectations (Table 2.1). It will likely take you at least 5 years to reach the level of independent funding (discussed further below); however, it is unlikely that your startup package will fully fund your research for this length of time. Consider negotiating a 3-year commitment with the possibility of obtaining further support when you demonstrate appropriate progress, which may be defined as publications or applying for/obtaining extramural funds. Careful planning and goal-setting will give the leadership confidence in your strategy, and they may be more willing to commit resources if your goals are tied to realistic milestones. Of course, this strategy does have some risks; if the timetable is not met, you must be prepared to either give up your support and your laboratory efforts or convince the chairperson that this timetable was not realistic.

Your institution or department will likely have funding opportunities set aside for junior faculty members on a competitive basis. These funds are generally flexible and can be used for specific projects or added to your laboratory working funds. These awards are also often for "pilot"-type studies that are hypothesis-generating and allow for attainment of skills and preliminary data that will support your future applications for extramural support. For example, at our institution, the American Cancer Society has an institutional support mechanism that provides $50,000 over 1 year to junior investigators in our cancer center.

Extramural Sources

There are numerous extramural sources available for funding. Table 2.3 is a non-exhaustive list of institutional, foundational, and society grants that a junior faculty member who is a surgical oncologist focused on gastrointestinal malignancies at

Table 2.3 Examples of funding opportunities available to a junior faculty member who is a surgical oncologist

Type	Source	Support (No. of "$" indicates funding amount)
Foundation	Disease-specific patient advocacy groups	$, 1–2 years
Society	Association for Academic Surgery American College of Surgeons Society of Surgical Oncology Society for Surgery of the Alimentary Tract American Society of Clinical Oncology American Cancer Society	$-$$, 1–5 years
State government	California Institute for Regenerative Medicine Cancer Prevention & Research Institute of Texas	$$$, 3–5 years
Federal government	Department of Defense Veterans Affairs National Institutes of Health	$$$, 3–5 years

our institution may consider. Rather than providing a list of all potential awards available to you, Table 2.3 serves as a starting point for your detailed search for available funding. Importantly, for any given funding source, there are often multiple mechanisms available, such as those for "high-risk" studies, career development awards, and long-term (i.e., 5-year) support.

Industry

As clinical surgeons, we have unique opportunities to partner with industry in research endeavors. For example, we may be asked to test new devices or obtain tissue for a company's research program; funds are provided based on the number of patients or samples accrued. Some funds require applications that are vetted by the company, akin to a competitive grant mechanism, while others may be a direct contract between the company and a principal investigator. Either way, it is essential to involve your institutional legal office to ensure that intellectual property and patient privacy are protected.

Nuts and Bolts of Grant Applications

General Concepts

Obtaining competitive awards requires you to convince reviewers that your science is innovative and impactful. Your reviewers may or may not be experts in your field, they may or may not be sleep-deprived due to other responsibilities, and they may

or may not be reading your application on a cross-country flight the night before a study section meeting. Obviously, you cannot control those external factors. However, there are many components of the grant application that are under your jurisdiction. For example, if the award requires a layperson abstract because there is a patient advocate reviewer, copying and pasting paragraphs from your Specific Aims page filled with scientific jargon will not help your cause. You can also choose whom you ask to review your drafts, and you control how much time you give them to provide comments. You must capitalize on all the variables under your control to maximize your odds of getting favorable reviews. The suggestions laid out below have been shaped by our experience with grant writing, critiques we have received for our proposals, or comments heard during our participation in NIH study sections.

Start Early

Obtaining funding, especially NIH K- and R-level funding, is a crucial inflection point on your career trajectory. Think back to other important junctures in your medical career, such as preparing for the Medical College Admission Test (MCAT) or United States Medical Licensing Examination (USMLE) Step 1. For these critical exams that determine the next step in our training, many of us took large dedicated blocks of time off to prepare. Similarly, you should carve out a significant block of time to prepare your grant applications. Obviously, the larger the grant, the more time you need. For a new K08 or R-level grant, we suggest 3 to 4 months. This dedicated block of time does not necessarily have to be consecutive. For example, you can set aside 4 straight weeks for intensive writing, and at the end of that period, send the drafts to your mentors and colleagues for their opinion. Then give them 4 weeks to review and comment. During that time, you may consider going back to your normal clinical schedule. The most important concept that you must keep in mind is that it is very difficult to write a competitive K08 or R-level grant during only nights and weekends. It is too easy to prioritize smaller daily tasks over grant preparation. Dedicated block time allows you to focus solely on your grant application.

Read and Follow the Instructions

Familiarize yourself with the funding organizations and their grant review processes. Format your proposal according to the organization's specific requirements. If you fail to follow the formatting rules for NIH grants, the application will be pulled from review, and you have no recourse until the next cycle. Talk early and often to the organization's staff members who can help guide you through the process.

Work Consistently

The Specific Aims page and the Research Strategy section are most important parts of your grant application, and the hardest ones to write. There will be days where you do not have the time or proper inspiration to make significant progress on these components. On those days, work on other required components that take much less mental energy but may still require a significant amount of time and thus, are best not left for the last minute. These include:

- Filling out lists of facilities and equipment—these may require emails to your department administrator or walking around your research facility to take an inventory
- Redrawing or editing figures so they are customized for your grant, rather than copying and pasting them from a manuscript or a lab meeting presentation
- Working on your budget or writing the budget justification—these may require emails or meetings with your finance department
- Editing your biosketch and those of your collaborators and drafting letters of support for your collaborators (see Grantsmanship section for details regarding this point)
- Sending emails to collaborators to remind them to send their science and their biosketches to you
- Setting up the software for your references

Doing SOMETHING every day that advances the application will help break the submission process into smaller and more manageable chunks.

Elicit Critical Feedback: From the Right People

You need to identify mentors, colleagues, and collaborators who will thoroughly review your proposal and provide thoughtful, frank, and substantive critiques and suggestions. "Looks good" is the worst type of feedback and rings of apathy. Expect and hope your application is returned to you covered with edits and comments so you that you can make your application as strong as possible before it undergoes external review. Having a mix of content experts and non-experts will be helpful for you to get a comprehensive perspective. Your application may have gotten too deep in the weeds with jargon and details that non-experts will notice, while superficiality will be pointed out by the experts. Remember that it requires a significant commitment to read a grant in detail and provide thoughtful commentary. You must provide your readers ample time, on the order of 2 to 4 weeks, while also retaining enough time for you to consider their suggestions and rewrite your application as needed.

Early Stage Investigator Status for R01 Applications

The NIH defines early stage investigators (ESIs) as researchers who are within 10 years of their terminal research degree or end of postgraduate clinical training (i.e., fellowship) and have not yet competed successfully for a "substantial" NIH independent research award (e.g., R01 or U01 grants). ESIs have a higher payline than established investigators and are prioritized for funding. For example, the National Cancer Institute (NCI) regularly provides ESIs 5–7 extra percentiles on the payline. Your R01 application receives ESI consideration only if all its principal investigators are ESIs. Thus, if you are seeking to team up with a senior colleague to bolster your chance at funding, remember that you will lose out on ESI points, which are crucial. Instead, consider designating your senior collaborator as a co-investigator. Such investigators do not have to be an ESI, and your application will retain ESI status. Details regarding ESI policies may be found here: https://grants.nih.gov/policy/early-stage/index.htm.

Asking the Right Questions, in the Right Way

Competing successfully for extramural awards depends on your ability to effectively communicate your scientific questions, and your plans to answer them. Your questions must be clear and framed in a way that upon successful completion of your project, the field will have been advanced in a substantive manner. Thus, in your application, you need to explain the gap in knowledge that currently exists in the field, why that missing knowledge is relevant, and how you propose to fill the gap.

Avoid asking "what" questions. These characterization type aims may be criticized as "fishing expeditions" and because you do not yet know results, it will be difficult for you to justify subsequent aims to follow up on the findings. For example, avoid statements such as: "In our preliminary work, we found that male mice with colon cancer treated with FOLFOX had shorter lifespans than female mice. In this aim, we will characterize the difference between male and female mice with colon cancer treated with chemotherapy with single-cell sequencing, spatial transcriptomics, and CyToF." In this case, we have asked "What is the difference between the two groups?"

Instead, you need to present the "what" as preliminary data and ask "why" or "how" questions. For example: "In our preliminary work, we found that male mice with colon cancer treated with FOLFOX had shorter lifespans than female mice. We analyzed tumors with spatial transcriptomics and found that male mice had more intratumoral immunosuppressive regulatory T cells. In addition, we found that the tumor microenvironment in male mice had higher levels of IL-10. In this aim, we will test the necessity of IL-10 in mediating the sex-difference response to FOLFOX." Here, we are asking how the difference that we observed is being carried out (i.e., the mechanism that explains the observation).

Obviously, the difference between the two examples is not just wording, but completed experiments that provide the data to justify the "how" and "why" questions. Asking these mechanistic-type questions is increasingly essential as you apply for larger and more competitive grants, and they are absolutely mandatory for R-level applications. Generally, for a given question, go "deeper" rather than "wider." From our example, you would be better off proposing more mechanistic studies related to regulatory T cells and IL-10 production rather than asking if FOLFOX also had sex-associated differences for other cancer types, or if other chemotherapy regimens had the same effects.

Grantsmanship

Your science is the foundation of your application, but your success in convincing the reviewers that your science is interesting and impactful (and thus fundable) is contingent on your ability to convey your ideas in a clear and compelling manner. Grantsmanship, especially formatting, is low-hanging fruit that must be exploited to maximize your odds of success. Style will not get you funded, but it can get you NOT funded.

First, present only data that directly justify the significance of your application and the aims you are proposing. Adding extraneous data to make your application seem more robust will likely have the opposite effect. Unnecessary data provide more targets for criticism, waste space that can used for making other figures larger and more legible, and distract or confuse your reviewers from the focus of the application.

Second, make your figures legible. Copying and pasting or screenshotting from your previous papers or presentations will not suffice. You should make the effort to redraw or reformat figures so that they fit within the confines of your current application. We also recommend printing your grant with figures in both color and black and white to determine if the data are easy to read and to understand without color as some reviewers may read a black and white printout of your application.

Third, use your biosketch to advocate for your grant. Section A of the NIH biosketch is a personal statement where you can discuss your training and how these experiences have prepared you for your current project. This is particularly essential for junior investigators. You should also emphasize your past publications that have data supporting the current proposal. If you are submitting with collaborators, take the time to review and edit everyone's biosketches. Senior collaborators often have out-of-date biosketches that do not include their most recent work and have vague personal statements. Take the time to review everyone's biosketches to ensure that all personal statements are aligned with the goals of the current proposal and define the unique skillset that a collaborator will contribute to the project. Discuss papers that you and collaborators have published together to document previous productive collaborations—and mention this in both your and your collaborator's biosketches. Using generic biosketches is a missed opportunity to advocate for yourself.

Finally, draft all the letters of support yourself to ensure that your name is spelled correctly, your project details are accurate, and the letters will be returned to you in a timely fashion. Do not be afraid to use superlatives, and do not undersell yourself. When we email our collaborators for their letters and biosketches, we attach the versions that we prepared for them to edit and expand upon, or simply sign off on if they choose.

Preliminary Work

As detailed above, for R-level applications, a substantial amount of preliminary data to support the proposal is mandatory. The data do not have to be published. For K08 applications, it is very helpful to have published a paper related to your current proposal. We have participated in multiple career development award study sections for the NCI and in our experience, applications from candidates who did not have a paper in press related to their proposals were dead on arrival. Other institutes are not as competitive, but having a paper puts you at the front of the pack. It does NOT have to be a paper that is published in high-level journals like *Cell*, *Nature*, or *Science*; any publication related to the topic of your proposal will suffice. A published manuscript speaks volumes to grant reviewers about your ability to ask clearly defined questions and carry out the work it takes to answer them.

Feasibility

One of the main criteria that reviewers consider, especially for junior investigators, is the feasibility of the proposed aims. There are both technical and logistical issues to review. For example, if you propose cutting-edge techniques such as spatial transcriptomics or CyTOF, you need to explain where the machines are located, who will be running the experiments, and who will analyze the data. If you and your collaborators have already performed these experiments and have published the results, then you can just say "CyTOF will be performed and analyzed as we previously have done" and provide the reference. However, if this is a new technique for you, then you will need to go into much more detail and include letters of support from collaborators or the director of the core facility where the experiment will be performed.

Logistical feasibility may come in many forms. For example, if you propose a multi-institutional collaboration to study patient samples, you need to convince reviewers that you can overcome the logistical challenges of working outside your institution. Preliminary "data" that is convincing would be already having material transfer agreements set up or having collected a handful of samples from other sites. Similarly, if you propose studying a genetically engineered mouse model that has multiple transgenic alleles, which would take many years to breed, document that you already have these mice in hand or are in the process of acquiring them from a famous scientist and provide a letter of support from that person.

Biostatistics, Potential Problems and Alternative Plans, and Sex as a Biologic Variable

A lack of biostatistical justification for your experiments and a discussion of potential problems and alternative plans are easy areas for reviewers to critique. Similarly, sex as a biologic variable is a required component of an NIH application review. You do not need to write expansively on these topics, but you need to put something in your Research Strategy section that explicitly addresses these areas.

Submission Process

All governmental grants (NIH, Department of Defense, etc.) need to be processed and submitted by your institution. Therefore, you must familiarize yourself with institutional due dates that will be days to weeks sooner than the official submission deadlines. Your institutional contracts and grants administrators must complete a significant amount of administrative paperwork that has nothing to do with your science. You may need to turn in your proposed budget and justification weeks before the grant deadline so the research office can check the numbers. For subawards to collaborators located at other institutions, your grants team will need a substantial amount time to work with their counterparts at the other programs. As you start working consistently with your departmental and institutional grants and

contracts officers, you will learn what the "hard" deadlines are, and which ones can be skirted. For example, at our institution, we are required to submit all grant-related documents 3 days prior to the NIH deadline, but we can still swap out an updated version of the scientific components of the application, such as the Specific Aims page, 2 days later.

Budget

For NIH R grants, you may ask for a modular budget of $250,000 per year for which you do not have to provide a budget justification. However, with a detailed budget justification, you may request $250,000–$499,999 per year. In our opinion, there is NO reason to restrict yourself to a modular budget. As we discussed at the beginning of the chapter, running a lab is a costly endeavor, and the modular budget, which was set in 1998 and has remained unchanged to account for inflation in the subsequent 25 years, will not be sufficient. The budget justification is usually less than two pages long and is not scrutinized in detail. The budget is not a score-driving component for the NIH study section, and it is only discussed AFTER the panel members have inputted their final scores for the application.

The NIH salary cap was $212,000 for 2023. This means that when you ask for 10% effort, you will receive $21,200 of direct support from the NIH, even if your institutional salary is higher than that. However, based on your institution or departmental regulations, the salary that your contracts office uses for your application may be lower than the cap. For example, your base salary may be $70,000 for being an assistant professor, but your total salary is much higher than that based on clinical augmentation, support for administrative work, etc. Your contracts and grants office may be required to put only your base salary into your application. You need to clarify with your institutional financial team how your salary is considered in terms of grant support.

Finally, from a grantsmanship standpoint, you need to give some nominal salary support to your scientific collaborators who have a significant role in your application even if they do not need the money. Even 1%–3% effort is sufficient to convince reviewers that your collaborators will truly be engaged. Analogously, if your project requires biostatistical analyses, you must provide financial support to a biostatistician, who is ideally a faculty member at your institution. The more rigorous the biostatistical needs of your project, the more mandatory it is to include a biostatistician as a funded collaborator. The 3% amount suffices for projects with straightforward biostatistical needs.

Responding to Rejection

For fiscal year 2024, the payline for R01s at the NCI is set at the tenth percentile, and for ESIs, the payline is the 17th percentile [5]. Details on the K08 payline are not publicly available, but it is likely at the 20th percentile or below. Other institutes will have more generous paylines, but they will still be in similar ranges. This means

that the large majority of submitted proposals will not be funded. However, the fear of failure should never deter you from applying; you will never receive competitive grants that you do not apply for. Over the course of your research career, you will experience many more rejections than successes in grant applications. Thus, establishing a process to cope and then respond to the critiques in a positive manner is essential.

After a rejection, your options are to: (1) revise and resubmit, (2) redirect to another funding mechanism or study section, and (3) abandon the current idea and start afresh. Different funding agencies and study sections have different emphases and missions. Speaking to program officers and having them review your Aims page may provide helpful direction for where to redirect your proposals. You should also elicit the feedback of your mentors, collaborators, and funded colleagues to determine if your proposal is worth resubmitting—again, finding the right people who will give you honest assessments is essential. The decision to resubmit should be driven by how close to funding your initial submission was, whether there are new published findings that have rendered your proposal obsolete, and your interest in pursuing your project.

Resubmission

Reading the summary statement for your unfunded application is an infuriating experience. As you go over the critiques, thoughts such as "I explained that!" or "What the **** is this person talking about?" will undoubtedly go through your mind. First, you should never take the comments personally, even if the critiques are unprofessional. Unless the comments truly cross the line in terms of professionalism or bias, you have no recourse. At best, the program officer may ensure that the reviewer will not be part of your NEXT application.

After some time has passed (usually a week or so for us), re-review the comments thoroughly and objectively to identify broad critiques that multiple reviewers had. If more than one person pointed out an issue, then it clearly must be addressed. Often each reviewer will have comments that do not overlap with other reviewers' critiques. Think critically about each point and consider whether you can and should address it. It is essential to remember that addressing each critique does not ensure you will be funded on a resubmission, since the reviewers are instructed to consider a resubmission on its own merits. A second thing to keep in mind for a resubmission is that the original reviewers may have written down only the most salient criticisms and not included other ones. Third, a resubmission may have new reviewers who have their own disparate opinions. Thus, you must rewrite and review your resubmission with a critical eye and not simply be satisfied with addressing the original reviewers' comments.

Identify comments that require you to make substantive changes to your aims or proposed experiment or to do new experiments to generate novel preliminary data to further bolster your proposal. These are the most time-consuming comments to address, but if accomplished, they are also the most convincing and welcomed by the reviewers on a resubmission. You should start on these as soon as possible; you

may also need to invite new collaborators who bring the requisite expertise to address these comments. Next, address critiques that may be due to confusion stemming from your muddled writing or the reviewers' lack of familiarity with existing literature (remember that the reviewer may not be a content expert). You may need to restructure your figures, rewrite part of the text, or provide some relevant references. Finally, if there are irrelevant comments that you choose not to address substantively, you should still mention these in the introduction, which is a one-page document that is required for NIH resubmission proposals. You need to frame your lack of action in a positive way, such as by diplomatically explaining that the reviewer may have misunderstood something, and you have rewritten the text to better clarify the point. Leaving comments completely unaddressed may cause reviewers to double down even if they are wrong. Thank the reviewers for their thoughtful critiques and respond respectfully and professionally.

As is the case with surgical skill, grantsmanship improves with repetition and persistence. Grants are iterative processes that improve with each version because of new data, better grantsmanship, and new collaborators.

Learn About the Review Process

The NIH review process is an intimidating gauntlet. All your mentors and colleagues who have applied for NIH funding will have horror stories about how they were shortchanged by a study section. Learning the ins and outs of the review process will put you ahead of the competition in terms of knowing what factors influence reviewer comments and scores. The NIH has an Early Career Reviewer (ECR) Program that invites junior faculty members who have yet to serve on study sections or held R-level grants to participate in a study section as a reviewer. We strongly urge you participate in the ECR program. Reviewing other people's grants and sitting on a study section will help you understand how reviewers determine overall impact, what the panel discussion sounds like, and what criteria are truly important to reviewers. This experience also gives you the opportunity to read other people's grant applications, which will allow you to compare what funded grants look like compared to ones that are scored poorly. As you will likely be assigned to study sections related to your interests, you will also see what is considered cutting-edge science, what models are acceptable or passe, and what scientific concepts are exciting. You can find more information here: https://public.csr.nih.gov/ForReviewers/BecomeAReviewer/ECR.

Summary

Securing funding for a basic or translational research laboratory is a substantial and daunting undertaking. The majority of your funds will need to come from competitive extramural sources, in particular the NIH. Obtaining external funding requires dedication, persistence, thorough planning, supportive mentorship, committed

institutional leadership, and luck. Most applications do not get funded; thus, without persistence, you will never compete successfully for extramural funds. Grant writing is an iterative process, and your proposal will improve with each version because of new data, better grantsmanship, and new collaborators. We hope our advice will help you on your journey.

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Choosing a Good Scientific Mentor and Being a Good Mentee

3

Cody Tragesser, Daniel Scheese, Mark L. Kovler, Benjamin D. Shogan, and David J. Hackam

Abstract

Being an effective mentor requires the acquisition of a set of skills, emotional intelligence, time and resources in order to identify the needs of the mentee, and to deliver on those needs. In parallel, being a good mentee also requires time, effort, and thoughtful engagement. Here we will describe the critical elements of being a mentor and mentee.

Keywords

Surgeon-scientist · Mentorship · Mentor · Mentee · Generosity · Academic surgery

Introduction

The role of a "surgeon-scientist" entails a surgeon engaged in clinical or basic science research who is dedicated to discovery and innovation. Given the challenges and complexities of the role coupled with the financial resources required for success, it is widely accepted that the surgeon-scientist is under tremendous pressure. These challenges not only complicate the ability of faculty members to achieve a balance between clinical practice and scientific research, but they also diminish the

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pool of available mentors. One of the essential foundations of a career as a surgeon-scientist is effective mentorship. We now seek to define the essential attributes of mentorship and present a framework for selecting a good scientific mentor and, as importantly, being a good mentee.

Defining Mentorship

There are many potential definitions for a mentor, and in fact, the word arises from Greek mythology, in which Odysseus entrusts his son Telemachus to the care of the character "Mentor" as he goes off to fight the Trojan War. Fortunately, today's mentors are not preparing their charges for physical battle (although at times academic surgery may seem to be a contact sport). Rather, the current mentor-mentee relationship involves the transfer of knowledge, skills and wisdom to prepare the mentee not for battle, but for career success.

In academic surgery, mentors therefore play several roles. These include counsellor, sponsor, and coach. The overriding principle of mentorship in surgical science is that of a more practiced and veteran individual or team of individuals who guides a mentee in the development and establishment of a successful clinical practice, creation of a scientific center and academic advancement.

A mentee, in contrast, is a person who is directed and advocated for by a mentor or team of mentors. While much has been written on mentorship, less has been discussed regarding "menteeship." The mentee plays the most active role in guiding the mentor-mentee relationship because at the root of the product of this relationship is the mentee's career.

While the above language describes what a mentor is, it is also important to describe what a mentor is not. From the point of view of academic surgery, a mentor-mentee relationship is focused on the professional and career goals of the mentee. The mentor can offer emotional support and guidance, but is most effective when this is offered within the context of appraising, and seeking to achieve, the mentee's career goals. The mentor should have content expertise in the needs of the mentee, and while the mentor should be accessible to the mentee as needed, such access is most effective when structured and goal-oriented. For this reason, mentees are most successful when they have multiple mentors, each of which able to offer specific guidance within their areas of expertise and skill.

Mentorship by Career Stage

While this chapter is dedicated to be a framework for junior faculty members aspiring for success in academic surgery, mentorship is critical at all career stages. Mentors play critical roles for medical students and residents. Mentors help develop the beginnings of a network for these students and trainees, help guide decisions on which specialty or residency the mentee will pursue, and advocate for the mentee via letters of recommendation or direct phone calls. Indeed, a well-written letter

from a dedicated mentor is often cited as a top selection criterion by residency program directors.

Many students participate in research with their mentor(s). Medical students may also find mentorship from junior and senior surgical residents who recently went through the match process themselves. One of the most important values of performing research with a mentor is not only the scholarly activity and the publications that ensue, but also the opportunity to expand on this personal research journey during the interview.

Surgical residency is another career stage when mentorship plays a critical role as evidenced by the literature that up to 70% of residents go into the specialty of their mentor. Again, surgical residents seek mentors who are both senior residents and faculty. Most junior residents will describe a senior resident or two who have taken a special interest in them or have been "taken under their wing." Senior resident mentors provide guidance not only in acquiring technical skill, but also in grasping fundamental clinical knowledge. Further, they are a very important resource for identifying faculty mentors and opportunities for academic development. It is also important for surgical residents to develop mentor-mentee relationships with faculty. Guidance during residency includes post-training career plans and ideas for academic development. Like the medical student residency match, dedicated mentors play a significant role in fellowship matching.

Perhaps at no stage in the career of a surgeon-scientist is mentorship more critical than for the junior faculty member. Junior faculty in academic surgical departments face daunting challenges regarding limited funding and resources. At the same time, they are working to launch a robust clinical practice and establish a reputation. Additionally, they are frequently faced with the challenge of navigating a new institution with its own norms. The mentor team assists in guiding the junior faculty member through the "politics" of the department and institution, helps with obtaining independent funding, and assists with scientific efforts. The effective mentor will be described further below.

Finally, while senior faculty provide mentorship, they also gain insight as mentees themselves. Senior faculty may seek out mentors especially when they branch out from their role as surgeon-scientist to include more administrative responsibilities. Promotions may lead to a change in institutions when mentorship is critical to navigate their new environment.

It should also be stated that while a good mentor is an important component of the success of students and faculty, it is by no means the only component. Just as there is no perfect mentee, there is no perfect mentor, and plenty of students and residents succeed without the benefit of a committed, experienced faculty member in their corner. There is no substitute for hard work, commitment and academic success on the part of the mentee. No mentor can make up for these critical components if they are absent on the part of the mentee.

In summary, throughout the career of a surgeon-scientist, mentorship has an influence on career choice, promotion, and productivity.

When the Mentee Becomes a Mentor

As a mentee moves through the ranks of academic surgery, at some point, that person will find themselves as a mentor to someone else. Often this happens early in the mentee's career, as for example when a subintern is teaching third-year medical students during their clerkships. More daunting is when a junior surgeon-scientist becomes the primary research mentor for a student or resident. This can be particularly stressful as the junior faculty is simultaneously building their career and advising the mentee. While these times of growth can be challenging to the newly minted mentor should remember that it takes practice and experience to be a successful mentor. Further, they should understand that what worked for them as a mentee will likely not work for everyone. These times also provide new opportunities for the new mentee to discuss mentorship strategy with their own mentors.

Choosing a Good Scientific Mentor

Defining an Effective Mentor

We believe there are certain prerequisites for effective mentors that should be sought after when pursuing a mentor-mentee relationship.

Experience

Depth of experience is an indispensable quality in an effective mentor. For aspiring academic surgeon-scientists, it is important to seek out mentors with experience in academic surgical departments and science. Because no single mentor will fit the exact career aspirations of a mentee, they can look for a team of mentors with different experiences, as discussed below. Experience and gained wisdom are an important entity that the mentor possesses that the mentee lacks, and much of mentorship involves the transfer of that gained knowledge from mentor to mentee. Additionally, a differential level of experience is one of the factors that distinguishes friendship from mentorship. So, in choosing a good scientific mentor, one should look for those with more depth of experience. Though most trainees naturally gravitate toward research mentors who have spent the most clinical time with them, they should be introduced and encouraged to consider outstanding research mentors with less clinical exposure. Mentees should focus on those mentors with the passion, commitment, and experience to serve as excellent mentors, beyond that which they experience on their clinical rotations.

Personal Dedication

The mentor-mentee relationship is built on personal dedication, investment, and trust. While both parties certainly benefit from the relationship, on the surface, the mentee benefits more than the mentor. Therefore, an effective mentor must show a

high level of commitment and an element of altruism. The motivation for mentorship comes in many forms. Shared personal experiences, an interest in giving back to their profession, and a gratitude for mentors in their own career are some of the reasons why effective mentors devote time and resources to their mentee. In truth, however, an effective mentor-mentee relationship should be as beneficial to the mentor as it is to the mentee.

Content Expertise

It is important to note that for a mentor to be effective, they must have content expertise in the domain in which the mentor-mentee relationship is based. This expertise should ideally be tied to the specific set of goals around which the mentor-mentee relationship is based. For a mentor who serves as a career guide, they should have expertise in the ins and outs of the specific career around which they are providing guidance. Similarly, for a mentor who is offering guidance on mental/emotional issues, they should have specific training in mental health. Selecting a mentor with incomplete or tangential content expertise to the goals of the specific relationship will lead to shortcomings and frustration.

Understanding

Understanding encompasses a spectrum of qualities—such as empathy, tolerance, and forgiveness—essential for a mentor guiding their mentee. Understanding built upon these qualities allows the mentor to provide tailored guidance.

There is increasing awareness and embrace of the diversity which exists among surgical trainees, considering a range of attributes such as social and ethnic background, physical ability, gender, sexual orientation, national origin, and political beliefs. Accordingly, the mentor must make every effort to empathize with and respect mentees, especially when there are differences along these lines. This understanding fosters more effective guidance and enhances rapport. Additionally, mentors should take a posture of curiosity, humility, and respect toward individuals of different backgrounds. This not only cultivates a secure, supportive environment where all individuals can flourish and achieve success but enhances diversity in academic surgery overall.

Furthermore, emotional understanding is pivotal; a mentor should gauge and respond to their mentee‘s emotions to offer feedback that both motivates and educates without jeopardizing the mentor-mentee rapport. Recognizing individual differences in how feedback is received is crucial; mentors must discern the limits and tailor their approach accordingly, mindful not to overstep boundaries.

Lastly, concerning understanding, a strong mentor is attuned to the life goals of their mentee and avoids imposing their own aspirations. Understanding that not every mentee shares the same vision, a mentor refrains from imposing personal ideas or plans. They keenly observe beyond surface agreements, discerning the mentee‘s genuine alignment with the guidance provided.

Generosity

Generosity in mentorship comes in many forms. As stated, the transfer of experience and expertise is one of the defining characteristics of a mentor-mentee relationship. For this transfer to occur effectively, a mentor must be generous with sharing their knowledge. For example, a scientific mentor who is independently funded can share previous grant applications with a junior faculty member, giving them insight into the process and what a fundable grant looks like. Similarly, a clinical mentor may assist in developing the mentees practice by asking them to participate in interesting cases or deferring referrals to their junior partner. Both instances require generosity of experience—on both occasions, the mentor is sharing a resource that took years or decades to develop with an unseasoned mentee.

Generosity also comes in the form of generosity of recognition. The effective mentor defers credit to their mentee to elevate the mentee's status. As discussed, this requires that the effective mentor no longer *requires* full credit for an accomplishment. They must reach a status in their career that allows them to be generous with recognition for this to occur. But simply reaching that status does not ensure shared recognition. A mentor who appears greedy or selfish with credit should be avoided, but a mentor who is known for recognizing their mentee's accomplishments publicly and through authorship should be sought after.

Similarly, generosity of financial support can be especially important for junior faculty members. The goal of the junior faculty member interested in a career as a surgeon-scientist is early independent funding. To accomplish this, they will need preliminary data and evidence of independence. An effective mentor can foster this through "start-up funding" in the form of financial backing and allocation of lab space and resources.

Finally, time is a scarce resource for all surgeon-scientists. By definition, an effective mentor is a busy surgeon-scientist themselves and often have burdensome administrative responsibilities as their career has progressed. Generosity of time is an important consideration when identifying a good scientific mentor and can come in several forms: regularity, accessibility, extent, and value of time spent mentoring.

Experience, recognition, financial support, and time are scarce resources in academic surgery. The effective mentor must not only have an abundance of these means to generously share with a mentee, but a level of altruism as the distribution may come at some level of personal or professional sacrifice.

Roles of an Effective Mentor

The effective mentor plays several roles. As discussed, transference of experience and knowledge is the overriding principle in mentorship. But not every decision a mentor made in their life or career is appropriate for every mentee. It is therefore important for the mentor to provide career guidance but not necessarily dictate a mentee's career. The effective mentor helps their mentee create a plan (5-year, 10-year, career) and outlines interval attainable achievements to meet that plan. For

the aspiring surgeon-scientist, these include concrete actions such as grant preparation and achievement of independent funding, peer-reviewed publication, and presentation at institutional and national meetings. The effective mentor also provides sponsorship and advocacy. This occurs on an institutional level in the form of promotional support and nationally in the form of backing for positions within societies. The effective mentor also assists with planning work-life balance and providing opportunities for networking.

Establishing a Team of Mentors

Given the complexities and demands of a career as a surgeon-scientist, it is unlikely in the current environment that a single individual can satisfy all the mentoring needs of a junior faculty member. It is challenging that a single mentor can effectively mentor a large number of mentees in a department. For the sake of efficiency, it is important to establish a team of mentors and mentoring networks. For example, the junior faculty member beginning their career oftentimes requires a clinical and a scientific mentor. A clinical mentor will guide them in establishing a practice, help with obtaining referrals, and assist with difficult clinical decisions and operations, and a scientific mentor, will guide them in establishing a lab, a focused line of research, obtaining grant funding, and providing introductions to cross-disciplinary collaborators.

Being a Good Mentee

Discussions of mentorship are often dominated by the description of a meaningful mentor, and while prioritizing mentorship is currently at the forefront of our profession, the role of the mentee is less appreciated. However, we believe that the success and productivity of a mentor-mentee relationship is reliant on being a good mentee. For the mentee, "the more you give, the more you get." A good mentee demonstrates dedication, reliability, and a strong work ethic. Consistently punctual and well-prepared, they approach mentorship meetings with openness and a willingness to receive guidance. Further, the mentee guides and facilitates the mentor's efforts. A good mentee enables the working relationship by planning meetings, asking important questions, and inviting constructive feedback. While this may appear self-explanatory on the surface, and sometimes is, for those struggling to find a good mentor, a focus on the process of being a good mentee can make for a more satisfactory mentor-mentee relationship.

Wellness of the Mentee

In the last two decades, "burnout" has been identified as one of the greatest threats to surgeons and surgeon-scientists in the modern era. The career of a surgeon-scientist adds additional challenges—including the need to publish, succeed in

grant writing, and discover new knowledge—further increasing susceptibility to burnout. Definitions of burnout vary widely, but it is characterized by exhaustion, depersonalization, and a low sense of personal accomplishment. “Wellness” is an inseparable term from the discussion of burnout and encompasses several strategies for combating burnout, though disparate perceptions of wellness between residents and faculty have made it somewhat of a charged term. To the newer generation of trainees, wellness tends to evoke a fair balance of work and personal life, while to preceding generations it may be seen as a threat to the rigorous training which is needed to produce competent surgeon-scientists. We propose that it should instead be viewed as a state of being which, when optimized, helps the mentee realize their potential and continue to perform for sustained periods, and when ignored, produces a competent mentee who is unbalanced in life and ultimately unsatisfied with their work. The mentor-mentee relationship can enhance the wellness of both parties when there is a shared understanding of this term. A mentor who aims to fulfill the multiple roles outlined above cannot simply ignore the mentee’s wellness as a secondary concern.

While the exact meaning of personal wellness is best defined by each mentee for themselves, certain guiding principles may be useful. Wellness can be conceptualized in multiple “realms” that reinforce one another and contribute to an overall state of wellness; key realms include the academic, social, physical, and emotional. Wellness in the academic realm may consider the mentee’s time management habits, ability to meet deadlines, support from colleagues, and overall success in research productivity. Wellness in the social realm considers the health of the mentee‘s personal relationships and the sufficiency of local and distant support systems. Physical wellness considers the mentee’s ability to attend to physical health through regular exercise and access to healthcare when needed. Finally, emotional wellness considers the mentee’s emotional state in the face of the varied experiences they encounter in their surgical training, and whether they have access to sufficient mental health resources. The mentee should reflect carefully on these areas of their life and set goals to maintain or improve wellness in each. This is where conscious effort on behalf of the mentee is essential.

Careful self-reflection allows the mentee to then have productive ongoing discussions pertaining to their wellness with the mentor. Such discussions are crucial, because the inability of the mentee to voice their needs to the mentor may lead to frustration and resentment from the mentee. The frequency and formality of such discussions is entirely the preference of both parties, but it is essential the mentee should feel empowered to honestly share with the mentor their perceived wellness in each area, and their goals pertaining to each. Ideally, honest discussion on this topic gives the mentor a holistic understanding of the mentee’s psychosocial context, which informs the patterns they may be observing in the mentee. The mentor should provide support for most stated personal goals formed through careful self-reflection, but the mentor should also consider providing constructive feedback if their experience suggests it is needed. When the mentor is first a listening ear, their constructive feedback is likely to be received much more warmly and effectively by the mentee.

In summary, a mentor-mentee relationship which openly addresses the wellness of the mentee establishes trust, upon which expectations and goals relating to research and training can be set.

Nuts and Bolts of the Priorities of a Surgical-Scientist Mentee

For a surgical trainee aspiring to embark on a career in basic science without a background in PhD studies, the transition poses considerable challenges and stress. This shift becomes particularly demanding after primarily concentrating on clinical medicine throughout their education and training. Proficiency in basic sciences demands a significant investment of time and dedication to lay the groundwork for a successful academic career.

The rigors of surgical training, notorious for their time-intensive nature, barely allow for an in-depth understanding of basic sciences without a dedicated hiatus, often requiring two to three research-oriented years. Securing a position in a reputable basic science laboratory under a skilled mentor can immensely aid in navigating this steep learning curve.

Surgical residents are accustomed to enduring strenuous, extended hours, a preparation that mirrors the perseverance needed to establish a foundational understanding necessary for a successful surgical scientist. During dedicated lab periods, the mentee‘s primary focus must pivot towards grasping basic science intricacies. This encompasses extensive literature reading, acquiring fundamental laboratory techniques, and engaging with experienced lab members for guidance and knowledge.

Given the concurrent pursuit of surgical training, it remains pivotal for the mentee to sustain involvement in surgical education conferences, Morbidity and Mortality conferences, and self-directed studies. Effective time management becomes imperative, enabling the mentee to fulfil these commitments while prioritizing their basic science training and research efforts.

Beginning the Mentor-Mentee Relationship

Initiation of the mentor-mentee partnership can occur by either the party and by a variety of fashions. Recently, several innovative strategies have been put in place by surgical departments around the nation to encourage initiation of mentor-mentee partnerships. Whichever way this occurs, the mentee will always play a significant role in seeking out mentors. Mentees should be proactive and set up meetings with potential mentors that have similar interest to discuss their goals. The mentee should come to these meetings prepared to discuss their aims including 5-year, 10-year, and career goals and a potential timeline for achievement. Meetings should include direct discussion of the mentor’s past mentoring experience. Etiquette for these meetings may seem obvious for some, but it is important to note that a good mentee will prepare an updated curriculum vitae before the meeting, familiarize themselves with the clinical and research interest of the mentor, express appreciation for the opportunity to meet, prepare questions, and accept feedback.

Additionally, mentees should seek mentors with like-minded interests and work styles. A good mentee will be honest about professional chemistry and the appropriateness of a personal match and not simply seek out any successful surgeon-scientist in their department. A good mentee should also be mindful of initiating and continuing mentorship relationships that are not compatible. A failed mentor-mentee relationship can be a significant setback for both parties involved—the mentee feels disheartened and defeated, and the mentor is left with a negative impression of the mentee and may be less willing to mentor in the future.

Initiation of mentor-mentee relationships should occur early. A good mentee seeks mentorship at all levels of their career from medical student to senior faculty. As discussed, the need for mentorship is fluid and changing as a career develops. Additionally, as mentioned, a team of mentors is frequently necessary. Further, in academic surgery, mentors may leave an institution for promotion and opportunity elsewhere. For all these reasons, a good mentee needs to stay *active* in seeking mentors. The stagnant mentee will find themselves failing at inopportune times.

Maintenance of the Mentor-Mentee Relationship

The responsibility for maintaining the mentor-mentee partnership falls on the mentee more so than the mentor. The principles of effective communication, respect, and responsibility can guide a good mentee in protecting the relationship.

Communication between mentors and mentees occurs through a variety of mechanisms. In today's constantly wired world, phone calls, text messages, emails, and informal "drop-in" chats all occur regularly, although the more structured the interactions, the more likely they are to be effective. A "good" mentee will discover the mechanisms by which their mentor likes to communicate. Effective communication also implicates keeping a mentor "up to date." The "good" mentee will correspond regarding career progress, project status, grants, and papers. Further, the good mentee responds at the earliest time possible to a communication from their mentor.

A good mentee demonstrates respect for an effective mentor in various ways. This includes valuing the mentor's time as a precious and scarce resource. Acknowledging that the mentor's time is taken away from their personal life and career, the exemplary mentee is mindful of this sacrifice during their interactions. Therefore, meetings and discussions should be focused. The "good" mentee prepares for these opportunities by proposing a focused agenda and bringing directed questions. It is also important to point out that the mentor's opinion should be respected. This includes a response to criticism. The "bad" mentee responds defensively and becomes argumentative when criticized by their mentor. In contrast, the "good" mentee tries to see their mentor's point of view and perspective and politely asks clarifying questions.

There are additional, more nuanced characteristics of an effective mentee. The good mentee meets deadlines. Therefore, time management is a skill that a "good" mentee attains. In being accountable, it is also the good mentee's responsibility to

decline a mentor's request if they cannot meet expectations. While the good mentee often feels the need to say "yes" to every opportunity to contribute, repeated failure to meet deadlines and responsibilities puts the mentor-mentee partnership at risk.

Summary and Conclusions

Mentorship has always been a critical aspect of the surgical-science career. However, with the current challenges in academic surgery, there may be no more crucial time for mentorship in academic surgery than the present. It is incumbent on our profession to meet the mentoring needs of aspiring surgeon-scientists if we are to maintain our role in the advancement of academic surgery, and the young surgeons of today must be active mentees.

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Effective Time Management Strategies for Conducting Laboratory Research

4

Evie Carchman and Lily S. Cheng

Abstract

When it comes to research as a clinician, especially basic science research, time is a precious commodity and organization is essential to utilize time effectively and efficiently. For surgeon-scientists who treat patients and spend considerable time and effort in the laboratory, effective time management is a critical skill to master. This includes detailed, advanced planning of experiments and grant submissions, efficient collection of data for grant proposals and timely publication of results, proper utilization of personnel and funds, fulfillment of commitments to both clinical and research pursuits, and the establishment of balance—to the extent possible—between work and life. Ineffective time management can delay your academic advancement, increase personal stress, decrease quality of work produced, decrease chances of maintaining funding and personnel, and decrease personal satisfaction. Instead of trying to create more time in a day, or more days in a week, effective time management allows us to use our time wisely.

Keywords

Time management · Organization · Prioritization · Productivity · Efficiency

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Definition

Good time management, the process of organizing and planning how to divide time between specific activities, enables us to work smarter, not harder. While new technologies allow us to stay connected to our work and to work virtually anywhere, this convenience can also take a toll on work-life integration. Time management is an increasingly popular subject due to the dissolving boundary between work and personal time, and our appropriate desire to maintain a personal life outside of work. As we seek to accomplish more in the same or less time, we can utilize effective time management skills to achieve both day-to-day and long-term goals. The secret of time management lies in the understanding that it is all about your behaviors and how to modify them. It is about self-discipline and self-awareness. Time management skills improve with practice.

Benefits

The clear benefits of effective time management include:

- **Maximum productivity and efficiency.**
  - When time is not wasted, you can get more work done in a day.
  - By accomplishing more in the same period of time, there is more time for other activities that will increase your productivity.
- **Enhanced professional reputation.**
  - When others recognize that you do not waste time, especially theirs, you build a reputation as a person who gets things done. People will trust that when you make a commitment, you will follow through with quality work. This reputation is important for the academic surgeon. Researchers seek collaborators whose contributions will maximize time and effort.
- **Less stress.**
  - Along with greater productivity and efficiency, you will experience less stress regarding deadlines and work-life balance. Decreased stress improves work satisfaction, prevents procrastination, and reinforces effective time management strategies.
- **Greater opportunities to achieve important life and career goals.**
  - With evidence of your productivity due to effective time management, more opportunities for advancement in both work and your personal life will surely follow.

Failing to Manage Your Time Effectively

Drawbacks to ineffective time management include:

- **Missed deadlines.**
  - When you do not prioritize and schedule your work time appropriately, it is likely that you will be working up to deadlines or even missing them—an investment of work and time without any reward. This time and effort could have been utilized to complete another project successfully.
- **Inefficient work flow.**
  - False starts impede an efficient work flow. You need to plan your activities for the day, accurately estimate the time required to complete them, and create a backup plan for when things come up unexpectedly.
- **Poor work quality.**
  - Without effective time management, you are likely to be working on proposals and projects right up to the deadline. Rushed work is bound to be incomplete and contain mistakes. Finishing your work ahead of a deadline allows time for checking facts, ensuring that formatting is correct, getting feedback from editors/experts, and making revisions. Work completed according to a well-planned schedule is more likely to represent your ideas accurately and to be a version that you are proud to present to others. Similarly, rushed work in the laboratory leads to mistakes with experimental design (forgetting appropriate controls) and experimental execution (calculating incorrect dilutions and dosing). Sub-standard work is obvious to others, can delay publications, prevent you from getting grants, and require the repetition of experiments—a waste of resources, including the valuable time of the people who work in your lab.
- **A tarnished professional reputation and a stalled career.**
  - Professional colleagues will take notice of low-quality work and an inability to complete projects. Signs of ineffective time management contribute to a poor reputation is that is hard to overcome. This reputation can significantly stall your professional advancement, making colleagues less likely to consider you for committees, collaborations, book chapters, invited lectures, and promotion.
- **Higher stress levels.**
  - Ineffective prioritization of projects, missed deadlines, and a skewed work-life balance create unnecessary stresses that can significantly impede work and affect your personal relationships which further perpetuates the issues above.

How To

Your best chance for successful time management is to begin with an introspective survey of your current skills and weaknesses. By understanding where you are now, what you are good at, and what you do poorly, you will be able to make improvements and succeed. The following are time management strategies (Fig. 4.1):

1. **Set Clear Goals/Priorities.**
   Begin with the end in mind. You want to match your time use with your priorities. First, determine your short-term and long-term career and life goals. These goals should be specific, attainable, and measurable. A fair amount of time should be spent on this activity, as it determines the direction of the steps that follow. Clear goals will allow you to more easily identify the tasks needed to attain them. Larger goals can then be broken down into the discrete actions. For example, your overarching goal may be to attain tenure at your institution. Underlying this goal are the components of research, teaching and service outlined by your institution's tenure review process. The research goal may be comprised of NIH funding, publications, and abstracts. Your stated abstract goal may consist of submitting four abstracts this year. Under your publication goal, you may plan to submit two basic science and three clinical papers this year. Under the basic science papers goals, you might place two of your current research projects. Under these research project goals are the experiments needed to complete the paper, and so on. You will need to use your limited time in a manner to meet the goals that have been set above. You should ask yourself frequently: *How does each of my actions bring me closer to my goals?* If your activities during the day are not bringing you closer to your goals, you may want to reconsider your current activities and commitments.
2. **Prioritize.**
   Next, prioritize the goals and the steps that are required to attain them. This action will allow you to focus most of your time on those goals that need to be

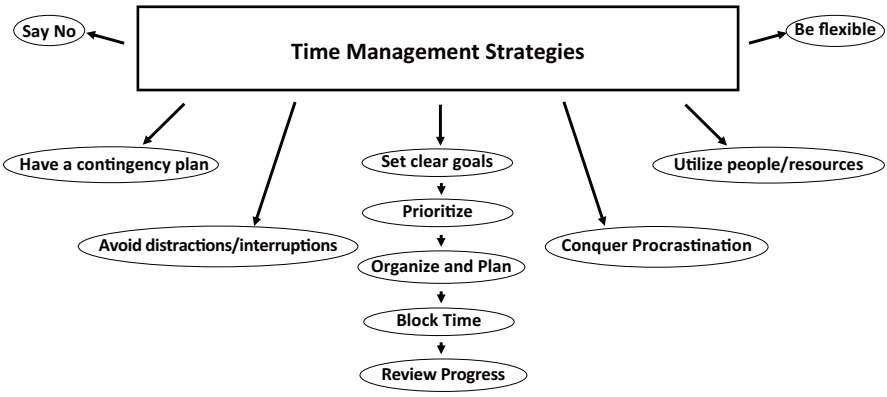

Fig. 4.1 Time management strategies. This figure demonstrates strategies for time management

completed soon, or on those that will require a significant amount of time to complete. Prioritization is not focused on getting more stuff done, but determining which of the necessary tasks should be tackled first. Then you can direct your time toward completing the most valuable tasks with the time you have. Prioritization is especially important when you have too many good ideas—without prioritization you may end up jumping around from idea to idea. The only way that you can carry good ideas to fruition is by prioritizing your goals.

3. **Spend Time Organizing and Planning.**

   Two minutes of planning saves 10 minutes of task execution. Once you have set your goals and prioritized the steps necessary to attain these goals, spend time planning and organizing your schedule/time and even your workspace in a fashion to reach them. Take the time to arrange your emails, documents, and office in a way that makes sense for you. For example, I organize my drafted papers and grants in folders in Box, a content management and file-sharing service provided by my institution. I make these folders accessible to our department's editors, grant managers, and graphic designers to review, make edits, and monitor the progress of projects. Box also allows for me to invite collaborators who can also work on projects alongside me in real time. I also have a 3'x4' whiteboard in my office with sections delineated for grants, papers, abstracts, teaching, clinical trials, mentoring, and lectures. Underneath each section I write my goals, along with applicable deadlines. These deadlines are also placed in my Outlook calendar with a reminder a week before. When a goal on my whiteboard has been reached, I write the date. This then allows me to add completed projects to my CV.

   During the planning/organization process, identify a convenient mechanism for listing your goals and tasks. In addition to a well-made plan, a to-do list will save you time. It is important to note that to be most effective, the to-do list should be easily accessible in various environments (office, laboratory, operating room, home, etc.) to allow you to refer to it at any time. This could take the form of a calendar on your phone, or a notebook that you always carry with you. I use Trello, a web-based project management tool, which is accessible on my computer and my phone and shareable with family and co-workers. It allows me to create separate boards for home, lab, and clinical practice. I can share boards and lists with my lab members to delegate tasks to them. Each of the tasks on a list should be discrete and attainable. Beside each task, note your estimation of the time that will be required to complete the task, bearing in mind that we often underestimate the amount of time it takes to complete a task. I set deadlines for others 2–3 days prior to the actual deadlines to buffer potential delays. This proactive instead of reactive mentality decreases stress and mistakes. Of note we understand there is a need for a balance between organization but also creativity.

   – To-do lists can be very effective, allowing you to focus your mind on important objectives. As prioritization and organization may change based on the progress of projects, these lists should be updated or reviewed at least weekly. The ability to keep lists by memory alone is temporary

- To-do lists also make you less likely to forget tasks, which is easy to do with a very busy academic career where you wear multiple hats (surgeon, teacher, clinician, scientist, parent, etc.). I put things on my to-do list that may seem obvious at the time, but serve as a great reminder several days later.
- The act of writing the list can help you organize your thoughts and prioritize things further.
- Creating a list allows you to see the bigger picture while also making sure the details are not forgotten.
- Creating lists can also save you time in the future, as you can refer to the completed lists when you catalog your academic accomplishments for promotion/tenure review. Create lists that allows you to track checked off items.
- You are less likely to be side-tracked when referring to a list.
- The act of crossing things off of your list is also extremely rewarding and can reinforce efficiency.

In the organization of your time, it is important to understand and accept that there will be instances when one task cannot be finalized without completing another task first. This needs to be considered during the organization phase to prevent wasting time and effort. Thus, you need to be able to envision all of the steps that are required to complete the task, and tasks should be listed in order of priority.

4. **Block Time.**

Next, you should schedule time explicitly towards working on the tasks necessary to achieve the goals you have prioritized. This means that your calendar should have *actual time blocked out* for drafting papers, writing grants, reviewing data, meeting with your lab, going to dinner with your partner, etc., just as for any scheduled meeting. Your clinical staff should know that this time is protected: NO, you cannot add a patient in clinic during that time; NO, you cannot add one more case during that time. To manage your time effectively, you need to OWN it. You should always block a little more time than you think you need, to allow yourself to get in right frame of mind, and prevent the feeling of being rushed. Administrative tasks such as emails, phone calls, and signing charts can fill up your day quickly and cut into your allotted research time. You should schedule separate times during the week to complete these tasks. In addition, try to group similar tasks on the same day. It is hard for anyone to write a grant when they are worried about the notes for the 17 patients they saw in clinic that morning. Therefore, try not to block your research time on the same day as your clinical responsibilities. I avoid context switching by scheduling my protected research time on Monday and Tuesday, followed by operating room and clinic on Wednesday and Thursday. This leaves Friday for catch-up and administrative tasks. By putting my research days at the beginning of the week, I can review last week's progress and results in our scheduled lab meeting on Monday, and then prioritize the experiments, data analysis, etc. that I and my staff need to do in the lab in the current week.

I block time every weekend to sit down with my significant other to review the week ahead to make sure that we have everything planned for next week. I

block time every month to sit and review my calendar and my significant others calendar for the following month to make sure we are not out of town at the same time or on call at the same time. This proactive activity is able to identify conflicts.

5. **Be Flexible.**

   Even with blocked or protected time, in some cases you will need to be flexible. There will be instances when you are on call and an emergency case comes in on your research day. Even if you are not on call, one of your postoperative patients may need your attention during the time you have blocked out to write a grant. Unavoidable personal life events will also occur. This is the reason to plan for a "buffer zone" of 1 or 2 days prior to the actual deadline to have everything completed. To prevent anger and frustration, and to address these acute and important surprises, you will need to cultivate flexibility, an important coping skill for when things go wrong. Instead of being paralyzed by disruptions and complications, you need to determine the next course of action and move forward. Remember that to be effective—be flexible.

   Finally, it is important to note that time management is not only a technique, but also a frame of mind. Your ability to deal mentally with disruptions and setbacks reduces the amount of time wasted when they occur.

6. **Have a Contingency Plan.**

   When we juggle multiple roles (surgeon, researcher, teacher, parent, etc.) there are likely to be interruptions that require immediate attention. Therefore, you should always have a contingency plan in place. For example, parents should establish a backup plan in case a regular caregiver gets sick. Since my spouse and I are both surgeons, we found a nanny who is available to take "call" on the days that we are both on call. We pay our nanny a little extra during that time, and more if she must come in. Having a contingency plan allows you to be flexible.

7. **Review Your Progress.**

   You should routinely (weekly or at least bimonthly) review your progress on various projects to ensure that you meet your goals in the desired timeframe. In other words, take a moment to examine your goals for the year (abstracts, manuscripts, lectures, grants) that are listed on the white board, or on whatever form of list you have chosen. To remind yourself to do this, add these periodic progress reviews to your organizing system of choice (calendar, list, etc.). At this time, you may need to make adjustments in your calendar to allot more time to projects that are lagging; such recalibration is appropriate and allows for maximization of your time.

8. **Utilize Resources/People.**

   You cannot and *should not* do everything yourself. Therefore, you should be intimately aware of all of the resources and people available to help you at your institution (grant support staff, editors, statisticians, graphic designers, administrative assistants, etc.) and in your region (food delivery services, nannies, babysitters, landscapers, electricians, plumbers, housecleaning services, etc.).

   All available resources should be used to maximize your efficiency and productivity. For example, the office of grants and contracts at your institution may

be able to provide assistance with biosafety or animal protocols, and there may even be boilerplate documents already available to adapt for grant applications.

By delegating tasks that would normally require your time, you can free up time for other activities. Delegation is especially important when there are others available who are better suited for that task than you are (e.g., graphic designers). If possible, have an administrative assistant add activities to your calendar (send emails with deadlines to put on your calendar) and schedule your professional meetings, travel, and lodging. Decide which tasks can also be delegated at home (someone to clean the house, mow the lawn, prepare meals, grocery shop, etc.). Organizing your daily, monthly and annual priorities in a list form will allow you determine the things that you will need to delegate.

When delegating tasks to others, it is very important to communicate the necessary components of the work that you want them to complete, so that you are not wasting their time or yours. You not only want them to carry out the task, but to do it correctly. The quality of your communication regarding the delegated task is usually directly related to the quality of work that you receive. Furthermore, you should check in with these individuals often to follow up on the progress of the work. If check-ins do not occur, is likely that you will be frustrated to find out later—often right before a deadline—that one of the delegated tasks was not done, or needs to be redone in a rush because it was not done correctly. Choose the right person for the task, provide clear instructions, and follow up on their progress often.

You should also share what you are working on or problems you are experiencing with your colleagues, family, friends, and mentor, as they can potentially provide time-saving advice for completing these tasks or addressing specific problems.

9. **Effective Communication.**

In a busy work environment, especially with electronic communication, it is easy to misinterpret rushed communication as being rude or unprofessional with their notorious unintended tone. These types of misunderstandings are a time waster. When managing your ever-growing email inbox consider the following: 1. Delete clearly unnecessary items and junk first. 2. Delegate emails that can be handled by someone else. For example, if the billing office is asking for which funding string should be used for a service you can forward this email to your lab manager to address. 3. Respond to urgent emails with a clear, concise response or call the person directly. 4. Defer emails that are not urgent or require a nuanced response to later. Emails are not time-efficient if they are misunderstood. If you are having to explain your first email you should probably call since email correspondence is not working. Also, emails are a lasting record and can be shared with others. If you are dealing with a sensitive issue a phone call or face-to-face meeting is likely more appropriate.

You should organize your emails into folders that make it easy to find them. You should delete these folders when they are no longer relevant.

Use descriptive and clear subject lines so that the receiver can triage.

Don't send emails when your emotions are high.

Be careful when forwarding emails that the email string before the message that is being forwarded is something that you want to include.

10. **Avoid Distractions or Interruptions.**

Surgeon-scientists often have competing responsibilities (clinical, teaching, administrative and familial). To use your allotted time efficiently, make an effort to avoid interruptions when working. One strategy is to try to block time early in the morning to complete activities—as the day progresses there are more opportunities for unexpected distractions. Other tricks include putting a "Do not disturb unless it is an emergency" sign on your door, turning off email pop-ups and alerts, and closing the electronic medical record (EMR) when working. It is tempting to check your email or the EMR. You should block off time for this, and stick to the activity that is scheduled during the current blocked time.

Regardless of the number of things you need to complete, if you cannot focus on the task at hand, it will not get done or it will get done haphazardly. Rather than trying to accomplish many things at once, you have to identify what is the most important at the time, and focus on that. Try to finish one task before jumping into the next. This requires using the prioritization techniques you performed previously, and blocking out the other tasks. Some rare individuals have the capacity to multitask and produce quality work, but for most of us this is not the case. Multitasking is possible with select activities to maximize efficiency; you might evaluate your progress on these tasks to determine if this is improving efficiency or not. I am a fan of checking my emails while on the elliptical machine at the gym, but obviously this should not be done while driving.

11. **Conquer Procrastination.**

This step requires self-awareness about when you are the most effective. Are you an early bird, or a night owl? You should block your time accordingly. It is also important to review your progress to make sure that your self- assessment is actually correct. When you are on a streak, try to identify the factors in your surroundings that may have enhanced your productivity. That way, you can recreate the conditions in the future. For example, I've discovered that ambient noises are very distracting to me; a white noise machine helps me focus. I am also distracted by people walking by my office, so having my door closed is helpful in me focusing on the task at hand. Learning to recognize and avoid potential distractions is also helpful.

Then there are those days when you are tired from being on call, depressed about a recent grant or paper rejection, or just don't feel like working. On these days self-motivation is key. You need to realize that the tasks at hand are the building blocks for what you are trying to achieve in your life. It could be that the task seems daunting and you don't even know where to start. This is especially common in large tasks, such as writing an NIH grant for an RFA that just came out and is due in a month. To accomplish the goal, divide it into discrete parts (specific aims page, animal section, human subjects, etc.) and then allocate time for each of these parts. You can delegate ancillary documents to administrative grant individuals so that you can focus specifically on the grant writing.

12. **Say No.**

When others recognize your productivity, you will be invited to join committees, review papers, mentor students/residents/junior faculty members, contribute to book chapters, and assist your mentors with their projects. It is important to determine if these activities will contribute to your short-term and long-term career goals. If these requests do not help meet your goals, then you should turn them down. Sometimes this can be very hard, especially if it involves your mentor, chair, or senior faculty member. But in order to have time to meet the goals you have set; you will need to abstain. In the end, there is just not enough time to do everything that is offered to you. Knowing your daily, monthly, and annual priorities in a list form will allow you to determine the things that you will need to turn down. Remember, quality of work is more important than quantity of work.

How Time Management Relates to Laboratory Research

All of the above strategies are relevant for time management in any situation, but especially when conducting laboratory research. You should set clear short- and long-term goals regarding your research program [1, 2]. These goals should then be prioritized, as it is not uncommon to have many good ideas with limited time, resources, and personnel.

Once prioritized, organize your approach to achieving these goals. This includes good communication with research staff regarding experiments: confirm that the experiments will be conducted in a correct fashion, follow up on the execution, and discuss ways to solve problems that arise. You should block time on your calendar to meet with your personnel to discuss the above; to read and review new literature regarding your area of research; to write abstracts, grants, and papers; to meet with individuals managing your research funds (I recommend quarterly); to go over research presentations with your staff; and to meet with collaborators. This blocked time should be kept separate from clinical, teaching, and administrative duties.

Given the unpredictability and frequent frustrations of laboratory research, you have to be flexible to adapt to changes in your research plan based on unexpected results, difficulties with assays or models, and problems with research personnel, funding, etc. One should also have a contingency plan for any set of experiments, anticipating potential limitations and pitfalls (a requirement of many grant submissions) to prevent wasted time when challenges occur. You should frequently review the progress of experiments and your research program to address areas that may be falling behind schedule, or unexpected results.

As much as possible, you should utilize the resources and people that are available to you at your institution. This is especially true with laboratory research, as we cannot be experts in all experimental techniques. For example, use core facilities with expertise in assays/experiments that are technically challenging (example: electron microscopy) or that require a large amount of time (example: histology core facilities for sample processing and cryosectioning).

Delegation is an important tool in successful laboratory research. You do not have the time to write grants, papers, abstracts, and to review data in addition to conducting the day-to-day activities of the lab. Therefore, weekly lab meetings should be scheduled to review the progress of the experiments and plan out the experiments and tasks for the next week, month, or year.

When it comes to writing grants and papers, it is important to avoid distractions and interruptions, to use your time efficiently, and structure your day to avoid procrastination. Finally, it is not uncommon to be asked to be involved in other research projects. If these projects will require your time and intention but are not directly in line with your research goals—a polite "NO" is advised.

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Maintaining an Effective Lab Notebook and Data Integrity

5

Andrew J. Murphy and Jussuf Kaifi

Abstract

Efficient, organized, and detailed data maintenance are the cornerstones of a successful laboratory. Furthermore, institutional and federal requirements mandate proper maintenance, documentation, and dissemination of experimental data in a way that is rigorous and reproducible. The complexity of data generated in the modern laboratory setting presents a significant challenge to these principles of proper record keeping and data integrity. This chapter will focus on the elements of the scientific method, data maintenance, and paper and electronic record keeping that can be used to facilitate successful laboratory operations for the surgeon-scientist conducting basic research. In addition, there is recent increased emphasis on measures to ensure experimental rigor and reproducibility supported by the scientific community and National Institutes of Health. This chapter will introduce the surgeon-scientist to the critical aspects of these requirements to ensure compliance with grant submission guidelines and common author instructions for manuscript submission.

Keywords

Notebook · Laboratory · Electronic · Data integrity · Rigor · Reproducibility Data archiving

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Introduction

The academic surgeon is intimately familiar with the importance of a detailed operative note. A properly dictated operative note allows the surgeon to revisit the indication, decision-making, technique, and potential shortcomings of an operation even years after the procedure was performed. The same attention to detail must also be utilized in the keeping of an effective laboratory notebook and maintaining data integrity in the academic surgical laboratory. Efficient, organized, and detailed data maintenance are the cornerstones of a successful laboratory. Furthermore, institutional and federal requirements mandate proper maintenance, documentation, and dissemination of experimental data in a way that is rigorous and reproducible. The complexity of data generated in the modern laboratory setting presents a significant challenge to these principles of proper record keeping and data integrity. This chapter will focus on the elements of the scientific method, data maintenance, and paper and electronic record keeping that can be used to facilitate successful laboratory operations for the surgeon-scientist conducting basic research.

The Challenges of Record Keeping

Data in the modern scientific laboratory may come in the form of handwritten notes, computer printouts, gel images, blots, spreadsheets, photographs, slides, computer files, and even massive electronic datasets related to genomic or proteomic investigations. These data can be tangible or electronic. Electronic data can be accessed using a wide variety of computer software. Furthermore, these electronic data can be stored on local hard drives, USB drives, or on cloud storage. The obvious challenge is to integrate these data into a decipherable system that can be shared among laboratory members and collaborators [1]. Lapsing into slack record-keeping practices can make manuscript preparation and internal or external review of scientific data problematic and can therefore directly impact the success of a laboratory. All team members must participate in rigorous record-keeping practices to optimize the reporting of confident, accurate, and transparent scientific data. For these reasons, the record-keeping practices and organizational skills of a candidate lab member should be strongly considered prior to their recruitment.

Legal Aspects

The laboratory notebook guidelines discussed in this chapter are also designed to facilitate legal determination of the exact timing of a scientific discovery in the event of a patent dispute. For much of recent history, US patent law favored a first-to-invent rather than a first-to-file approach. However, the America Invents Act, passed in 2011, aligned the USA with the rest of the world by converting to a first-to-file approach for patents filed after March 16, 2013. This law has rendered the legal utility of laboratory notebooks in patent disputes more limited [2]. However,

investigators are now encouraged to be much more proactive in filing for patents related to their research, and appropriate record-keeping practices can greatly facilitate gathering the appropriate information for the application process. Investigators should consult their institutional offices of technology licensing regarding recommendations for this process upon initiating their research program or when preparing for publication of a significant manuscript.

Paper Laboratory Notebooks

Although it is essentially impossible for a traditional paper laboratory notebook to contain the entirety of data related to an experiment in the contemporary laboratory, the notebook can still contain essential details about the scientific method and can also serve as the index of record keeping for results that are not amenable to a paper format. In fact, a paper notebook is often the easiest way to record routine research results during the actual conduct of an experiment. Each lab member should maintain their own laboratory notebook. The notebook should have bound, serially numbered pages to alleviate concerns about data omission. Loose leaf notebooks without serial numbering of pages, while convenient, can raise concerns about possible tampering of data or proper maintenance of data integrity. Pages should not be skipped or left blank. Prior entries should be referenced by page number and date when continued. Notes should be recorded in permanent ink. All entries in the notebook must be legible and in the primary language selected by the primary investigator so that they can be reviewed by all current and future lab members when necessary. The notebook should be kept in the laboratory and should never leave the institution, even temporarily. Loss or theft of data can result in catastrophic loss of productivity and progress for the scientific laboratory, and thus this point must be stressed to each new laboratory member during their orientation period. Laboratory notebooks should be backed up by photocopying or scanning when possible. Laboratory notebooks should not depart with trainees when they have completed their involvement in the laboratory. Rather, departing trainees can make copies of their laboratory notebooks when appropriate. Laboratory notebooks are the property of the laboratory and institution. Completed notebooks should be kept in a designated, secure location within the laboratory for a specified number of years.

The bound notebook should contain a table of contents at the beginning, and each experiment should be dated and entered in chronological order. The title, primary investigator and co-investigator names, purpose, hypothesis, and methods should be explicitly written in the notebook at the beginning of an experiment. Specific reagents should be listed in the description of methods along with the manufacturer and catalog numbers for future reference. Reagents from collaborators or other investigators should be noted for proper acknowledgment on publication. Recipes for buffers or other reagents should be explicitly written into the notebook at least once. The best practice is to write out the experimental methods in granular detail once they have been established and may be generalized to a laboratory protocol [1]. A collection of laboratory protocols should also be kept separately within

the laboratory for the benefit of all members. Future experiments can simply reference the detailed entries of methods when appropriate.

Experimental results and impressions should be catalogued in the laboratory notebook on an ongoing basis [1]. Keeping results in the notebook often involves taping or gluing images of gels or other data into the notebook. In addition to the cataloguing of raw data in this manner, orienting comments and information should be written into the notebook to ease data review after time lapses or when revisiting a project. Make sure to label all gel images in the notebook at the time they are placed onto a page. An effective way of maintaining proper research results is to summarize the day's work and results into a paragraph in the lab notebook at the end of each day, accompanied by a written brief experimental plan for the next day.

Paper laboratory notebooks cannot possibly contain the entire spectrum of research results, particularly those from instrument-generated computer files and other large electronic datasets. However, the pages of the laboratory notebook can be used to refer to file locations and computer programs which can be used to review the referenced data. In the contemporary laboratory, most every notebook page should explicitly refer to an electronic file location in which related results are stored. These references should be confirmed by the primary investigator or laboratory manager. The ideal file location is on a password-protected, shared drive accessible to all appropriate individuals in the lab rather than on personal or single hard-drive storage. Automatic and regular backing up of files should be utilized at regular intervals (daily and automatic if possible).

For industry and federally regulated laboratories, recorded experiments require signatures by the researcher and a witness. The witness is typically someone who is not directly related to the project but can understand the work that is presented. The witness can attest to the authenticity of the research performed, which is extremely important should legal defense of research integrity become necessary. While this is typically not formally required or practical in an academic setting, it is good practice for the primary investigator or laboratory manager to personally review and sign off on all lab members' notebooks on a scheduled basis (either biweekly or monthly). During review and sign off, the primary investigator or laboratory manager should also make sure that all references to electronic data locations have been recorded properly and can be readily accessed. At the time of manuscript preparation, all data pertaining to a manuscript should be kept in a distinct file that is easily accessible or linked to the manuscript.

Electronic Laboratory Notebooks

As mentioned above, large "omic" or high-throughput electronic datasets have essentially rendered the traditional paper laboratory notebook obsolete as a comprehensive record-keeping method. An electronic laboratory notebook (ELN) allows for consolidation of fragmented laboratory records into a single interface which may also directly link to large electronic datasets [3]. ELNs can be "specific ELNs" that are designed to work with specific applications, instruments, or

data types, or they can be "generic ELNs" designed to support access to a variety of data types and information needs. A generic ELN aims to function similar to a modern-day version of the traditional laboratory notebook. ELNs may exist as web-based, free and open-source, or proprietary software. Some ELNs function similar to a blog, while others aim to represent an evolved interface based on a traditional lab notebook [4]. ELN software can be designed specifically for the management of scientific data, or more general note-taking software such as OneNote (Microsoft, Redmond, WA) or EverNote (Evernote Corp, Redwood City, CA) can be used as an ELN [5]. ELNs are also called by a variety of acronyms including ERN (Electronic Research Notebook), ERMS (Electronic Resource Management System), LIMS (Laboratory Information Management System), and SDMS (Scientific Data Management System). LIMS and SDMS are software that may aim not only to replace the traditional laboratory notebook but also to address broader laboratory management, inventory, cataloguing of experimental protocols, and sample storage issues [6]. Comprehensive lists of ELNs, associated costs, and product reviews are publicly available, and some are listed at the end of this chapter.

The goal of nearly all ELNs is to document scientific data in a centralized, legible, easily searchable, historically accurate, and legally rigorous fashion. ELNs also can promote secure collaboration among investigators or lab members sharing the same program. The ELN can facilitate review of the laboratory record by the primary investigator or laboratory manager. A properly implemented ELN can decrease errors and potentially reduce total laboratory costs. Because data can be accessed or generated using a variety of electronic devices including computers, scientific instruments, and handheld devices, ELNs offer a secure way to integrate data between these devices using a single record. The ELN eliminates the need to remove the paper laboratory notebook from the laboratory. ELNs allow for easier backing up of data and enable efficient importing or exporting of data to facilitate collaborations or review. In industrial labs involved in medical device or drug development, ELNs are subject to Food and Drug Administration (FDA) regulations 21 CFR 820 and Title 21 CFR Part 11 which pertain to data security and integrity [7]. These regulations do not apply directly to academic laboratories, but similar assurances and protections should be sought from the manufacturer of the ELN being considered for use in the laboratory.

ELN or LIMS also can offer the ability to integrate sample information and management into daily experimental notes. Samples can be catalogued and recorded within an experimental protocol. This can allow the investigator to quickly link to the data sheet for a reagent being used. The sample age, aliquots, and other background information can be readily referenced during the experiment. For example, the investigator can pull up the data sheet for an antibody, determine the number of remaining aliquots, and determine its expiration date readily during an experiment. These features of LIMS can be used to catalog and organize freezers and link to this information during the experiment. This can save time, prevent running out of reagents, and reduce the need to look through sample boxes to find an intended reagent [8].

Although ELN use has increased in the scientific industry and despite the numerous advantages of ELNs, a small portion of academic scientific researchers currently utilize this format. Identified barriers to adoption of the ELN include budgetary concerns, time needed for conversion to an electronic format, disruption of existing workflow, concerns about data security, superfluous features, and software being offered in a limited number of languages. Concerns regarding Information Technology (IT) support also have limited adoption of ELNs [9]. Furthermore, because so many varieties of ELN exist, many academic researchers are concerned about adopting a particular type of ELN that is not universally utilized across their institution, thereby limiting the ability to share or transfer data. Therefore, adoption and support of the ELN may need to be considered on the departmental or institutional level to address these concerns.

Archiving of Scientific Data

New trends in data availability are improving the sharing of scientific data among investigators across the globe. Funding agencies and an increasing number of high-impact peer-reviewed publications are requiring that primary scientific data be made available to the scientific community. For routine experiments with limited datasets, uncropped or primary images from which figures were generated are often required as supplementary files to be submitted at the time of peer review. Thus, it is important to keep track of the location of all unprocessed, raw data even after figures are generated. Spreadsheets containing raw data may also be required to accompany manuscript submission. These files may appear as online supplemental files associated with the primary publication. For larger "omic" datasets, requirements often mandate that raw data be deposited into a publicly available repository such as the database of phenotypes and genotypes (dbGAP; National Center for Biotechnology Information (NCBI), Bethesda, MD) for sequencing data or the Gene Expression Omnibus (GEO; NCBI, Bethesda MD) database for gene expression, array, and functional genomic data [10, 11]. Specialized reagents, plasmids, novel cell lines, patient-derived xenografts, and genetically engineered mouse models may also be required to be made available to the scientific community either directly from the investigator or by deposition of the model system into a commercially available repository such as Addgene, The Jackson Laboratory (Bar Harbor, ME) or ATCC (American Type Culture Collection, Manassas, VA) [12, 13]. A statement attesting to the availability of data or reagents known as a data sharing plan is required for NIH grant submission and often required when a manuscript is submitted to a journal for publication [14]. Keeping reagents, models, and data meticulously organized is therefore critical in light of these requirements.

Rigor, Reproducibility, and Data Integrity

A 2016 survey of 1576 researchers by the journal *Nature* determined that more than 70% of scientists have tried and failed to reproduce results from another laboratory and more than half have failed to reproduce their own work [15]. Moreover, 52% of

researchers felt there was a significant crisis of reproducibility in the scientific community [15]. The scientific community, scientific journals, and grant funding agencies now refer to this widespread problem as the "reproducibility" or "replication crisis." Key factors contributing to the scientific reproducibility crisis include: (1) Increasing complexity of contemporary science including complex experimental and computational data, (2) The advent of artificial intelligence (AI), and (3) Over reliance on the classic $p = 0.05$ measure of statistical significance as the single figure of merit.

Scientific journals and funding agencies have adjusted policies and requirements to address this crisis of reproducibility and ensure the integrity of scientific data in the published literature. The National Institutes of Health (NIH) revised the language of review instructions for research grants and mentored career development awards to promote rigorous and transparent research. While these changes are specifically designed to address rigor and transparency in NIH grant applications and progress reports, by extension these guidelines are indicative of increased emphasis on rigor and reproducibility by the scientific community overall [16, 17]. The NIH guidelines contain four areas of focus: rigor of the prior research, scientific rigor (design), biological variables, and authentication (Table 5.1) [18]. These areas must be specifically addressed in each grant proposal to receive funding. It is worthwhile for the surgeon-scientist to consider each of these areas when designing and conducting research projects or applying for federal funding. Additionally, the NIH and journal editors from over 30 preclinical scientific journals including *Science* and *Nature* reached consensus in 2014 on required principles and guidelines for reporting preclinical research (Table 5.2). Ultimately, the desired product is data with integrity and that which most closely approximates the truth.

The rigor of the prior research for a grant application or research project is derived from prior published research or preliminary data justifying the scientific question and supporting the application. The investigator should consider and specifically discuss the strengths and weaknesses of the rigor of the prior research when prioritizing projects for grant application or scientific inquiry. If the rigor of the prior research is weak, researchers may be tempted to over-interpret or misinterpret data to align with preliminary findings and concur with a faulty scientific premise. A research career with long-term success cannot be based on a faulty premise, and thus it is important to consider and openly discuss the quality of observations that constitute the background or preliminary data for any research endeavor. As such, an explicit discussion of the rigor of the prior research is required in the Significance and Approach sections of the Research Strategy for NIH grant proposals.

Scientific rigor (design) is the stringent application of the scientific method to ensure robust experimental design and eliminate bias from the interpretation and reporting of results. Thus, the objective, hypothesis, specific aims, long-term goals, inclusion and exclusion criteria, experimental methods, variables, statistical methods, potential problems, and alternative approaches should be determined and documented a priori. Plans to repeat experiments should be made from the outset of the project. If possible, results should be confirmed by two or more experimental assays (orthogonal approaches). When appropriate, formal statistical power calculations

Table 5.1 NIH requirements for rigor and transparency

Area of focus	Description of requirement	Section of grant
Rigor of the prior research	Careful assessment of the rigor of prior research with identification of weaknesses Describe strengths and weaknesses in rigor of prior research. Describe plans to address weaknesses in rigor of prior research that serves as key support for proposed project	Research strategy Significance Approach
Scientific rigor (design)	Scientific rigor is the strict application of the scientific method to ensure robust and unbiased experimental design, methods, analysis, interpretation, and reporting of results. Emphasize how experimental design, methods will achieve robust and unbiased results	Research strategy Approach
Biological variables	Sex, age, weight, underlying health conditions are critical factors affecting health or disease. Describe how relevant biological variables, including the above are factored into research design, analysis, and reporting in vertebrate animal and/or human studies. Strong justification is required if proposing to study only one sex.	Research strategy Approach
Authentication	Key biological and/or chemical resources: cell lines, speciality chemicals, antibodies, other biologics Describe methods to confirm the identity and validity of above resources in proposed study. Authentication plan should state in one page or less how key resources will be authenticated including the frequency.	Other research plan section Include as an attachment Do not include in the research strategy

Adapted from "Enhancing Reproducibility in NIH Applications: Resource Chart" https://grants.nih.gov/policy/reproducibility/guidance.htm

should be employed to determine the number of replicates or animals that should be used for a given experiment [17]. These calculations are also often required by institutional review boards and animal care and use committees.

The research plan should also explicitly consider relevant biological variables, including sex. Sex is a biological variable that was historically not sufficiently considered in studies using vertebrate animal models or cell lines. This resulted in a paucity of data in the basic science literature related to sex-based differences in disease development, disease phenotype, and treatment responses. Specifically, both male and female mice should be included in an experimental design unless there is an overwhelmingly strong scientific justification (e.g., the study of ovarian development or testicular cancer) for limiting the study to only one sex. Cell lines established from both males and females should also be utilized when available [19, 20]. In addition, other variables including age, weight, and underlying medical conditions should be appropriately considered when they could be relevant to the scientific question.

Authentication of key resources is important to ensure that proper, intended reagents are being utilized for experiments and that they are achieving their targeted

Table 5.2 NIH Principles and Guidelines for Reporting Preclinical Research

Recommended journal requirements	Description
Rigorous statistical analysis	Provide guidelines for statistical analysis. Check statistical accuracy of submissions when appropriate
Transparency in reporting: Core standards	Eliminate unreasonable limitations on length of methods sections. Provide a submission checklist including core standards to authors
Standards	Encourage or require use of standards in scientific community (nomenclature standards, animal research reporting standards such as ARRIVE guidelines)
Replicates	Indicate number of times experiments repeated. Distinguish between biologic and technical replicates
Statistics	Require full reporting of statistics: Test used, value of *N*, mean, median, SD, SEM, confidence intervals
Randomization	State whether samples were randomized and methods used for randomization
Blinding	State whether investigators were blinded to group assignment during outcome assessment
Sample size estimation	Report power analysis if used. If not, describe how sample size was determined
Inclusion and exclusion criteria	Indicate criteria for exclusion of any data or subjects
Data and material sharing	All de-identified datasets must be made available upon request. Recommend submission of data to public repositories and link between manuscript to this data. Encourage submission of all data within manuscript or as supplemental material. Encourage sharing of software and code.
Consider refutations	If a paper is published, the journal must consider publication of refutations subject to the same standards
Best practice guidelines	
Image-based data	Journals encouraged to establish explicit guidelines for image-based data in author instructions
Antibodies	Report source, characteristics, dilutions, and validation methods
Cell lines	Report source, authentication, rule out mycoplasma contamination
Animals	Report source, species, strain, sex, age, breeding strategy, inbred and strain characteristics of transgenic animals

ARRIVE Animal Research: Reporting of In Vivo Experiments; *N* number of samples; *SD* standard deviation; *SEM* standard error of the mean
Adapted from https://grants.nih.gov/policy/reproducibility/principles-guidelines-reporting-preclinical-research.htm

chemical or biological effect. These resources must also be free from contamination. Authentication is particularly important for cell lines, specialty chemicals, antibodies, and other biological reagents used for experiments. Both the method and frequency of authentication should be specified at the outset of a project. Short tandem repeat DNA profiling of all cell lines should be performed at the outset of an experiment and after significant passaging has occurred to ensure the profile matches the

parent cell line [21, 22]. Cell lines should be tested to ensure absence of mycoplasma contamination [23]. Genetically modified animals or cells should be validated by confirming the genomic alteration using PCR amplification or other genotyping assays. Chemical reagents that are key to the research plan should be obtained from reputable manufacturers and/or also may need to be validated by liquid or gas chromatography or mass spectrometry. Validation of antibodies involves showing that the antibody is specific, selective, and reproducible in the desired experimental context with appropriate positive and negative controls. Adopting algorithms for antibody validation can facilitate this process in the laboratory setting [24].

Efforts to identify the scope of the reproducibility crisis and improve it are contributing to the growth and advancement of a field of study called metascience. Metascience is defined as the use of the scientific method to study science itself. Many of the specific above recommendations pertaining to rigor, reproducibility, transparency, and reporting of results were developed to address issues uncovered by metascience [25]. In addition, metascientific efforts have resulted in initiatives for pre-registration of scientific studies and clinical trials. Pre-registration of scientific studies before they are conducted involves submitting a registered report to a journal, which is then evaluated for publication according to theoretical justification, experimental design, and the planned statistical analysis [26]. Although this is not a common contemporary practice for most basic science literature, the purpose is to reduce publication bias (e.g. not publishing negative results) and it may become more widely adopted in the future. In addition, the evolution of the field of metascience has led to the founding of organizations such as the Enhancing the Quality and Transparency of health research Network (EQUATOR) who issue guidelines for methodology and reporting standards that apply to a wide array of scientific studies and are adopted or recommended by an increasing number of scientific journals [27].

An emerging challenge in scientific reproducibility is the advent of artificial intelligence (AI) and machine learning [28]. Machine learning and other types of AI are powerful computational tools that can identify patterns in data that may not be recognized by conventional methods or tools used by human researchers. Although AI is undoubtedly poised to significantly advance almost every scientific discipline, it is already contributing significantly to the reproducibility crisis. Machine learning classification algorithms are typically developed using a "training" or "discovery" set of data and then confirmed using a second set of data known as a "validation" set. One emerging problem and significant challenge to reproducibility is data leakage, in which there is insufficient separation between the training and validation datasets that leads to an erroneous classifier which can then not be reproduced externally. Due to the complexity of AI algorithms, data leakage is often unbeknownst to the investigators. The issue of data leakage is compounded by generative AI approaches such as Large Language Models (LLMs) which have the ability to create new data (text, images, etc) using models derived from their training data. Checklists have been developed and adopted by scientific journals regarding the use of AI and machine learning to combat these issues, although significant challenges remain unaddressed and unsolved [28].

Overreliance or sole reliance on the p-value as the single measure of statistical significance has contributed to the scientific reproducibility crisis. Although a detailed discussion of p-values and statistics is beyond the scope of this chapter, there are several consistent features of overreliance on p-values that can contribute to poor scientific reproducibility. First, depending on the data type and application, a p-value of 0.05 may not be a particularly rigorous threshold because it implies there is a 5% probability the null hypothesis could be falsely rejected. The more tests performed, the more likely the null hypothesis is falsely rejected. Second, because of the emphasis placed on p-values, a practice deemed "p-hacking" in which multiple varied (often similar) hypotheses are proposed and tested until one of the test reaches the threshold of $p = 0.05$ can lead to erroneous interpretation of scientific findings and failure of reproducibility [29]. Third, the cutoff of $p = 0.05$ is somewhat arbitrary and, for example, rejecting results with a p value of 0.051 with a large effect size or accepting results with a p-value of 0.049 with a small effect size could lead to accepting false positives or rejecting false negative results. In response to the above and other related issues, the American Statistical Association issued the following statement in 2016, which is directly relevant to data reproducibility: *"Good statistical practice, as an essential component of good scientific practice, emphasizes principles of good study design and conduct, a variety of numerical and graphical summaries of data, understanding of the phenomenon under study, interpretation of results in context, complete reporting and proper logical and quantitative understanding of what data summaries mean. No single index should substitute for scientific reasoning."* [30].

Conclusions

The complexity of data generated in the modern scientific laboratory setting presents a significant challenge to the principles of proper record keeping and data integrity. Efficient, organized, and detailed data maintenance are the cornerstones of a successful laboratory. These processes may be facilitated by a variety of electronic laboratory notebook or lab management software designed to accommodate and manage the large, complex datasets and procedures typical in today's laboratory. A data sharing plan including appropriate archiving of large datasets is often required for federally funded studies and by many high-impact journals. There is now a sustained, increased emphasis on measures to ensure experimental rigor and reproducibility supported by the scientific community and National Institutes of Health. The academic surgeon-scientist conducting basic research can benefit from stringently incorporating these measures into their research projects from the outset of their career. Requirements pertaining to data archiving and data integrity should be reviewed when planning applications for funding or manuscript submission.

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NIH Website for Rigor and Reproducibility

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Statistics for Bench Research

6

Timothy W. King

Abstract

Success in academic surgery requires a basic understanding of statistics. Without this understanding a surgeon-scientist will not be able to interpret scientific articles, develop a hypothesis, or analyze the data they collect in their research program. The purpose of this chapter is to provide a brief overview of the statistical methods Statistical methods which are used in bench and clinical research.

Keywords

Statistics · *t* test · Type 1 error · Type 2 error · ANOVA · Correlation · Parametric Nonparametric

Introduction

The use of statistics is a fundamental part of any research program. Without statistics, a scientist is unable to appropriately interpret their data. Therefore, in order to be a successful scientist, a thorough understanding of statistics is critical. While a thorough review is beyond the scope of this text, the goal of this chapter is to give you a basic overview of the statistics needed for success in your laboratory and provide resources to further explore this topic as it pertains to your research.

Since there are multiple statistical computer programs currently available, it is very easy to enter data into a program and erroneously select a statistical test which

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provides a "significant" result. Thus, before you ever start an experiment, you need to formulate a hypothesis. Your experiment will then test this hypothesis. The "null hypothesis" assumes that there is no difference between the two groups, and the "alternative hypothesis" assumes that a difference exists. You are trying to prove the alternative hypothesis. Formulating your hypothesis will define what the controls and variables of the experiment are and will lead you to the statistical analysis that is most appropriate for that experimental dataset.

The first step of the analysis is to understand the types of data and variables that you are investigating. Therefore, the first question to ask is what types of data are you collecting?

Types of Data

There are two types of data: continuous and discrete. Continuous data are quantitative and can have any value within a defined range (e.g., age, heart rate, tumor size). There are three types of discrete data, which are all qualitative:

1. Nominal: the data falls into defined categories with no implied order (e.g., eye color, gender, race).
2. Binary: the data is a yes/no and is usually coded as a 1 or 0 (e.g., disease present (diabetes/no diabetes), family history of cancer).
3. Ordinal: the data falls into defined categories with an implied order (e.g., tumor staging, GCS score).

For example, if you were studying melanoma, Breslow depth would be a continuous dataset and Clark staging would be an ordinal discrete dataset. Applying a discrete statistical analysis to the Breslow depth would be inappropriate.

Once you have defined the type of data, you need to determine the independent variable(s) from the dependent variable(s). The independent variable is what you control and change in the experiment, while the dependent variable is what happens as a result of the change you made (e.g., a drug treatment would be the independent variable, and the survival after treatment would be the dependent variable).

Descriptive Statistics

Descriptive statistics are what we use to analyze the data that has been collected. Commonly, we then use this data as a sample of the whole population to make generalizations about the populations from which the samples were drawn. This is called inferential statistics. If we want to make inferences about a population, then it is important to ensure that the sample we are analyzing is a fair representation of the population we want to study. Measures of central tendency help us determine if this is true.

The mean (average), median (middle value), and mode (most common value) are three commonly used measures of central tendency. Determining the distribution of the data is also very important as this will determine the statistical analysis that you used to analyze that data. If the data follows a "bell-shaped curve," you can conclude that it has a normal distribution. In a normally distributed (or parametric) data, the mean, median, and mode will all be the same value (Fig. 6.1). The mean can be affected by the sample size and outliers within the data. As the sample size increases, the effect of the outliers will decrease. If the data is not normally distributed, it is considered skewed. A positive-skewed dataset has the long tail of the curve on the positive side of the peak. This is also called a right-skewed distribution and will have the mean to the right of the median. A negative-skewed (or left-skewed) distribution will have the long tail on the negative side of the peak, and the mean will be to the left of the median.

It is also important to measure the variability of the data. The *range* is the difference between largest and smallest values. In a normally distributed dataset, the variance (S^2) is a measurement of dispersion and is derived by taking the difference of each individual variable (x) from the mean, squaring this difference, summing all the differences together, and then dividing the sum by the total number of data points (n). Mathematically, it is represented by the following formula:

$$S^2 = \frac{\sum(x - \text{mean})^2}{n}$$

The standard deviation (SD) is defined as the square root of the variance, and the standard error of the mean (SEM) is the standard deviation divided by the square root of the n. SEM is commonly used in calculating confidence intervals and in studies with a smaller sample size. For a normally distributed dataset, 68% of the data will be within one SD of the mean; 95% will be within two SDs of the mean;

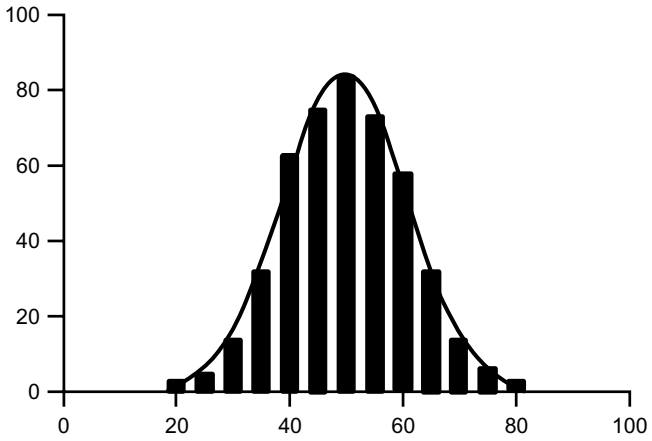

Fig. 6.1 A normally distributed bell-shaped curve. The mean, median, and mode are all equal to 50

Table 6.1 Summary of Type 1 and Type 2 errors

Study results	Truth/reality	
	Difference (null hypothesis false)	No difference (null hypothesis true)
Difference (Reject null hypothesis)	Correct decision (Power)	Type 1 error (α)
No difference (Accept null hypothesis)	Type 2 error (β)	Correct decision

and 99.7% will be within three SDs of the mean. If the data is not normally distributed, then it should be analyzed with nonparametric methods.

Type 1 and Type 2 Errors

When testing a hypothesis, there is a risk that the wrong conclusion is made from the interpretation of the data. The probability of a scientist concluding that a difference exists and in reality it does not (i.e., incorrectly rejecting the null hypothesis in favor of the alternative hypothesis) is called a Type 1 error (α) (Table 6.1). The probability that the scientist concludes that a difference doesn't exist when in reality it does (i.e., incorrectly failing to rejecting the null hypothesis) is called a Type 2 (β) error. In general, a Type 1 error is set at 5% and a Type 2 error is set at 20%.

Sample Size and Power

The power of a study is the probability of detecting an effect when there is an effect to be detected. It is defined as $1 - \beta$. In general, scientists want the power of a study to be at least 80%. As the power of a study increases, the risk of making a Type 2 error, or concluding there is no effect when, in fact, there is one, decreases. The power of a study is directly related to the sample size because the larger the sample size, the smaller the SEM. Power also depends on the how much of a difference the investigator is trying to detect. If you are trying to detect very small difference, you will need a very large sample size to find that difference. This is important to determine before beginning an investigation to ensure that the study is feasible. For example, if you were trying to detect a small difference in tumor growth (e.g., 2 mm) after a drug treatment, you may need a very large sample size which might make the study impractical or too costly to do. However, if you changed the outcome to be a moderate difference (e.g., 2 cm), the sample size would go down significantly while maintaining the same amount of power, and the study could be done.

Data Analysis

p Value

The p value is the probability of concluding a difference exists due to chance. In most studies the p value is set at <5% (<0.05). This means that 5% of the time when we see a difference it is due to random chance and not due to a true difference. If the p value is <0.05, then we would conclude that there is a statistically significant difference.

Confidence Interval

The confidence interval (CI) is another common way to display your data. A 95% CI means that 95% of the values are approximately ±2 SD of the mean. A 95% CI is calculated as the mean ± 1.96 × SEM. For example, if the mean survival rate was 9 years and the SEM was 2, the CI would be (5.08, 12.92). This indicates that while we measured the mean survival to be 9 years, we are 95% confident that the real value is between 5.08 and 12.92 years.

Statistical Tests

Table 6.2 shows a list of the most common statistical analysis performed in the surgery literature, and Fig. 6.2 shows a flowchart to help determine which statistical analysis to use. We will now review each of these tests:

t Test/ANOVA/Mann-Whitney *U*/Kruskal-Wallis Test

A *t* test is used to compare one variable between two groups when the data is normally distributed and continuous. A *t* test compares the means between two populations. A paired *t* test assesses the significance of mean difference between paired data variables within a sample population. This is commonly used in drug treatment experiments as the before treatment and after treatment are paired for each individual. The significance of mean difference measured by the *t* test is dependent upon sample size and standard deviation within each data group.

If you have three or more groups being compared, you would need to perform an analysis of variance (ANOVA) test . Like the *t* test, an ANOVA compares the mean, it just does it for multiple groups. ANOVA can include factors between or within study subjects or experimental samples.

Table 6.2 Most frequently used statistical tests used in the surgical literature

Test	Application
Chi-square test	Nonparametric; used to compare frequencies between groups at baseline (i.e., percent male)
Logistic regression	Model; used to determine odds of an event occurring, controlling for confounders (i.e. odds of death); often used in the analysis of observational studies
Student's *t* test	Parametric; used to compare group means at baseline (i.e., age); used for analysis of normally distributed data
Fisher's exact test	Nonparametric; used to compare frequencies between groups at baseline; often used as an alternative to the chi-square test when frequencies are small
Mann-Whitney *U* test	Nonparametric; used to compare population distributions between groups at baseline using medians (i.e., length of stay); used for analysis of non-normally distributed data
Kaplan-Meier curve	Nonparametric; used to summarize time-to-event data (i.e., 5-year disease-free survival)
Log-rank test	Nonparametric; used to compare survival distributions between two samples (i.e., time to event)
Cox proportional Hazard model	Model; used to summarize time-to-event data, controlling for covariates (i.e., assess the effect of risk factors on disease-free survival time)
Analysis of variance (ANOVA)	Parametric; used to compare means at baseline (i.e., age) when there are >2 groups; used for analysis of normally distributed data

Adapted from Williams et al. [6]

A Mann-Whitney *U* (or Wilcoxon Rank Sum) is the "*t* test" for data that is not normally distributed but is continuous and independent. Rather than compare the means (like a *t* test), this test compares medians. This reduces the impact of outliers in your data. If the data is paired (dependent), then you would use the Wilcoxon Sign Rank test . The ANOVA equivalent for data that is not normally distributed is the Kruskal-Wallis test . It is used to compare the ranks across three or more groups.

Chi-Square/Fishers Exact Test

If the data are binary, the most commonly used test for statistical analysis is the chi-square test. The chi-square will test if two categorical variables are related in some population. The null hypothesis of the chi-square test is that there is no relationship between the categorical variables in the population. An example research question that could be answered using a chi-square analysis would be does smoking cause lung cancer? The chi-square for this is shown in Table 6.3. The proportion of smokers that develop lung cancer is $\frac{A}{A+C}$, and the proportion of non-smokers that develop lung cancer is $\frac{C}{C+D}$. If smoking causes lung cancer, the proportions should be different. Using a chi-square table, you can also calculate the odds ratio (OR).

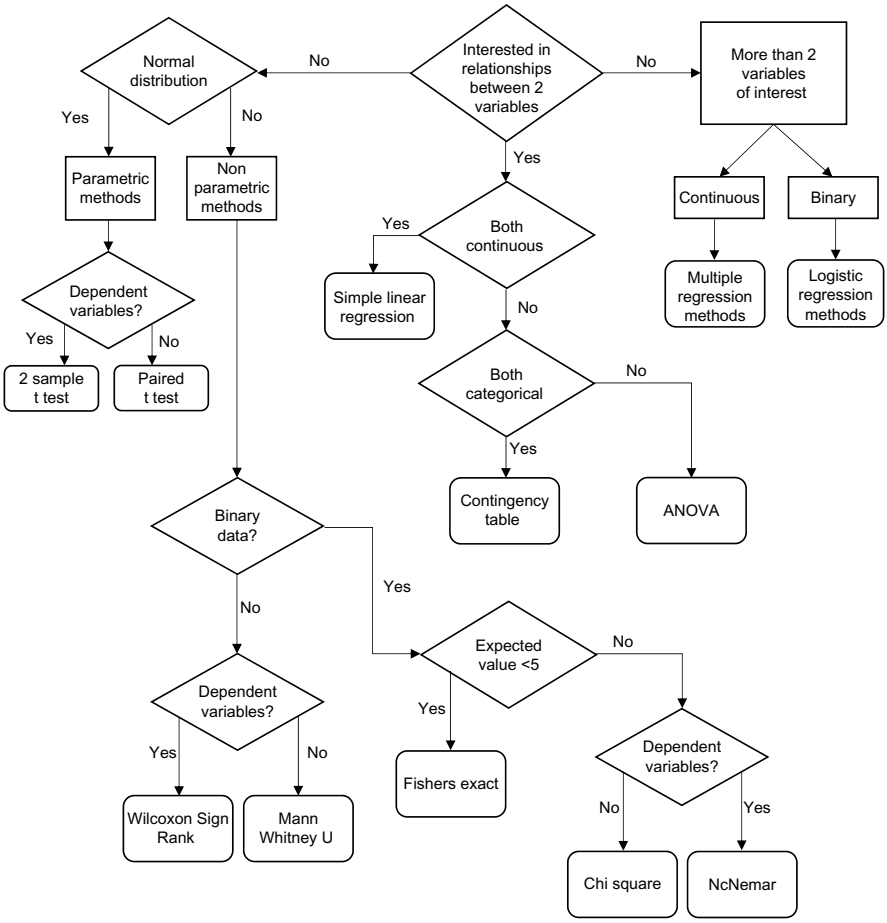

Fig. 6.2 A flow chart for selecting the appropriate statistical method. (Adapted from Cassidy 2005)

Table 6.3 A contingency table (chi-square) for smoking and lung cancer

Smoker	Lung cancer	No lung cancer	Total
Yes	A	B	$A + B$
No	C	D	$C + D$
Total	$A + C$	$B + D$	$N\ (A + B + C + D)$

The OR measures the association between an outcome and a treatment/exposure, or, in our example, a comparison of lung cancer given two different groups (smoking vs. no smoking). The OR is calculated as $\frac{AD}{BC}$. If the OR = 1, then there is no difference between the groups (i.e., the odds of getting lung cancer are the same in smokers and non-smokers). If the OR is above 1, this is statistically significant.

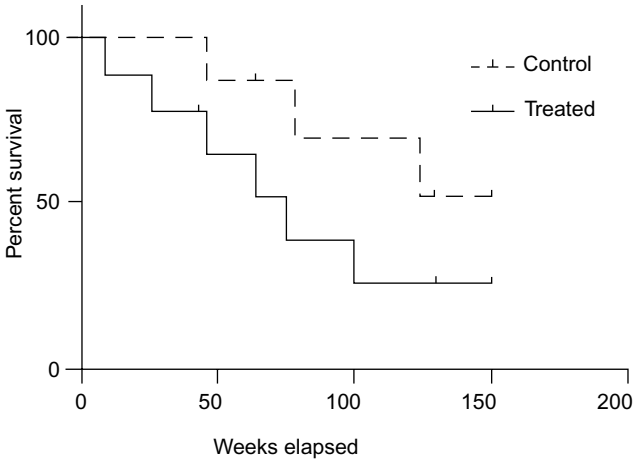

Fig. 6.3 A Kaplan-Meier survival curve

It is important to calculate the expected value for each cell in the table. The expected value is the totals for each row and column of that cell multiplied together and then divided by the *N*. Thus, for cell *B* the expected value would be $\frac{(A+B)(B+D)}{N}$. If the expected value is less than 5 for any cell, then the sample size is too small for the chi-square analysis, and the Fisher exact test should be used. If the data are dependent, then the McNemar test should be used.

Kaplan-Meier Curve/Log-Rank Test/Cox Proportional Hazard Model

The Kaplan-Meier curve is one of most common tests used to measure the fraction of subjects living for a certain amount of time after treatment (Fig. 6.3). The log-rank test is used for comparing the survival of two or more groups (such as different treatment groups in a clinical trial). The log-rank test only assesses the effect of one variable at a time on prognosis. If there are multiple variables that need to be evaluated, the Cox proportional hazard model should be used.

Correlation and Regression

Correlation indicates the degree to which two variables are linearly related to each other. A positive correlation means that as one variable gets bigger, so does the second variable. A negative correlation means that as one variable gets larger, a second variable gets smaller. The correlation coefficient (ρ) quantifies the strength of the relationship between the two variables and exists between -1 and 1. If $\rho = 0$, then

there is no correlation. If $\rho = -1$, then there is a perfect inverse linear relationship (and, likewise, if $\rho = 1$, that represents a perfect positive linear relationship).

Linear regression analysis is used when one variable is dependent upon another variable. Linear regression models the relationship between two variables by fitting a linear equation to the observed data. For example, a scientist might relate the drug dose to serum drug concentration of individuals using a linear regression model. Multiple regression is conducted to determine the outcome variable with changes in multiple input variables. This is commonly used to predict a patient's prognosis based on several variables such as age, sex, cancer stage, family history, etc. Logistic regression analysis is applied to a binary outcome with multiple variable inputs. For example, the risk of death for a patient with a lung cancer diagnosis (yes/no) could be modeled with the input variables of the patient's age, number of years smoking, stage at diagnosis, family history of cancer, etc. From this model predictions can be made. Logistic regression can generate an OR, *P* values, SD, and 95% CI. Using the lung cancer model above, you might determine that the OR for smoking is 4 and the CI is (3, 5). This would mean that patients who smoke are four times more likely to die of lung cancer than non-smokers and that we are 95% sure that the true OR is between 3 and 5. Since the OR is above 1, this is statistically significant.

Sensitivity/Specificity

Sensitivity is the ability of a test to correctly classify an individual as have the disease, while specificity is the ability of a test to correctly classify an individual as disease-free. A contingency table as shown in Table 6.4 should be created for analysis. Referring to Table 6.4, the sensitivity and specificity of a diagnostic test is defined as:

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}} \qquad \text{Specificity} = \frac{\text{TN}}{\text{TN} + \text{FP}}$$

A test with 90% sensitivity will identify 90% of patients who have the disease, but will miss 10% of patients who have the disease. A highly sensitive test can be useful for ruling out a disease if a person has a negative result. The acronym widely used is SnNout (high *Sen*sitivity, *N*egative result = rule *out*).

A test that is 90% specific will identify 90% of patients who do not have the disease. Tests with a high specificity (a high true negative rate) are most useful when the result is positive. A highly specific test can be useful for ruling in patients who have a certain disease. The acronym is SPin (high *Sp*ecificity, rule *in*). Thus, in

Table 6.4 Sensitivity and specificity

	True state	
	Has disease	Does not have disease
Test positive	TP	FP
Test negative	FN	TN

TP true positive, *FP* false positive, *FN* false negative, *TN* true negative

general, you would want a screening test to be highly sensitive and then a secondary confirmation test to be highly specific.

The positive predictive value (PPV) is the percentage of patients with a positive test who actually have the disease, and the negative predictive value is the percentage of patients with a negative test who do not have the disease. These are mathematically defined as:

$$\text{PPV} = \frac{\text{TP}}{\text{TP} + \text{FP}} \quad \text{NPV} = \frac{\text{TN}}{\text{TN} + \text{FN}}$$

Conclusion

Statistical analysis of your scientific data is very important to the success of your laboratory. However, if the wrong statistical methods are used, the conclusions you draw may be invalid. Therefore, it is critical that you formulate a valid hypothesis, collect your data accurately, and understand your data well so that you can select the appropriate statistical methods to use. In addition, it is important to consider bias, confounding factors, and clinical relevance when evaluating your data. Finally, it is highly recommended that a biostatistician be involved in your complex investigations from the beginning to ensure the appropriate study design, data collection, and analysis are done.

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Ethics in Laboratory Research

7

Dae Guang Chung Kim and Luke Brewster

Abstract

Ethics in laboratory research has become a formalized training course, and the reasons for this are multiple. Given the multiple unique pressures experienced by surgeons, the concepts taught in these ethic courses are particularly relevant. Here, the concepts will be discussed in a case-based format including issues highly relevant to surgeons.

Keywords

Ethics · Integrity · Objectivity · Reproducibility · Honesty · Surgical scientist · Openness

Introduction

Ethics in lab research is a traditionally important topic that has recently become more formalized. Resnik has recently presented a summary on this topic with useful links to federal resources, including the National Institutes of Health (NIH), National Science Foundation (NSF), and others [1]. Here, he delineates several themes that

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are relevant to this chapter. The most pertinent ones to laboratory research reside under the umbrella of research/scientific integrity and include the following subheadings of Honesty, Objectivity, Carefulness/Reproducibility, Openness, Animal Care, Human Subjects' Protection, and Responsible Publication.

This chapter will focus on these topics with particular attention to how they may impact developing scientists and physician-scientists in the surgical field. We will use fictional cases that are purposefully absurd to illustrate the ethical problems, and then we will present some tools or solutions for investigators to consider. Finally, we attempt to add salt to these important topics that can be perceived as stale, because research ethics, like medical ethics, is done best when the laboratory work itself is done well.

A Personal Introduction to Research

The thrill of discovery hits us all at different times in our lives. For me, it began in a research methods seminar during my undergraduate education. We read and presented landmark papers in the scientific literature, including the seminal publication on DNA by Watson and Crick [2]. While I was attracted to the rigor, logic, and discovery of scientific research, it was the mentorship of these professors, and that of my future surgeon-scientist mentors, that led me to be a surgeon-scientist.

Thus, I want to emphasize to the readership, that more than the words describing appropriate conduct, mentors can and do set the tone and provide parenteral teaching to mentees for scientific integrity and discovery. While research experiences can help build our mental acuity and may provide the building blocks of future surgeon-scientists, they may also attract others into the privileged field of surgery.

Mentoring

Case Story

Before your lab's weekly meeting, your mentee comes to you in frustration. You ask him to present the data that he has been working on for the last few weeks, but he reluctantly discloses that he does not feel comfortable presenting the data. When you ask him why, he states that he has had trouble analysing the data correctly as he is not confident in his interpretation of the tissue slides.

1. You tell him to look at these papers from the lab to help him and set up a follow up meeting.
2. You empathize with him and set aside time to meet and review the slides to help him understand better the histology and tissue architecture.

A one-on-one meeting can be less pressuring for the mentee to share issues than a lab meeting. While the scenario above has the mentee coming to you, this may be uncomfortable for them. I myself have underestimated the reticence of some

learners with regard to sharing data in a peer environment. This can lead to frustration of both parties and undermine the fabric of the lab community. For mentees who conduct a research procedure for the first time, it is understood that they will make mistakes and need to perform analyses multiple times. The way mentors respond to the mentees' errors can shape the emotional perspective mentees have towards their research and themselves. Thus, it is imperative for mentors to create a lab culture that fosters growth rather than stress and pressure for perfectionism.

The mentor-mentee relationship is a special bond. Mentors play a crucial role in shaping their mentees, scientifically and ethically. In ancient times, the choice of where you received your education was akin to entrusting your soul to another. To some degree, this still holds true. The goal of many mentees is to use their research experiences to assist them in the next directed step forward for them (e.g., medical school or a certain fellowship), while the mentor wants their assistance in developing a research project and furthering their life's work, not in a selfish way but in the way a child carries on the family name. These mentors are better found than assigned [3], but exceptions surely exist. Some characteristics of a good and beneficial mentor-mentee relationship include having open communication and the mentor enabling such discussions. This environment is enabled by clear expectations and mutual respect between the two parties.

In addition, the education/formation of moral character and what is ethically correct and incorrect are important lessons for the mentor to teach the mentee [4]. Educating developing scientists and/or medical students about the methods of research and manuscript writing may also impact scientific misconduct. Daniele Fanelli mentions early-career scientists should receive proper education and training on implementing research and conduction research data in appropriate methods because they are most likely to receive retractions. In a recent research study on cheating students, there have been five implications to why students chose to cheat: first was stress of strong performance, second is very high stakes for his/her position, third is extrinsic motivation, fourth is low success rate, and lastly peer culture with accepting of cutting corners to achieve desirable results [5]. Obviously, a strong mentor can help one avoid all of these perils.

Research Integrity

As I entered the laboratory during my general surgery training, I received a book entitled *Scientific Integrity* [6]. In preparation for this chapter, I have reviewed this book and my notes, and I ask the readership to consider reading the Nuremberg Code of Ethics and the Declaration of Helsinki (has multiple updates). I will attempt to highlight key points in the relevant sections, but these documents are well constructed and may be useful to readers as they begin to understand the structural underpinning of research protocols and protections.

Case Story

A person who had left your mentor's laboratory just as you entered submitted a manuscript for publication based on work done in the lab, but without any of the lab members as authors. To make matters worse, there are several authors from another institution that were most certainly not involved. You know all this because a journal editor asked you to review the manuscript as your expertise overlaps in this research field.

- You do the review based on the science and notify the editor that you (and others from the lab) should be included as authors.
- You do something else.

There are a number of ethical problems with this scenario and doing something else is required. First, even if the person did work on the paper, there are shadow authors, omissions of relevant authors, and the omission of proper funding source and senior authorship. So there is a lot to deal with here.

My mentor had something along these lines occur to him, so we turned it into an ethics' case for more general discussion [7]. My mentor alerted the journal editor, and the submitting institution was alerted. We never heard from the submitting author.

"Integrity is what you are when no one else is looking." I cannot find the origin of this quote but it is nicely illustrated by the following event. Bobby Jones was a famous golfer in the first half of the 1900s. During the 1925 US Open, his club inadvertently touched the golf ball prior to his formal stroke. By all accounts, there was no advantage gained by him from this act, and his caddy was the only other witness of this event. This caddy reported later that because no advantage was gained, the act did not require a penalty, nor did it need to be reported to the Marshall. To add pressure to this scenario (like that of a critical experiment for an important paper), Bobby Jones was in contention to win the US Open that year; he was or would become champion of this tournament in 1923, 1926, 1929, and 1930. As you may have guessed, he chose to ignore the caddy (who was not correct in his interpretation of the rules) and do the "right" thing. He knew the rules and the rules required reporting the event and taking the penalty. He went on to lose the tournament, and that penalty was the difference in the score. When word got out about his truthfulness, he was congratulated broadly for his honesty, he responded that "you may as well praise a man for not robbing a bank [8]." "Integrity is what you are when no one else is looking."

Honesty

Science, as an enterprise producing reliable knowledge, is based on the assumption of honesty [5].

Case Story

You cannot get a Western blot to work right. Sometimes the control blot works, other times, the experimental protein works, but they never seem to work at the same time. Your PI is frustrated with you because he has not seen data from you in over a month. He asks you to bring the Western blots to the lab meeting. You make a beautiful powerpoint presentation and use the best of the best pictures to illustrate the control and the experimental protein, but you do not tell him of your problems with the assay.

- You are to be congratulated, you have brought a new level of groundsmanship to the lab years.
- You should report to your PI what you did and ask for help in performing the experiments properly.

Below, we will discuss some tools to assist developing investigators maintain honest approaches in their work. Lüscher proscribes investigators that "(1) trainees need supervision by an experienced mentor; (2) the results obtained by researchers should be discussed regularly at research meetings with presentation of the raw data; (3) all authors should carefully read and approve papers they are involved in; (4) ethical and animal research approval should be available and checked by a responsible person in the department at the beginning of each study; (5) certified courses on good clinical practice and animal experimentation for clinical and basic researchers should be mandatory; (6) creation of an ethical board composed of a few wise elder statesperson for the journal family be devoted to the promotion of proper scientific conduct; and (7) last, but not least, we must stress that science is a commitment to honesty and the pursuit of truth—nothing else."

If an investigator follows these simple instructions, not only is honesty fostered but the research retains its value. These practices save time and money and may help alleviate some of the stress of not obtaining the right result. In our case study, the young investigator has a technical issue that a more experienced technician may be able to point out. If he were to ask for help, he may be seen as a problem solver rather than a problem child. Bringing the raw data to the lab meeting would also prevent the discomfort and frustration he would otherwise experience the first time he presents "partial" results in the lab meeting.

Objectivity

Case Story

You think you see a pattern in the data that is quite exciting. If there is a pattern, you have a pretty good paper that is innovative and may get you to the podium at an important meeting. You start "looking" at the data. By simply deleting some of the lower value points in the treatment group and removing one of the high values in the

control group, you have a statistically significant result. You decide to bring this methodology to a statistician in your department and see if these data points can be removed.

- While it seems like you are fishing around to cherry pick data, the use of an unbiased statistician is scientifically sound.
- You should not go to the statistician (and simply make the pattern work) because they do not understand the physiology behind the experiment and the deleted data points look like outliers to you.

Statistical analysis and data interpretation are crucial and integral components in research. There are various data interpretation tests available to investigators. However, science should not search out the *P* value; rather the *P* value helps provide investigators and the scientific readership with an objective likelihood of the results being non-random. The *P* value is not magic, nor does it tell you that something is true [9]. The use of a statistician can be very helpful to researchers rigorously testing their data but also in designing future experiments to best test their hypothesis. As surgeons, laboratory experiences can be exceedingly important to our training [10]. While not always obvious, surgeon-investigators are becoming more rare. As such, we surgeons need to be as thoughtful in our statistical analyses as we are in our experiments. This includes proper use of statistical methods and not simply relying on what we know or think we know [11].

Rigor and Reproducibility

Case Story

Your best friend "in the whole wide world" shares with you that his new protein stops angiogenesis. He says the paper (8 years of work) is almost accepted, and it will change the world for many patients. You study angiogenesis, and he gets you some of the protein. After the paper is published, you test this protein in your angiogenesis assays. It does not work.

- You should keep performing replicate experiments until it works.
- Call him a liar and stop being his friend.
- Look at his protocols and see where the differences may be.

The NIH have made reproducibility a priority in funded research [12]. The lack of reproducibility of an assay/test/experiment has been encountered by all of us with gray hair. This is why a single promising experimental result is not included in publications. Sometimes the reasons for this are clear, other times they are not. Certain approaches, like blinded histology scoring and replicating results are common and help improve scientific rigor. Well-done science that does not yield the expected result can be scientifically impactful to those who keep their eyes open [13]. Sloppy

science is simply sloppy, and those who employ this sloppiness are not only poorly utilizing precious funding resources but also staining the scientific reputation of their laboratory. These actions can lead to retractions and infamy [14].

Transparency

Case Story

Your spouse is a famous scientist with several successful commercial ventures, and you both have retained distinct last names. You are publishing a paper using one of his/her protocols that has recently been licensed. You are asked to put relevant disclosures on the disclosure form. Since you only know about the licensing from her (and it is not public knowledge), you can keep this information to yourself for now. Further, it is unlikely that anyone will put it together that your spouse was involved because of the different last name.

- That approach is easily defensible in today's world.
- You should ask the journal's editor whether this is a relevant disclosure for this manuscript.

Since conflicts of interests occur in science [15], the proper method of handling this is to disclose any conflicts of interest in a standardized and habitual manner. This allows decisions to be made by the editors or deciding body with the best information possible. Some conflicts can be managed (disclosure or otherwise); others cannot. Importantly, not all conflicts are financial. Conflict is not always obvious and may be subtle [16]. Here, we will keep the discussion about those that can be managed, as the handling of disclosures that cannot be managed is a much more complicated process and beyond the scope of such a chapter.

Without proper disclosure, one's reputation is in peril. Recently, the newspapers have suggested that the conflict of interest disclosures from employees at a prominent hospital were not adequate or reliable (sometimes included/other times not included). This hospital has subsequently started a task force to ensure proper disclosure and investigate protocols when conflict precludes moving forward [17]. Unfortunately, the under-reporting or variability in reporting may be more widespread than one would think [18]. Given the relative ease of the public discovering your conflicts of interest from open access reporting, one would think that this behavior could be intelligently modified by both authors and journals. This may seem a bit farfetched to a young investigator, but it is important to verify with your co-authors when submitting your work for publication. Here, either you or your PI should ensure that all authors have listed their disclosures.

The disclosure, mitigation, or recusal due to conflict of interest also applies to those judging the work (study sections, manuscript reviewers, etc.). In order to minimize bias, there have been recommendations to doubly blind reviewers from manuscripts and grants [19]. As with any simple solution, it does not typically fit the

needs of a complex problem. An alternative approach is the open review process, in which all information would be transparent. This unblinding of the review would likely be helpful to the authors [19], who could use this information to suggest names for future reviewers, but it may impair the completeness of the reviewers' response. Blinded reviews on the other hand lack transparency and can hide potential bias.

Protection of Human Subjects and Animal Research

As these are politically charged times, it is probably no surprise to readers that human and animal subject research often is discussed in the public arena along strongly emotional lines or presented as sensational events. However, if you do things right, you should be able stay out of the papers.

Animal Research

Proper animal care is an important part of reproducible research. While there are federal rules that assist institutions, in general, mammalian animal research regulations are divided according to whether the animals are rodents or something else.

Regardless of the animal used, the local animal use committees are vital to your research success. As such, I have become "that" investigator who goes to them both in advance to an amendment but also sticks close to them afterward to be sure it works. The paperwork involved and time commitment needed from a young investigator can be excessive, so one must guard against complacency in this work. I have found a free and downloadable resource that comprehensively outlines proper procedures for working with your animal use committee and performing operations on animals [20]. Further reading suggestions for the Animal Welfare Act (rodents and NIH guidance cited here) [17]. For those of you more bold or interested in this subject, Degrazia has tried to moderate the academic discussion and provide some stimulation of further thoughts and action [20, 21].

Human Subjects Research

Much of the codification of human research norms came from notorious events. Thus, it is not surprising that the Nuremberg Code came from the atrocities of the Nazi regime with regard to experimentation on certain ethnic groups including Jews and Gypsies. It may be surprising that the USA did not sign onto this code. Our country has a number of scandalizing events that are similarly notorious. One of these is the Tuskegee Syphilis study, which started prior to antibiotic therapy but continued for 42 years. This was a scandal as the patients did not have access to readily available proper antibiotic care. This led to the Belmont Report, which is another foundational document in medical ethics. These landmark documents

continue to occupy and frame discussions today [22–24]. This infamous event led to the Belmont Report, which continues to influence research norms today.

Current approaches to human research are largely legalistic and center on a patient consenting to the protocol. Certainly, the degree of risk inherent to the protocol is important to the internal review board (IRB) process. This is fairly prevalent in the literature, and since these IRBs can be local or central, I leave it to the readership to learn more on this important topic.

A more challenging ethical scenario for young surgeon-investigators is properly representing yourself as an investigator to the subject. Here, the patient may know you as part of the care team, and as such he may not understand your role as an investigator, but rather just see you as his surgeon. As such, the patient he/she believes this is part of his medical decision plan. This is very difficult to combat. In these circumstances, I will discuss the idea of the research protocol with the patient (as my patient), and then if they are interested, have our research coordinators discuss the specifics of the protocol and consent process with him (as a research subject). If it is only me, I will clearly state that this is research and not part of the clinical plan. Further, I explain that I will care for them regardless of whether they participate or not. Similar guidance is available from the American Medical Association [25].

Responsible Publication and Reporting Corrections

Case Story

During the final revision on the paper that took you months to complete, you reminisce about all the arduous work you put into each step of your research and decide to look back at your initial data files. While glancing through the data, you realize that certain numbers portrayed in the final draft do not align with the data you collected. Upon further inspection, you realize that certain data files had very similar names, causing you to use the same numbers for the analysis of multiple experimental groups by mistake.

1. You ignore the error, include the erroneous data in the paper, and keep that a secret to your grave.
2. You inform your PI of this error and present corrected data to her prior to the next laboratory meeting.

Certainly, it is attractive to get the paper submitted. One could simply state, at any point in the future, that they were unaware of the problem with the data; it is certainly difficult for another to know one's heart. However, the mentee in this case did find the error, and contrary to what he or she may think, the author group would much rather find out an error before submission than afterwards. As such, A+ work would include not only alerting the PI, but also having the corrected data available for review at the same time.

While research misconduct can be committed by any personnel working in the lab, it may be difficult to know the best way to proceed when you suspect that someone else is conducting fraudulent research, or even worse, if you know you did. Research on this topic suggests a slightly higher prevalence of reporting misconduct when it was non-self (14%) compared to self-reporting (2%) [11]. While not enviable, I have benefitted from internal audits of our research protocols. These have certainly allowed me to avoid such hazards in my laboratory.

Retractions are indicators of scientific misconduct, and there have been an increase in retractions since the 1990s [14]. Again, Fanelli's work not only has linked retractions to science misconduct but also to data fabrication, plagiarism, and falsification of data [26].

Be honest, be objective, be careful, be open, provide sound conditions for animal and human research, and engage in responsible publication of your work. These are in many ways, the best days of your life, spend them in the best manner possible.

Conclusion

Scientific integrity is not a theoretical idea, but the result of sound research processes. Mentors and mentees are the lifeblood of scientific progress, and the long game is the only game in town. Research is a privilege that is well paired with surgeons. Let no one tell you otherwise. When you know right from wrong, do right, no matter the cost. If you are confused or conflicted, be honest and open. Use your mentors, statisticians, and others without a dog in the fight. Their objectivity will help allay your concerns and protect the integrity of your work.

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Modern Techniques for DNA and RNA Assessment

8

Mehves Ozel, Jurgis Alvikas, and Matthew D. Neal

Abstract

Advances in molecular and cellular biology have led to an in-depth understanding of the basic mechanisms of life and disease. Elucidation of principles of genetic information storage in DNA, transcription of this information into RNA, and translation of RNA into proteins have led to an exponential growth of techniques useful for screening, diagnosis, prognostication, and treatment. In a wide range of medical and surgical specialties, many of these techniques are already standard of care and are used daily. In an emerging model of precision medicine, data from DNA and RNA of an individual patient will be used to guide their care. While much progress has been made, our understanding of majority of surgical diseases is incomplete. An academic surgeon with a career of scientific pursuit must have a thorough understanding of the current state of laboratory techniques for DNA and RNA and of the knowledge gaps that future investigations should address. This chapter reviews the basics of genetic, genomic, and transcriptomic analyses.

Keywords

DNA · RNA · Sequencing · Chromosome · Chromatography · Southern blot Northern blot · Single cell sequencing · CRISPR/Cas

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Introduction

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the building blocks of hereditary information. The transmission of DNA from generation to generation, its transcription to RNA, and translation to proteins form the basis of molecular biology. The understanding of the flux of information from DNA to RNA to protein and eventual function have revealed the universal molecular processes of life. At the core of many diseases, derangements somewhere along this pathway have been identified, from inborn errors in the DNA, to errors arising from dysfunctional transcription, to pathologic translation of the proteins or their aberrant function. Each of these impairments is an opportunity to develop assays for early diagnosis, to inform patients of their prognosis, and to devise more effective treatments.

Deoxyribonucleic Acid (DNA) Assessment

DNA Introduction

At the most basic level, DNA is composed of chains of repeating modular 2-deoxyribose sugar moieties and nucleotides, named adenine, cytosine, guanine, and thymine, connected by phosphodiester bonds. In contrast to RNA (discussed later in this chapter), DNA lacks a 2′ hydroxyl group and therefore maintains greater stability and is less susceptible to damage from environmental forces (i.e., ultraviolet (UV) light, heat), making it more suitable for long-term genetic storage. DNA was identified as the basic building block of human genetic code, and its structure was described in 1953 by Watson and Crick. Decades of research revealed the human genome to be composed of 20,000–30,000 genes, and its sequencing, completed in 2001, was one of the major scientific achievements of the twentieth century [1–3]. DNA sequencing, or determination of its exact nucleotide sequence, was made possible by the development of techniques such as the chemical degradation method and chain-termination sequencing in the 1970s [4, 5]. Each gene has a complex network of promoters, regulatory sequences, enhancers, and silencers that regulate its expression across different stages of development and across different tissues. The double helix structure of DNA means it has two polymers of DNA running in opposite directions and connected by weak hydrogen bonds allowing the two strands to be separated for transcription or duplication. This property is exploited in many investigative techniques [6].

In humans, DNA is organized into 23 pairs of chromosomes which are modified and tightly packaged into *chromatin* inside a cell's nucleus. Chromatin contains both DNA and various proteins which together greatly influence gene expression. Only a small fraction of the entire genome is expressed in any given cell. The expressed genes determine the function of the cell and how it interacts with its environment. Methylation of cytosine bases can inactivate segments of the genome that are not needed for a particular cell type. Inactive DNA is further condensed into non-expressed regions of the chromosome with the help of *histones*, specialized

DNA-binding proteins, and other components of chromatin remodeling complexes. DNA also has many other protein interactions that allow for its storage, unwinding, transcription, and duplication.

Measuring and Identifying DNA

Southern Blot

Developed by Edwin Southern and published in 1975, the *Southern blot* allows for identification of specific DNA sequences. Purified DNA samples of interest are digested by endonucleases into smaller fragments, separated by size using gel electrophoresis, and transferred onto nitrocellulose or nylon membranes. DNA is then permanently attached to the membrane by either heat or UV radiation to decrease its loss in subsequent steps. Labeled hybridization probes of either complementary DNA or RNA are then used to detect sequences of interest on the membrane [6]. Imaging of hybridization and detection of results are dependent on the labeling techniques used during probe development. Typically, probes are labeled with radioactivity, fluorescence, or chromogens and can be detected by autoradiography or phosphorimaging and fluorescence or color change, respectively. Although it has been supplanted by techniques described below, Southern blot can be used for screening of DNA libraries in homology-based cloning when the amino acid sequence of a protein is known. A recently developed dual labeling technique has allowed for detection of two regions simultaneously [7]. Southern blotting is additionally useful for detection of methylated DNA [8].

DNA Sequencing

Chemical Degradation Sequencing

Chemical degradation sequencing, developed by Maxam and Gilbert in 1977, at its core relies on chemical modification of DNA and cleavage at specific base sites [4]. This technique allows purified samples of double-stranded DNA to be sequenced without the need for further cloning. One of the 5′ ends of the DNA to be sequenced is radioactively labelled, and a complex chemical treatment is used to create small breaks in the DNA with the concentration of the chemicals used dictating cleavage. The fragments of DNA are then separated by gel electrophoresis, developed by autoradiography, and the sequence determined by inspecting the image. Major limitations of this technique are the need for radioactive labelling and the complexity of the protocol.

Chain-Termination Sequencing

Chain-termination sequencing developed by Sanger and colleagues in 1977 [5] is relatively easier and more reliable. Automation and development of DNA sequencers have made this the main methodology of sequencing in most systems. The basis of chain-termination sequencing is the stopping of DNA chains using

chain-terminating nucleotides or dideoxy-nucleotides that lack the 3′ hydroxyl group. Early chain-terminating sequencing methods involved four reactions, which required single-stranded DNA templates, DNA primers, DNA polymerase, unmodified nucleotides of which one is labelled, and modified chain-terminating nucleotides. The use of the chain-terminating nucleotides allows for fragments of DNA of various lengths to be produced. The random incorporation of chain-terminating nucleotides as well as four separate reactions with the four separate modified nucleotides ensures that all combinations of termination will be produced. Fragments generated are subsequently denatured by heat and separated by gel electrophoresis with four reactions run in parallel. Fluorescent labelling allows the original DNA template sequence to be read following the size of fragments across the gel. Since its development, chain-termination sequencing method has become even easier and more reliable [9–12].

Massive Parallel Sequencing

Massive parallel sequencing is a more recent and currently the most widely used DNA sequencing technology. It incorporates many of Sanger's original techniques and includes several novel ones. They are known as *next-generation sequencing*, *high-throughput sequencing*, or *second-generation sequencing* and include pyrosequencing, reverse dye terminator sequencing-by-synthesis (SBS), sequencing by chained ligation, sequencing by unchained ligation, ion-sensitive SBS, and single-molecule sequencing [13].

These techniques vary in their biochemical approach, but their principles are similar. Randomly fragmented segments of DNA from the sample of interest are ligated to a platform-specific adaptor at the end. The adaptors allow for a solid surface use of a polymerase to amplify the attached fragmented DNA, although some of the techniques rely on solution-contained amplification. Amplified fragments are then spatially arranged on arrays before sequencing. Most of the sequencing processes automate a series of enzyme-dependent biochemical steps to achieve fluorescent readouts of the sequence, though newer platforms have the ability of electrical detection [13]. In older sequencing protocols, biochemical reactions themselves were the rate-limiting step, but the use of the newer platforms has shifted the rate-limiting step to data preparation and analytics. These technologies can generate orders of magnitude more data than the original Sanger techniques. Advances in computing and information technology have helped make analysis of this data more efficient.

Massive parallel sequencing platforms all have similar principles but vary in their strengths. For example, pyrosequencing has long read lengths and high sequencing capacity making it ideal for de novo sequencing, re-sequencing of prior genomes, and metagenomic studies [14–18]. Reversible dye terminator SBS platforms offer shorter read lengths and are suitable for re-sequencing, transcriptome sequencing, and sequencing of DNA fragments from protein-DNA interfaces as well as full mammalian genomes [19–23]. Sequencing by chained ligation platforms has low error rates, short read lengths, error correction systems, and massive data output and can therefore be effectively used in

re-sequencing projects and transcriptome sequencing [13, 21, 24, 25]. Finally, ion-sensitive SBS offers the advantage of electrical detection for sequence analysis which increases sequencing speed [26–28] and is currently well suited for sequencing of smaller bacterial genomes and target sequences of larger genomes but has the potential to sequence entire human genome in the matter of hours in the future [29, 30].

Whole Exome Sequencing

Massive parallel sequencing technologies are a powerful tool for study of disease and, clinically, for diagnosis of genetic disorders. However, while whole genome sequencing, as described above, can be used to generate a large amount of genomic data from which aberrant genes can be deduced, the effects of these aberrations of a patient's phenotype are not always clear. This is because, although a patient may have mutations in the critical genes, the phenotype depends on the gene expression. To investigate the expression patterns in samples of interest, *whole exome sequencing* was developed [31].

Briefly, this technique starts by obtaining genomic DNA samples, just like whole genome sequencing, followed by enrichment of exons. Several enrichment strategies are commercially available and include both array-based [31] and in-solution capture [32]. The enrichment increases the amount of exome DNA in the sample relative to non-exome genomic DNA. The sample is then sequenced using next-generation sequencing methods, and computational methods are used to derive the exome sequence.

Applications of the *whole exome sequencing* technology are an active area of research. It has been used to find rare variants in complex diseases with multiple derangements at single base-pair, gene, or pathway level [33]. Another application is diagnosis of patients with rare Mendelian disorders and identification of the mutations that underlie them [34].

Whole exome sequencing is a pragmatic approach to search for genetic aberrations in areas where such abnormalities are most likely to result a phenotypic change. This technique, although powerful, will likely be replaced by whole genome sequencing, as the latter becomes cheaper and bioinformatic tools for analysis improve.

Third-Generation Sequencing

The most recent advance in DNA sequencing is the development of *third-generation sequencing*, also known as *long-read sequencing methods*. These techniques include *single-molecule real-time (SMRT)* and *nanopore sequencing* and allow a study of genomes, transcriptomes, and metagenomes and direct study of different DNA base modifications [35]. While next-generation sequencing techniques are very powerful, one of the major drawbacks is their inability to read long sequences. Fracturing a genome into sequences that are too long for next-generation platforms to sequence will lead to errors in final sequence assembly. Another limitation is the reliance of next-gen methods on PCR amplification, which can be difficult to efficiently perform on regions with high GC percentage.

SMRT requires less starting DNA, produces substantially longer read lengths, eliminates the need for PCR amplification, has faster turnaround times and can investigate individual DNA base modifications. Nanopore sequencing also has much longer read lengths, with up to 1 Mb reported in recent studies [36], does not require PCR amplification, and, thanks to new sequencer hardware technology, can be extremely small and portable as well as high throughput [35]. Although currently it still has high error rates [37], it is a promising technology that is likely to be in widespread use in the future.

In conclusion, DNA sequencing remains a field of intense investigation and progress. While many new sequencing technologies are emerging, challenges remain in pre-sequencing preparation and post-sequencing data analysis and storage. Automation, bioinformatics, and computer science are rapidly advancing fields that will be required for fully streamlined handling of the vast amounts of data produced by DNA sequencing.

CRISPR/Cas9 Genome Editing

DNA sequencing is cheaper and easier today than it has been even few years ago, and a vast amount of genomic data is either available to the public or is easily attainable for research purposes. The understanding of the exact base-pair sequences and their function helps researchers design experiments with precisely targeted DNA modifications. Classic molecular cloning techniques have performed this function for decades, but a new technology, called *CRISPR/Cas9*, can modify DNA with unprecedented ease and precision [38].

First described in 2012, *Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9* technology utilizes a system that confers adaptive immunity against viruses to prokaryotes [39]. It relies on an RNA-guided endonuclease, known as *Cas9*, to cut a specified DNA target. This guide RNA can be engineered to be complementary to virtually any DNA sequence allowing cleavage of DNA at sites of interest with single base-pair accuracy. After cleavage, existing genes can be removed, or new ones added, in an application termed *genome editing*.

Genome editing has already been adopted across a wide array of disciplines. In biology, individual cell lines or model organisms can be engineered to study the genes or diseases of interest. In biotechnology, crops, such as rice, wheat, or tobacco, can be grown more efficiently and more resistant to pest. In medicine, there is the potential to perform gene editing in both germline and somatic cells. This has generated enthusiasm that *CRISPR/Cas9* may have far-reaching biomedical applications, including genetic screening [40], gene therapy for genetic disorders [41], cancer [42], HIV [43], malaria [44], and others. However, because this technology can genetically alter human embryos and early fetuses, scientists, physicians, and politicians have raised bioethical concerns regarding its use in both the USA and the European Union [45, 46]. The laws governing genome editing are still in their nascence and will undoubtedly change as the public is educated and gains interest in the subject.

CRISPR/Cas9 is a versatile and powerful technology with wide-ranging impact. In medicine, it holds potential to advance our knowledge and offer novel therapeutic options. Surgical specialties, such as surgical oncology [47] and transplantation [48], may soon see the results of *CRISPR/Cas9* applications . Physicians, including the surgeon scientists, will need to balance what this technology is capable of in theory and in what circumstances it should actually be utilized in the clinical setting. *CRISPR/Cas9* is covered in-depth in Chap. 11.

Assessment of DNA-Protein Interactions

In the cell, DNA functions in constant collaboration with multiple diverse proteins. It requires histones for storage, transcription factors for transcription and helicases, and polymerase and ligases for duplication and repair, to name just a few. Techniques to assess the interaction between DNA and proteins are essential research tools for the surgeon scientist. Multiple methods have been developed for this assessment. In this section, we will review structural assays, bandshift assays, chromatin immunoprecipitation (ChIP-based methods), DNA footprinting, and computation prediction tools.

Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assays (EMSA), or *bandshift assays*, have become a standard technique for analyzing the potential of a specific DNA sequence to bind protein [49–51]. EMSA is a relatively simple, quick, and sensitive in vitro way of testing DNA-protein interaction on both qualitative and quantitative ways. This method is based on the theory that protein-bound DNA or RNA will have less mobility through an electrophoresis gel. The specific nucleic acid-protein interactions can then be investigated with radioisotope-labelled DNA probe. Combined with protein identification techniques, EMSA becomes even more robust. Though no longer a cutting-edge technology, modern EMSA can be combined with protein identification to create a high-throughput DNA-protein interaction assay platform [51].

Deoxyribonuclease (DNase) I Footprinting

DNase I footprinting is a valuable method for locating protein binding sites on DNA. Cleavage sites on a DNA molecule that are bound by a protein will be protected from DNase action. The DNA specimen of interest will be fragmented by DNase treatment at the unprotected cleavage sites but not the regions of DNA that are in association with proteins. Gel electrophoresis and autoradiography are then used to detect the binding sites [52]. DNase I footprinting can determine the individual binding curves for protein-binding sites on DNA. This method also allows for qualitative extraction of DNA-binding proteins since proteins can be protected from degradation by bound DNA. Quantitative DNase I footprinting is one of the few methods able to resolve the cooperative protein-DNA binding affinities.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is another well-established technique for investigation of DNA-protein interactions. This method is based on treatment of living cells with chemical cross-linking compounds that covalently link proteins to each as well as to their surrounding DNA targets. After the cross-linking, chromatin is extracted and fragmented. Cross-linking is then reversed, and the protein-DNA complexes can be isolated by specific antibodies against the target proteins. RT-PCR is then used on the associated DNA fragments to determine the specific sequences [51, 53]. This method is well suited for studying transcription factors, interactions between DNA sequences, as well as modification status of associated chromatin.

Combination of ChIP assays with microarray platforms, also known as *ChIP-chip* or *ChIP-on-chip*, can be used to survey the entire genome for DNA-binding sites for a particular protein of interest [54]. The use of ChIP-chip methods has helped find transcription factor-binding sites, investigate epigenetic modifications, and study a variety of factors involved in DNA repair and replication. Another technique involving ChIP combines it with next-generation sequencing, the so-called *ChIP-Seq*, and offers greater sensitivity and higher fidelity for genome-wide analysis compared to ChIP-chip platforms [55]. Multiple computer algorithms have emerged for the analysis of ChIP-Seq datasets improving accuracy and statistical power and reducing false positive rate. Additionally, developments that allow the identification of multiple proteins on a single DNA sequence have enabled investigation of histone modifications and higher-level chromatin changes associated with gene transcription [51].

Single-Cell ChIP-Seq

Single-Cell ChIP-Seq [56] is a sequencing technique that enables the investigation of protein-DNA interactions and allows for genome-wide mapping of histone modifications at the level of individual cells. Traditional ChIP-Seq methods involve the pooling of millions of cells, which can mask cellular heterogeneity and obscure rare or transient protein-DNA interactions. Single-Cell ChIP-Seq addresses these limitations by allowing researchers to analyze chromatin states and protein binding events in single cells.

Sample preparation starts with individual cell that are isolated and fixed to preserve the protein-DNA interactions within chromatin. Fixation is essential for maintaining the spatial organization of chromatin and preventing dissociation of proteins from DNA during subsequent steps. Antibodies specific to the protein of interest are used to selectively immunoprecipitate protein-DNA complexes within each cell. This step captures the protein-bound DNA fragments and enriches them for sequencing with PCR amplification to prepare the immunoprecipitated DNA fragments for sequencing using next-generation sequencing platforms, generating high-throughput data containing information on protein-DNA interactions at single-cell resolution.

Single Cell Assay for Transposase-Accessible Chromatin Using Sequencing (scATAC-Seq)

The ATAC-Seq [57] method is a genome-wide next-generation sequencing-based assay that characterizes chromatin states in individual cells. ATAC-Seq is used to identify regions of the genome that have open chromatin states that are generally

associated with sites undergoing active transcription. The sequencing of open chromatin regions that are being transcribed can lead to the identification of transcription factors that are active in the phenotype or conditions being investigated.

ATAC-Seq leverages the activity of the prokaryotic Tn5 transposase enzyme, which can only access open chromatin regions. The transposase inserts sequencing adapters into accessible regions of DNA, capturing fragments of chromatin that are not bound by nucleosomes. These accessible regions correspond to regulatory elements such as promoters, enhancers, and transcription factor binding sites. By sequencing the DNA fragments, researchers can map and quantify chromatin accessibility across the genome at the level of individual cells.

ChIP-Seq and ATAC-Seq are both next-generation sequencing techniques used in epigenetics research, but they serve distinct purposes and provide different insights into chromatin biology. The differences are displayed in Table 8.1.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

A novel method of studying DNA and protein interactions is *systematic evolution of ligands by exponential enrichment (SELEX)* . It is a biochemical technique that rapidly selects sequences of oligonucleotides with appropriate high affinity to a given molecular target out of a vast library of randomly generated oligonucleotides [58]. SELEX begins with preparation of a library of oligonucleotides that have the potential of interacting with proteins. This library is then incubated with a target ligand, either a protein or a small organic compound, and bound and unbound oligonucleotides are separated. Bound oligonucleotides are then amplified by PCR, in case of DNA, or RT-PCR followed by in vitro transcription, for RNA. A SELEX round is defined as a single cycle of binding, selection, and amplification. After multiple SELEX rounds, the amplified oligonucleotides can be selected for sequencing.

Table 8.1 Next-generation sequencing techniques

	Target	Input material	Antibody dependency and detection throughput
ChIP-Seq	Specific proteins such as transcription factors or histone modifications bound to DNA	Requires a relatively large amount of starting material, usually millions of cells	Requires good and specific antibodies. Provides high-resolution mapping of protein-DNA interactions but may be limited by the specificity of antibodies used for immunoprecipitation
ATAC-Seq	Accessible chromatin regions, which are indicative of regulatory elements like promoters, enhancers, and transcription factor binding sites, regardless of the proteins bound to DNA	Requires a small amount of starting material, often as few as a thousand cells	Not antibody-dependent. Provides high-resolution mapping of open chromatin regions and is not limited by antibody specificity, offering unbiased insights into global chromatin accessibility

SELEX and its newer variations, such as protocols combining it with serial analysis of gene expression (SAGE), provide a novel method for study of protein-DNA interactions. This method has vast potential for the identification of binding between nucleic acids and proteins or other molecular targets. It has been useful for defining regulatory loops of DNA that participate in chromatin packaging.

Scanning Probe Microscopy (SPM)

Another technique to investigate structure of DNA and its interaction with proteins, especially as it pertains to higher-order chromatin structure, is *scanning probe microscopy (SPM)* and its variations. First developed in 1980s and 1990s, SPM is a branch of microscopy that uses movement of a physical probe over the surface of a specimen to form an image. A probe tip is moved over a surface of a sample and is kept at a constant height above the sample through a feedback mechanism. This allows for nano- or atomic-scale measurement of surface properties [59]. The main advantage of SPM and its variants is their resolution. Differences in height as small as a few picometers can be determined by this method. In addition, unlike electron microscopy, SPM can be performed in standard laboratory air, temperature, and pressure conditions. The primary downside of SPM is its slow image capture time and the inability to precisely resolve liquid-liquid or solid-solid interfaces. This technology can be used to image different forms of DNA structure, from double helical DNA strand to tightly packaged chromatin. Many challenges need to be overcome, however, before SPM can be widely used for biomolecule imaging. One major challenge is that biomolecules, including DNA and DNA-protein complexes , are highly mobile and have dynamic structures which can change faster than a SPM image can be captured [60].

Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) spectroscopy is a highly sensitive and quantitative technique that can simultaneously measure both binding affinity and kinetics of biomolecular interactions [61]. One ligand, such as DNA, is immobilized on an SPR surface, and the second ligand, the protein of interest, is introduced. Binding between these two ligands will alter the refractive index of the surface used, which can then be quantified with precision. Because this change occurs due to ligand binding, kinetic, affinity, and thermodynamic properties of this interaction can be deduced. This technique does not require labeling and thus eliminates the possible change to the ligand molecular structure [62]. Additionally, unlike EMSA, it does not require stable DNA-protein complexes and, importantly, can measure both association and dissociation. SPR spectroscopy can effectively demonstrate dynamic differences between wild-type and mutant protein binding.

Laser Flow Cytometry

DNA of eukaryotes and other higher organisms are organized into chromosomes. As described earlier, chromosomal structure is of critical importance to activity of that region of the genome. The area of the chromosome that is to be expressed needs to be unpacked to allow for the transcription complex to gain access to the DNA. This

action is tightly regulated by a myriad of different proteins and can be difficult to study due to its complexity. Some older techniques, like karyotyping, have been key to studying transmission of genetic material between generations and its relation to pathology. Various staining techniques, from Giemsa staining to more modern fluorescent methods, have helped identify and analyze specific regions of chromosomes. However, these techniques lack resolution, and, recently, *laser flow cytometry* has been employed to separate genomes into single chromosomes for further analysis [63]. Combining of laser flow cytometry with techniques mentioned earlier for DNA and DNA-protein interaction analysis holds great potential for a deeper and more detailed study of DNA regions of interest.

Ribonucleic Acid (RNA) Assessment

RNA Introduction

Ribonucleic acid (RNA) is composed of repeating modular sugar moieties, similar to DNA, but is typically single stranded and with a hydroxyl group attached to the pentose ring at the 2′ position. This difference makes RNA a less stable molecule than DNA because it is more prone to hydrolysis. Although the difference in stability makes RNA more susceptible to degradation and environmental damage, it also makes it ideal for gene expression which requires a highly dynamic molecule that can be used in rapid transcriptional switches. Gene expression is dependent on transcription of DNA into RNA and the translation of RNA into protein. Because RNA is a key step in this process, its analysis is crucial for understanding of gene expression dynamics. This has led to better understanding of a wide array of diseases and is used in medicine for diagnosis, classification, prognostication, and therapeutics. The following section will explain the roles of different types of RNA in a cell and techniques for RNA assessment.

RNA exists several forms within the cell, both coding and noncoding, with each performing a distinct function. Coding RNA is called *messenger RNA (mRNA)* and functions as the template for translation of RNA into protein. mRNA accounts for only a small fraction of the total RNA in the cell. After transcription, it undergoes further modifications before being translated. Modifications important for translation are the addition of methyl-guanosine cap (5′ cap), addition of a series of adenine nucleotides to the 3′ end (poly-A tail), and the splicing of introns [64]. After modifications are complete, mRNA migrates to the cytoplasm from the nucleus, and translation begins. Translated mRNA is degraded by an enzyme called RNase, usually in the matter of minutes to hours, which terminates translation. Noncoding RNAs are not translated into proteins and instead perform other vital functions within a cell. Examples of noncoding RNA include transfer RNA (tRNA) and ribosomal RNA (rRNA), which are necessary for translation; small nuclear RNA (snRNA), which is involved in processing of precursor mRNA; small nucleolar RNA (snoRNA), which guides chemical modification of other RNA molecules; microRNA (miRNA), which regulates posttranscriptional gene expression;

Piwi-interacting RNA (piRNA), which is involved in both epigenetic and posttranscriptional gene silencing; and large intergenic noncoding RNA (lincRNA), which are important regulators of a variety of diverse cell processes [64–68].

Measuring and Identifying RNA

Given that mRNA is an intermediate step between DNA and protein, its measurement is a surrogate marker of gene expression. Many techniques of RNA analysis exploit the fact that RNA is single-stranded and is transcribed from a complementary section of DNA. Working with RNA, however, has several inherent challenges—the most important of which is its short half-life due to susceptibility to breakdown by RNase. Multiple methods of RNA analysis will be discussed in this section and include Northern blot, ribonuclease protection assay, in situ hybridization, complementary DNA (cDNA) arrays, microarrays, and sequencing.

Northern Blot

Northern blot, or *RNA blot*, is the technique developed and published in 1970s to detect presence of an RNA molecule and its size [69]. Purified RNA samples are separated by size using gel electrophoresis and transferred to a blotting membrane. Membrane is then incubated with labeled single-stranded DNA probes that are complementary to the RNA segment of interest. Once the DNA probe has annealed to the RNA, it can be detected by either chemiluminescence or autoradiography.

Ribonuclease Protection Assay (RPA)

Ribonuclease protection assay (RPA) relies on the binding of antisense RNA probes to their complimentary RNA segments of interest and subsequent formation of double-stranded RNA (dsRNA) molecules [70]. Following treatment with the RNA probe, the sample with dsRNA is incubated with ribonucleases which degrade and remove the unbound single-stranded RNA but preserves dsRNA. Gel electrophoresis is then used to separate the dsRNA complexes which can be detected by autoradiography.

In Situ Hybridization (ISH)

Northern blots and RPAs require extraction of RNA from tissue or cell samples prior to analysis. In situ *hybridization (ISH)* , on the other hand, can detect and quantify RNA in the tissues or cells of interest. This technique requires that tissue or cell section is first treated to fix target transcripts in place and permeabilized to improve access of the probe. Treatment with proteases is used to remove RNA-bound proteins. A labeled RNA probe is then hybridized to the sample and detected by autoradiography or chemiluminescence. The detection of probes with chemiluminescence with this technique is known as *fluorescent* in situ *hybridization* (FISH) [71, 72].

Measuring Gene Expression

While Northern blot, RPA, and ISH can detect and/or quantify the RNA of interest, they are not able to detect levels of expression of specific genes. However, sequencing of the human genome and especially which DNA sequences are actually transcribed created the possibility of specifically designing complementary DNA (cDNA) probes to areas of interest. The cDNA probes can be used to compare relative expression levels of several hundred genes at a time between two conditions. Early iterations of cDNA arrays required manual handling of probes resulting in high inter-experiment variability. Continued assay improvement has led to increased automation, improved reproducibility, and decreased quantity of RNA needed for experiments. In addition to cDNA arrays, microarrays, reverse transcription polymerase chain reaction (RT-PCR), and transcriptome sequencing have been developed as more powerful tools of gene expression assessment.

cDNA Arrays

cDNAs are amplified by standard PCR and spotted on a glass slide [73]. RNA is extracted from the two samples to be studied, isolated, and labelled with appropriate fluorescent dye [74]. Next, the samples are mixed and hybridized with the cDNA probes on the glass slide. This approach allows for comparison of gene expression between two conditions for as many genes as there are probes on the slide. However, one of the limitations is reproducibility since the cDNA probes must be manually spotted on the slide producing significant variability between experiments.

Oligonucleotide Arrays or Microarrays

Microarrays , similarly to cDNA arrays, also measure gene expression using cDNA probes fixed to a glass slide. Instead of researcher having to manually fix the probes, microarray probes are directly synthesized on the slides [75, 76]. Many commercially available probes are available and can be chosen to assess expression of a multitude of genes or gene clusters.

In the first step, RNA, representing transcripts of all active genes, also referred to as the *transcriptome*, is extracted from the tissue sample to be studied. mRNA from the sample is amplified, labelled by one of several available methods, and hybridized to the microarray. Following hybridization, the microarray is washed, and signal is detected by measuring fluorescence at each spot. In case of biotin-labelled samples, an intermediate post-hybridization step is needed to stain the array with fluorescently labelled streptavidin. A laser scanner can be used to excite the fluorophores and allow for computer capturing of the images. Because the amount of mRNA represents how active each gene is, the level of fluorescence in each well corresponds to the level of gene expression. Automation makes profiling of thousands of gene simultaneously possible [75, 76]. Although powerful, this technique is not well suited for detecting low levels of gene expression given background fluorescence and nonspecific binding.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a technique that synthesized cDNA using purified RNA from a tissue sample. This technique is used to synthesize cDNA for cDNA and microarray analysis but can be combined with quantitative real time analysis, known as *real-time quantitative RT-PCR (real-time qRT-PCR)*, to analyze expression of many genes in a single sample. cDNA is produced from purified RNA as above and then combined with DNA-dependent DNA polymerase and fluorescent primers [77, 78]. Fluorescence increases as the PCR cycles continue and eventually reaches the point at which it is significantly above background fluorescence. The cycle number when this happens, known as the threshold cycle, is inversely proportional to the original relative expression level of the gene of interest.

RNA Sequencing

RNA sequencing is a newer technique for measuring gene expression and quantification of RNA molecules. Also known as *RNA-Seq* or *whole transcriptome shotgun sequencing*, it uses next-generation sequencing methods to determine presence and quantity of RNA [79]. It avoids some of the pitfalls of microarrays, such as cross-hybridization artifacts, poor quantification of lowly or highly expressed genes, and the necessity to know the sequence a priori [80], and is capable of detecting alternatively spliced transcripts, posttranscriptional modifications, gene fusions, gene mutations, and single nucleotide polymorphisms (SNPs) as well as investigating different RNA populations including rRNA, tRNA, miRNA, and small RNAs [81, 82]. This technique is capable of analysis of many sequencing reactions occurring simultaneously and yields millions of RNA sequence reads [83].

Single-Cell RNA Sequencing (scRNA-Seq)

Although RNA sequencing is a powerful and unbiased technique of obtaining gene expression patterns of tissues, differences between individual cells within a tissue can have profound functional consequences. To better study these differences, *single-cell mRNA sequencing (scRNA-Seq)* technology was developed in 2009 and has gained in popularity through the mid-2010s [84]. The principle of this technology is essentially the same as *RNA-Seq*, but an additional step is added prior to sequencing that separates the individual cells. Originally, this separation was performed manually, but microfluidic circuits, nanodroplets, and, most recently, in situ barcoding have greatly improved the efficiency since then [85]. After individual cells are isolated and RNA is extracted, cDNA libraries are created, amplified, and sequenced. The single-cell gene expression profiles can be deduced and different cell populations identified within the original sample. Currently, 10X Genomics offers approved protocols for various cell types, including human and mouse cell lines (cryopreserved or freshly cultured), human blood cells (PBMCs, BMMCs), mouse blood cells (PBMCs, BMMCs, splenocytes), and dissociated tumor cells (DTCs). However, researchers should not feel restricted by these provided protocols, as

numerous custom-optimized protocols for different tissues are available. While some may be more common, such as those for mouse [86] and human tissues [87, 88], researchers have the flexibility to adapt and optimize protocols to suit their specific experimental needs.

Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq)

A new technology that builds on single-cell RNA sequencing is *cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq)*. Like *scRNA-Seq*, it yields single-cell gene expression levels but is additionally able to identify and quantify cell surface proteins of these cells. In essence, this method discerns RNA expression within individual cells via complementary DNA (cDNA) and identifies cell surface epitope expression using antibody-derived tags (ADTs). This is accomplished by treatment of the sample with antibodies conjugated to oligonucleotides that can be captured by specially designed primers and amplified by PCR. These oligos contain a barcode sequence, which, if amplified, indicates the binding of the antibody to its corresponding cell surface epitope [89]. This powerful technique is increasingly used to identify novel cell subtypes in fields of immunology and oncology and will likely continue to be applied even more broadly. Currently, ADTs can detect 163 different cell surface epitopes expressed by each cell.

Cell Hashing

After introducing CITE-seq in 2017 [89], researchers extended single-cell antibody-based multiplexing strategies by incorporating Hashing-Tag Oligos (HTOs) into their methodologies [90]. HTOs are short oligonucleotide sequences that are uniquely barcoded and conjugated to antibodies targeting ubiquitous surface proteins. This innovation allows for the simultaneous labelling of multiple experimental samples with distinct barcodes, enabling researchers to pool these samples together for analysis. By utilizing HTO labelling, experimental costs are further reduced, addressing a longstanding challenge in single-cell analysis.

Single Cell Multiome ATAC + Gene Expression

Traditional scRNA-Seq methods focus solely on gene expression data, missing crucial information about regulatory elements such as enhancers and promoters. Since enhancers and promoters do not produce mRNA, they remain invisible to RNA-based technologies. However, the combination with single-cell ATAC-Seq allows researchers to access and analyze regulatory networks comprehensively. Multiome Sequencing, a method described in 2020 by 10X Genomics, overcomes this limitation by integrating single-cell RNA-Seq and ATAC-Seq, enabling the identification of enhancer and promoter activity directly within the cell population. By combining gene expression profiles with chromatin accessibility data at the single-cell level, Multiome sequencing enables researchers to perform regulatory network analysis directly on the cell populations of interest, elucidating the relationships between epigenetic regulation and gene expression, and specifying the targets underlying the basis of diseases. Sample

preparation starts with the suspension of single nuclei extracted from the sample of interest. Transposition is carried out using the Tn5 transposase enzyme, which selectively cleaves DNA in open chromatin regions. Transposed nuclei are partitioned into droplets known as GEMs (Gel Bead-in-Emulsions), each containing a single Gel Bead with a unique 10x Barcode. Within each GEM, these unique barcodes are linked to available mRNA and transposed DNA fragments originating from a single nucleus. Two separate libraries are constructed from the same pool of GEMs: One library is prepared for sequencing RNA, providing gene expression data. The other library is prepared for sequencing ATAC, providing information on chromatin accessibility. After library sequencing, researchers receive fastq files for RNA (GEX) and ATAC libraries separately. Cell Ranger ARC, a specialized software, can be utilized to analyze and process the received data, facilitating downstream analysis and interpretation. After completing the necessary preprocessing and quality control steps, which will be detailed in upcoming chapters, researchers can utilize the SCENIC+ [91] pipeline. This tool, which utilizes both RNA and ATAC libraries of the same individual cells, reveals gene regulatory networks driven by enhancers, although this technology remains in a development phase.

Single Cell Multimodal Omics Sequencing

After the introduction of numerous single-cell sequencing techniques, which have become cost-efficient and established their significance in translational science research, there has been a notable advancement in multiomics approaches (Table 8.2). This advancement comes with the development of a powerful new technique called DOGMA-seq [92], introduced in 2021. DOGMA-seq is a method for transcriptomic (RNA/GEX), cell surface epitopes (ADT), and chromatin accessibility (ATAC) measurement from thousands of single cells on the 10x Genomics Multiome platform. It generates scRNA-seq, scATAC-seq, and cell surface epitope sequencing libraries linked by 10x cell barcodes for coordinated analysis. However, researchers should keep in mind that DOGMA-seq has been optimized for use with only peripheral blood mononuclear cells (PBMCs).

Analysis and Interpretation of Gene Expression Profiles

The use of novel techniques of RNA analysis described above has been increasing in both basic science and clinical laboratories. They are a powerful tool to study levels of gene expression among different physiological and pathological conditions. However, they have led to an exponential increase in the complexity of data produced and the difficulty of analysis. A scientist must be well versed in analysis and explanation of the results obtained. In this section, we will describe the general approach to interpreting gene expression profiling experiments, including data normalization, quality assessment, data analysis, multiple comparison problem, and final interpretation.

Table 8.2 Multimodal-omic sequencing techniques

	Target	Sample Preparation	Available Protocols
scRNA-seq	Used to analyze gene expression (RNA) at the single-cell level.	Cell Isolation Cell Barcoding RNA Extraction and Library Preparation Sequencing	Wide range of approved protocols for multiple and tissue cell types are available
CITE-seq	Combines scRNA-seq with the simultaneous measurement of surface epitopes (ADT) on individual cells. Antibodies conjugated to oligonucleotide tags are used to barcode cells based on the protein markers they express, enabling the investigation of cell surface protein-RNA relationships, cell states, and cellular heterogeneity profiles in RNA and ADT expression	Cell Isolation Antibody Staining Cell Barcoding and RNA Extraction Library Preparation and Sequencing	Approved protocols for multiple human and mice cell and tissue types are available
Multiome ATAC + Gene Expression	This technique captures information about both chromatin accessibility (ATAC-seq) and gene expression (scRNA-seq) simultaneously at the single-cell level allowing for the investigation of the regulatory landscape of individual cells, including the identification of transcription factor binding sites, enhancer activity, and their correlation with gene expression patterns	Cell Isolation Chromatin Accessibility Assay (ATAC-seq) Nuclei Isolation and Lysis RNA Extraction and Library Preparation Sequencing	Approved protocols for multiple human and mice cell types are available
DOGMA-seq	Simultaneously profiles transcriptomics (RNA expression), accessible chromatin regions (ATAC-seq), and surface epitopes (ADT) levels within individual cells	Cell Isolation Antibody Staining Cell Permeabilization Library Preparation and Sequencing	Sample preparation protocol only optimized for PBMCs

Normalization

As discussed earlier, microarray platforms require detection of fluorescence to infer level of gene expression. Normalization is a step of data analysis that adjusts individual hybridization intensities of each well so that they are balanced and meaningful comparisons can be made [93]. Changes in labelling efficiency, differences in starting mRNA, and variability of experiment preparation can all greatly impact output readings. Normalization adjusts the fluorescence levels across the wells allowing for comparison between different microarrays. It can be accomplished through computational methods such as scaling, quantile normalization, distribution adjustment, and locally weighted scatterplot smoothing (LOWESS) [93].

Quality Assessment

Quality assessment should occur both before and after normalization. In general terms, purpose of quality assessment is to ensure that all steps of the protocol were accomplished successfully and without error. Pre-normalization assessment should evaluate the microarray for mechanical issues like scratches, bubbles, or other artifacts. After normalization, the assessment should aim to identify outlier samples or significant unexpected differences between batches of microarrays. This will allow for an accurate statistical analysis, since outliers can be evaluated and excluded, if appropriate.

Data Analysis

Microarray experiments will provide image files with raw data files upward of 1 GB in size [83]. Large databases where both microarray data and clinical or experimental variables can be stored are necessary to streamline data analysis. The analysis can be accomplished in a variety of ways ranging from simple statistical methods to development of novel computer algorithms. A variety of computer software is commercially available and can be used to analyze differential gene expression, gene network analysis, class prediction, and class discovery.

Differential gene expression analysis is one of the most common tools used to analyze microarray data. It identifies the variability in gene expression between two conditions and is typically accomplished through use of t-test, analysis of variance (ANOVA), or linear modelling. Gene network analysis identifies potential new interactions between genes and their expression [94]. Class discovery, of particular importance in oncology studies, relies on the notion that every phenotype (e.g., tumor) can be classified based on its gene expression profile. Due to their scope, microarray studies provide an opportunity to identify tumors with novel gene expression profiles. These studies are known as class discovery studies. Another type of microarray-based analytic experiments, class prediction studies, aims to investigate experimental tissue and assign it to one of the already known phenotypic classes (e.g., tumor class) [95].

Multiple Comparison Problem

Unique statistical problems arise from analyzing large microarray datasets. With increasing number of statistical tests performed, the likelihood of a false-positive result increases. For example, if Type I error of an experiment is defined by the standard p-value of 0.05 and a microarray study produces expression levels of 5000 genes compared between two conditions, there would be 250 Type I errors.

Statistical methods can be used to lower the probability of Type I error, including Bonferroni correction, Benjamini-Hochberg procedure, and the Q-value. The Bonferroni correction lowers the Type I error probability by adjusting the p-value. The desired p-value is divided by the total number of tests, making it significantly less likely to have a Type I error but also less likely to identify real differences. Benjamini-Hochberg procedure utilizes the Q-value to control the FDR by providing a method to choose the appropriate p-value for the data interpretation given a researcher-defined acceptable FDR [96, 97].

Interpretation

Interpretation of the vast amount of information gained from microarray studies remains challenging. Microarray experiments allow for comparisons between expression signatures [98], older and newer gene probes [99], gene sets [100], gene pathway relationships [101], and human cell responses to treatment with various bioactive molecules [102]. Though microarray protocols have become efficient and can quickly provide large amount of information about gene expression, interpreting biological significance of this data remains a challenge. Many of the advances involve various novel computational methods to identify significant patterns, such as enrichment ranking, and gene set enrichment analysis (GSEA) [100, 101, 103]. In addition, the use of heat maps for analysis of related groups of genes helps visualize large numbers of gene expression profiles simultaneously [104].

Analysis and Interpretation of a Single-Cell Data

Run Cell Ranger

After completing single-cell library preparation, sequencing will be conducted at next-generation sequencing centers or platforms. Researchers will receive fastq files for each sequenced modality and library. They must download these files and prepare for running the appropriate Cell Ranger program. Cell Ranger Count is used for single-cell RNA-seq and CITE-Seq libraries and can process RNA, ADT, and HTO libraries. For single-cell multiome ATAC+gene expression assays, researchers should utilize Cell Ranger ARC. Those working with Dogma-seq libraries must employ a combination of different programs, as a single program capable of processing RNA, ADT, ATAC, and HTO (optional) libraries simultaneously is not currently available. Upon completion of the analysis, researchers will have features, barcodes, and matrix files they need for analysis.

There are multiple programs and packages that researchers can use after this step to analyze their data. Here we describe the most used pipeline:

Create a Single-Cell Dataset

Using the Seurat [105] package that is available in R studio, researchers should use cell ranger output which creates a unique molecular identified (UMI) count matrix and create a Seurat object that includes all the assays they sequenced including RNA, ADT, ATAC and HTO.

Quality Control, Data Normalization and Scaling

Commonly used QC metrics in the community include the number of unique genes detected in each cell. Low-quality cells or empty droplets typically manifest with very few genes, while cell doublets or multiplets may demonstrate an unusually high gene count. Another crucial metric is the percentage of reads mapping to the mitochondrial genome, which serves as an indicator of cell quality; cells with low quality or dying often exhibit significant mitochondrial contamination. Seurat internally computes mitochondrial QC metrics using the set of all genes starting with

MT- as a standard for mitochondrial genes. After the removal of unwanted cells from the dataset, the subsequent step involves data normalization, which standardizes the feature expression measurements for each cell based on total expression. This process involves multiplying by a scale factor (10,000 by default) and subsequently log-transforming the result. Next, we apply a linear transformation ('scaling') which is a standard pre-processing step before dimensional reduction techniques.

Visualization

After pre-processing, high-dimensional single-cell expression data is often reduced to a lower-dimensional space to facilitate visualization and downstream analysis. Various dimensionality reduction techniques, such as principal component analysis (PCA), uniform manifold approximation and projection (UMAP), and t-distributed stochastic neighbor embedding (tSNE), could be employed to capture the underlying structure and variability within the dataset effectively. The primary goal of these algorithms is to learn the underlying structure in the dataset, thereby clustering similar cells together in a lower-dimensional space. While 2D visualization techniques like tSNE and UMAP are valuable tools for exploring datasets, it's crucial to acknowledge that all visualization techniques have limitations, especially in fully representing the complex multidimensional nature of single-cell data solely on a two-dimensional figure. Hence, researchers are encouraged to leverage techniques like UMAP for visualization but caution against drawing biological conclusions solely based on visualization techniques. After generating the initial UMAP and identifying cell clusters, researchers can explore the differentially expressed genes that define each cluster or use canonical cell markers to assign the cell type identity to clusters.

Differentially Expressed Feature Calculation and Mapping

Calculating and mapping differentially expressed features is a key step in single-cell data analysis, providing researchers with valuable insights into cellular behavior and differentiation. Although it's not strictly mandatory, it has become a standard part of single-cell analysis pipelines due to its usefulness in understanding complex biological data. Many methods exist for identifying differentially expressed genes, but some are more reliable than others, particularly when it comes to reducing false positives—a common issue in this field as we mentioned earlier. To address this problem, researchers are encouraged to use 'pseudo-bulk' approaches. A recent study [106] compared the performance of 14 differential expression methods across a set of 18 gold-standard datasets. The results showed that the top most accurate and precise six methods all employed a common approach: they aggregated cells within a biological replicate to create 'pseudo-bulks' before applying statistical tests. This pseudo-bulk strategy has proven to be a robust way to identify differentially expressed features while minimizing false positives. Statistical methods for identifying differential expression must consider the inherent variability among biological replicates to ensure accurate results in single-cell data. By accounting for this natural variation, pseudo-bulk approaches can more reliably detect changes in gene

expression resulting from biological perturbations. Researchers aiming for high accuracy in their single-cell data analyses should consider integrating this approach into their pipelines. The packages employing the 'pseudo-bulk' approach, such as edgeR [107], DeSeq2 [108], and limma [109], are readily available for analysis. Methods following a 'single-cell' approach, include logistic regression, t-tests, Wilcoxon rank sum tests, MAST, likelihood ratio tests, negative binomial, and Poisson methods.

After performing statistical analysis to obtain p-values and log2 fold changes for each gene within a cell type, the next common step is to map these differentially expressed genes onto predefined biological pathways or gene sets. This mapping process uses pre-constructed reference maps, which may be selected by the researcher or come pre-built into the analysis method. These maps are built on a foundation of cumulative scientific knowledge and are frequently updated, often on an annual basis. Researchers can find such maps for a variety of species, including but not limited to humans, mice, rats, flies, zebrafish, yeast, and various strains of E. coli. Among the methods for mapping genes to these reference maps, two common approaches are Gene Set Enrichment Analysis (GSEA) and Over-Representation Analysis (ORA). Gene Set Enrichment Analysis (GSEA) takes as input both p-values and log2 fold changes. By analysing the distribution of these values across a ranked list, GSEA can calculate the statistical significance of gene set enrichment, providing insights into which pathways or gene sets are most likely to be affected by a given condition. GSEA's output includes a Normalized Enrichment Score (NES), which indicates both the strength and direction of the enrichment for each gene set. Over-Representation Analysis (ORA), on the other hand, uses a simpler approach, focusing on the count of significant genes within a given gene set. It compares this count to the expected count based on a random distribution to determine if there is a significant over-representation of differentially expressed genes in that set. This approach provides a straightforward way to identify which gene sets contain more significant genes than expected by chance.

Table 8.3 displays the usage, advantages, and disadvantages of both mapping methods:

In the previous discussion, we outlined the most common and widely accepted pipeline for single-cell data analysis. However, as this field has grown in popularity and complexity, additional advanced analytical methods have emerged. Table 8.4 provides an overview of these newer, sophisticated analytical approaches, allowing researchers to select the best methods based on their needs and research interests. Detailed information can be found in the corresponding papers for the common tools listed such as: Monocle [110] Slingshot [111], CellChat [112] and NicheNet [113], SCENIC [114], SCENIC+ [91].

Clinical Applications of DNA and RNA Analysis

The progressive development of molecular biology techniques of DNA and RNA analysis and their application has led to an explosion of information and insight into

Table 8.3 Advantages and disadvantages of single-cell analysis mapping techniques

Method	Purpose and Methodology	Advantages	Disadvantages	Usage
Gene set Enrichment Analysis (GSEA)	Determines whether a specific set of genes (pre-defined gene sets, like pathways or gene ontology terms) shows statistically significant, consistent differences in expression across a ranked list Uses statistical methods, such as the Kolmogorov-Smirnov, to assess whether these gene sets are distributed non-randomly along the ranked list	Considers All Genes: By analysing the entire list of genes, GSEA reduces bias from arbitrary thresholds Accounts for Gene Correlations: It acknowledges correlations among genes in pathways or biological processes Sensitivity to Small Changes: Detects shifts in gene set expression even with subtle changes	Complexity: GSEA can be computationally intense Requires p values and log-two fold changes	RNA assay of the single cell data After performing differential expression analysis and selecting a relevant reference map, researchers will have everything they need to run GSEA
Overrepresentation Analysis (ORA)	Check whether specific gene sets contain more differentially expressed genes than expected Typically involves setting a threshold for differential expression (e.g., p-value < 0.05) and then determining if a higher-than-expected proportion of genes within specific sets meet that criterion. Tests like Fisher's exact test or chi-square test are commonly used	Simple and Fast: ORA is straightforward to compute and less computationally demanding	Threshold Sensitivity: The choice of differential expression cut-off can affect the results Ignores Gene Correlations: ORA doesn't consider relationships among genes in a set Misses Subtle Changes: It can overlook gene sets where changes are consistent but small	ATAC assay of the single cell data ATAC data provides researchers with information about differentially accessible chromatin peaks and chromosomal regions, instead of specific gene names. To gain biological insights into epigenetic changes between treatment and control groups, researchers can determine the coding regions associated with these differentially accessible peaks and then conduct ORA on these coding regions Because the focus in ATAC-seq data is on number coding and non-coding regions, ORA is the preferred method for this type of analysis

Table 8.4 Novel single cell analysis techniques

Method	Purpose	Common Tools
Trajectory Analysis and Pseudo time Estimation	Models cellular differentiation trajectory Estimate pseudo-time for each cell which is a measure of cell maturity Identify branching points in the cell development and maturation trajectory	Monocle and Slingshot
Cell-cell interaction analysis	Quantifies and reveals changes in cell-cell communication and signalling patterns Identifies major signalling pathways and communication networks for each cell type	CellChat and NicheNet
Transcription Factor Analysis	Identifying transcription factors and their target genes, known as regulons Creates gene regulatory networks using transcription factors and their downstream targets	SCENIC SCENIC+ (requires RNA + ATAC assays)

biomolecular basis of normal physiology and pathology. Over the years, these techniques have been successfully applied to a myriad of diseases, and these applications have been translated into changes of clinical practice. For example, the field of pathology once relied on histopathology for diagnosis and prognostication but now frequently employs genetic profiling. Oncology has also benefited greatly from both hematologic and solid tumor genetic testing. Genetic tests are now routinely used for prognostication and clinical decision-making in breast, colon, and thyroid cancer to name a few.

The impact that the techniques described had on basic science research and clinical practice is broad, far-reaching, and ever evolving. Much of our understanding and approach to various diseases is due to technology of genetic information analysis. For an academic surgeon, their appreciation is imperative, both in the lab and in the clinic. It will be difficult to read a peer-review journal and not come across multiple techniques described here, whether it's the use of RT-PCR, microarrays, sequencing, or genetically engineered animals.

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Modern Techniques for Protein Assessment

9

Johanna H. Nunez and Vanessa Nomellini

Abstract

Despite the advances in modern medicine, our ability to adequately diagnose and treat a number of disease processes is still lacking. Key to our knowledge is the understanding of genetic regulation, intracellular activity, and cell-to-cell interactions. It is estimated that there are 20,000–30,000 genes that comprise the human genome code for greater than one million different proteins, any of which may be biomarkers for disease, targets for drug therapies, or insights into formulating better or more tolerable treatment strategies. With the expanding interest in basic and translational science in the field of surgery, it is important that the academic surgeon revisit the fundamentals of protein analysis to successfully formulate an experimental design. There are an endless number of ways to purify, identify, and classify the function of proteins. The major steps in protein analysis involve (1) determining the source, (2) extracting the protein, (3) purifying the protein, (4) identifying and/or quantifying the protein, and (5) establishing its function by assessing how it interacts with other proteins. This chapter reviews the current methods involved with each of these steps and acts as a guide to answering the academic surgeon's queries.

Keywords

Protein · Precipitation · Chromatography · Gel electrophoresis · Mass spectrometry · Western blotting · Enzyme-linked immunosorbent assay (ELISA) Multiplex assay · Fluorescence resonance energy transfer (FRET) flow cytometry · Immunohistochemistry

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Despite the advances in modern medicine, our ability to adequately diagnose and treat a number of disease processes is still lacking. Key to our knowledge is the understanding of genetic regulation, intracellular activity, and cell-to-cell interactions. The Human Genome Project has given us insight into the 20,000–30,000 genes coded by our DNA. However, it is estimated that these genes code for greater than one million different proteins [1, 2], any of which may be biomarkers for disease, targets for drug therapies, or insights into formulating better or more tolerable treatment strategies. With the expanding interest in basic and translational science in the field of surgery, it is important that the academic surgeon revisit the fundamentals of protein analysis to successfully formulate an experimental design.

There are an endless number of ways to purify, identify, and classify the function of proteins, making the process of choosing the most effective way to answer a scientific question seems daunting at times. The major steps in protein analysis involve (1) determining the source, (2) extracting the protein, (3) purifying the protein, (4) identifying and/or quantifying the protein, and (5) establishing its function by assessing how it interacts with other proteins [3]. This chapter reviews the current methods involved with each of these steps and acts as a guide to answering the academic surgeon's queries.

Protein Sources

To start, one must determine the source of the protein in question. The most common sources are whole tissue, bacteria, yeast, and cell cultures. The following is a discussion of when to consider choosing one over another.

Whole Tissue Samples

The most direct sources, although perhaps the most difficult to obtain, are samples from individual patients. When analyzing human proteins, one does not need to be concerned with differences in homologous genes or variations in disease pathophysiology, as when utilizing animal models or prokaryotic cell systems. However, in most instances, obtaining human samples is an elaborate process involving the Institutional Review Board [4, 5]. In addition, the procurement of certain tissues may be near impossible to justify, such as obtaining whole organs or even tissue biopsies. Blood, urine, sputum, and buccal cell samples, on the other hand, are useful, noninvasive methods for collection of human protein.

A widely acceptable alternative to obtaining human tissue is creating an animal model using a mammalian species with a known sequence homology to the protein in question. With this method, one must still undergo the process of an institutional review and approval. Such protocols typically allow for the acquisition of tissue biopsies, whole organ procurement after euthanasia, or other invasive procedures. A benefit to this system is that some of the animals available for research purposes can be bred syngeneic or of identical genetic backgrounds [6]. This eliminates the heterogeneity often found in the human population, which significantly lowers the sample size needed for an adequately powered test.

Bacteria

When large-scale production of the protein is required for analysis, recombinant DNA technology may be preferred. By exploiting the ability of bacteria to incorporate foreign DNA in the form of plasmids, or separate circular fragments of DNA, one may generate millions of copies of the desired protein for analysis [7, 8]. In this process, bacteria are transformed with the selected plasmid and are grown in culture. As the culture is incubated, the bacteria grow and divide. The protein is then synthesized using the bacteria's inherent mRNA transcription and protein translation machinery [9].

A number of plasmid vectors are readily available to purchase from commercial industries. Most vectors at minimum incorporate an antibiotic resistance gene to allow for selective growth as well as multiple restriction enzyme sites which allow easy insertion of the desired gene. This method is cheap, easy to manipulate, and creates millions of copies of the protein in question. In addition, it can allow for not only the translation of native protein but also a tagged version of the protein in question. For example, some commercially available plasmids have the ability to add a histidine or glutathione S-transferase (GST) tag to the end of the gene once incorporated into the plasmid. Translation of the protein thus yields a tagged protein, which is one method of purification, as discussed later.

The main disadvantage of this system is that prokaryotic cells do not undergo posttranslational modifications, as they lack an endoplasmic reticulum [10]. Therefore, one must recognize that the use of this synthetic protein may not confer the same cellular effects as the native human protein. In addition, this technique requires that a source of the DNA be available, either from a genomic DNA or cDNA library, as discussed in other chapters. Now that the Human Genome Project is complete, the sequences of all genes are known, making this process feasible regardless of the gene being studied.

Bacterial artificial chromosomes (BACs)—or large plasmids that can be incorporated into a bacterial cell—can also be used [11]. This system allows for the incorporation of very large DNA sequences, up to 300 kb. The Human Genome Project, for example, used BACs to generate large stretches of DNA containing multiple genes which were then sequenced [1, 11]. The disadvantage of the BAC is that, since the sequences are so large, only one or two plasmids may be introduced into a given bacteria. Consequently, the overall yield of DNA, and therefore protein, is lower.

Yeast

Similar to bacteria, yeast cells can incorporate DNA in the form of plasmids and can rapidly divide to generate large copies of the protein being studied. Again, this is a cheap and simple method for protein analysis. The main advantage over bacteria is that the yeast artificial chromosome (or YAC) can incorporate DNA fragments up to 3000 kb. In addition, since yeast are eukaryotic cells, synthesized proteins will undergo posttranslational modifications as in human cells. The disadvantage is that the proteins generated in this system are often unstable and may generate unpredictable protein sequences [12].

Mammalian Cell Culture

When the experimental design simply calls for the direct effect of a treatment on a particular cell type or assessment of the protein within the cell, a culture system may be more prudent. Cells may be cultured from direct isolation from tissue (ex vivo primary cell cultures) [13] or may be purchased from a biotechnology company. Primary cell cultures, whether directly isolated or purchased, tend to be limited in the number of times they may replicate before undergoing senescence [14]. With various genetic modifications, some cell lines have been engineered to be immortal and can be maintained indefinitely [15].

The main benefit of this system is that it most closely resembles the human environment. Options with this technique include lysis of the cells for analysis of intracellular proteins, assessment of cell surface proteins, and measurement of secreted proteins within the supernatant. In addition, the cells may be treated with a stimulant or suppressant to determine the effect it has on protein levels [16]. Certain eukaryotic cell lines may be used for incorporation of foreign DNA, but this process is much less reliable than the bacterial cell culture system, as these cells do not readily incorporate foreign DNA.

There is an entire methodology to maintaining a cell line that is beyond the scope of this chapter. However, one must recognize that cell culture has multiple limitations, requires the purchase of a number of pieces of equipment, and involves very particular maintenance. Primary cells undergo senescence rather quickly in culture and therefore are not useful when anticipating long-term experiments [14]. Many labs therefore prefer to use immortalized cell lines which have an indefinite growth curve [17]. The process of immortalization involves infection with viral oncogenes, overexpression of telomerase, or inactivation of tumor suppressor genes [17]. For example, one of the most widely used immortal cell lines are HeLa cells which are derived from a patient with cervical cancer as a result of human papillomavirus (HPV) infection [18]. As one would expect, the main disadvantage of using immortalized cell lines is that these cells do not mimic normal cells found in the body. Thus, many use this method as a starting point for determining the mechanisms of intracellular or cell surface proteins. Further studies are then needed to corroborate any significant findings.

Protein Extraction

Once the source of protein is established, one must decide the process by which the protein is removed from the system. In most instances, the protein is being derived either from a tissue homogenate or cell culture. Protocols for tissue homogenization are quite standard and mostly involve mechanical disruption of the tissue. This process often results in a variable yield of protein. Thus, other techniques may be added which help increase the amount extracted. Examples include sonication (application of ultrasound to the sample), enzymatic digestion, application of high-pressure nitrogen, and use of detergents or solvents [19].

Protein Purification

To isolate the protein for further analysis, there are a number of methods one may employ. The ideal method to choose depends on the nature and purity of the starting product.

Precipitation

One of the simplest ways to extract a protein from a cell lysate, cell supernatant, or tissue homogenate is by precipitation. The most widely used method of precipitation employs ammonium sulfate in a process known as *salting out*. With higher concentrations of ammonium sulfate, protein solubility decreases, yielding the formation of a precipitate. Centrifugation of the precipitate with subsequent dialysis or washout of the remaining components within the supernatant allows for isolation of a majority of proteins. This system is quite simple; the yield, however, is usually unpredictable. Other reagents may be used to precipitate protein, such as trichloroacetic acid, acetone, methanol, and chloroform. The decision to use these alternative compounds depends on the various chemical characteristics of the protein being purified [3, 20].

Centrifugation

Another method that may be employed to separate components of a cell lysate or tissue homogenate is *ultracentrifugation*, also known as *velocity sedimentation* [3, 21]. This process involves suspending a mixture within a liquid and applying angular acceleration to the sample. The particles with the highest mass experience the largest degree of gravitational force and move to the bottom of the sample tube most rapidly. Depending on the force applied, various components of intracellular organelles can be separated out. At very high speeds, proteins and other macromolecules can be isolated [21].

A variation on this method, known as *equilibrium sedimentation*, utilizes a gradient of various sucrose densities to separate out cellular components and macromolecules [21]. As the column is centrifuged, the molecules move through the gradient until they reach the sucrose layer that is equivalent to their density. After centrifugation, the molecular components separate out into visible bands within the sucrose gradient, which are easily collected. One can predict which layer of the column the desired protein lands based on its sedimentation coefficient, which is related to the size of the molecule. Compared with velocity sedimentation, equilibrium sedimentation takes days instead of hours, but it often yields a larger amount and cleaner separation of cells or macromolecules.

Chromatography

The process of *chromatography* compared with precipitation and centrifugation is much more specific in that it exploits a particular chemical property of the protein being studied for the purposes of purification (Fig. 9.1a) [3]. The basic principle involves exposing a protein solution to a column containing resin beads. Proteins bearing a particular chemical property either bind to or move slower through the column, while all other proteins quickly elute out. The protein of study is then extracted from the column by a second elution step which reverses its interaction with the column [3]. The chemical properties of proteins that are exploited in this method include size, ionic charge, hydrophobicity, and various types of affinity.

Gel filtration chromatography (Fig. 9.1a, left panel) simply involves separating proteins based on size. With this system, the column is composed of resin with certain sized pores. Small molecules fit inside the pores and are held up within the column, whereas larger proteins cannot fit in the pores and elute out quickly. As the solution slowly moves through the column, samples can be collected in separate tubes at various time points as they elute out the bottom. The different fractions collected each contain a relatively pure sample of proteins with similar sizes. If the size of the protein being studied is unknown, each fraction can be tested to determine which contains the desired protein by way of some of the methods discussed in the section on protein identification and quantification.

Hydrophobic interaction chromatography involves using resins within the column which have certain hydrophobic groups attached. As most proteins contain a variable number of hydrophobic regions, they will differentially adsorb to the resin, following the principal of "like dissolves like." This process is promoted by using a salt-based solution that neutralizes most of the ionic charges on the protein and allows for increased interaction with the hydrophobic column. Other factors that promote adsorption of the protein to the column include temperature, pH, and concentration of the salt solution. The protein can then be eluted off the column by decreasing the ionic strength or adding other agents, such as denaturing solvents or detergents.

Ion exchange chromatography (Fig. 9.1a, middle panel) utilizes the ionic charge of the protein of study. Proteins that have an overall negative charge can be separated from a solution using a column containing a cationic resin and vice versa. The proteins are then eluted off by applying solutions with increasing ionic charges that act to displace the proteins adsorbed to the column.

Affinity chromatography (Fig. 9.1a, right panel) involves capturing the desired protein by attaching an enzyme, antibody, or protein-binding partner to the column. When a protein-containing solution is passed through the column, the desired protein will selectively bind to the column, while all other components will elute through. Examples of this method include "lock and key" interactions between an enzyme and its ligand, binding of glycoproteins to lectin, metal binding, and antibody-mediated binding (called *immunoaffinity chromatography*) [22, 23]. As described above, recombinant proteins may be constructed with a histidine tag, which has a high affinity for nickel and cobalt. Columns may be purchased which contain beads that are

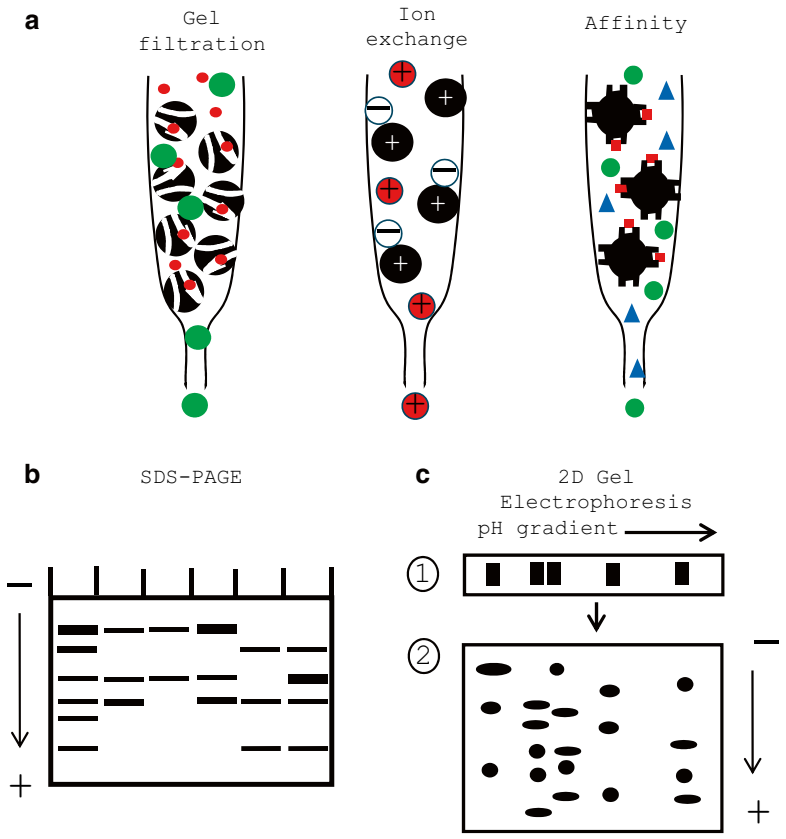

Fig. 9.1 Methods of protein purification. Various types of chromatography are shown in (**a**). *Gel filtration chromatography* (*left panel*) involves purification of proteins based on size. Small particles (*red circles*) become trapped in the small pores within resin beads, while larger particles (*green circles*) elute out quickly. *Ion exchange chromatography* (*middle panel*) involves using charged resin beads to purify protein. If the beads (*black circles*) are positively charged, the negatively charged proteins (*white circles*) will bind, while the positively charged proteins (*red circles*) will repel from the beads and filter through. *Affinity chromatography* (*right panel*) involves a lock and key-type mechanism of protein purification. The beads are coated with a binding partner or antibody to the target protein, which binds the desired protein to the column. All other proteins then elute out. The protein can then be dissociated from the column. (**b**) In *SDS-PAGE*, protein samples that have been exposed to SDS are loaded into the top wells of the gel. An electric field is then applied, and the proteins move toward the positive electrode. In the leftmost column is a protein standard which contains multiple proteins with known sizes. In this example, there are five remaining wells that contain unknown samples. The band corresponding to the protein of interest can be determined by comparing the bands to the ladder of protein standards. Since SDS-PAGE only separates proteins by size, each band may contain multiple proteins. To better separate an unknown sample, *2D gel electrophoresis* may be used as shown in (**c**). First, proteins are separated by allowing movement through a pH gradient down a narrow strip of polyacrylamide gel (1). Proteins will be separated by their isoelectric point, or the pH at which their net charge is zero. The gel is then turned 90° and placed adjacent to a new piece of gel. The proteins are then exposed to SDS and an electric field is applied (2). Proteins will move toward the positive electrode as in SDS-PAGE. Thus, the final 2D gel resolves proteins based on isoelectric point along its width and size along its length

coated with either of these metals in order to purify His-tagged proteins. Other premade columns are coated with antibodies to specific fusion protein tags, such as GST. These binding interactions are quite strong but can be disrupted—thus eluting the protein from the column—by washing the column with concentrated salt solutions or solutions with a different pH. Recent advancements in supramolecular affinity chromatography stem from improvements in synthetic, natural, and inorganic supports as well as new smart and nanomaterials. Advances in covalent immobilization, biospecific adsorption, and non-covalent immobilization in affinity chromatography have also led to improved techniques [24, 25].

High-performance liquid chromatography (HPLC) is a more modern method of chromatography that applies high pressures to the column to improve the resolution of the protein of study. In traditional chromatography, the flow of the protein-containing solution relies on the force of gravity. Thus, one of the main factors involved in the degree of purification is the spacing of the resin beads. If the beads are too tightly packed, the flow of the protein-containing solution is slower, and the degree of nonspecific binding increases. Alternatively, if the beads are not tight enough, the proteins will pass too quickly, and the yield of purified protein will be lower. In HPLC, the beads are packed exceedingly tight, making the flow of the protein solution close to zero. When high pressure is applied, the solution flows through the column in seconds as opposed to hours with traditional chromatography. Since the column beads are so compact, there is more surface area for protein binding, which allows for a much cleaner separation of the protein of study.

Gel Electrophoresis

Another preferred method of protein purification involves the application of electricity to a protein solution in a process entitled sodium dodecyl sulfate polyacrylamide gel electrophoresis (or *SDS-PAGE*) [3]. With this technique, proteins are first placed in SDS to unfold the proteins in solution. Most investigators also add a reducing agent, such as β-mercaptoethanol, to disrupt all disulfide bonds and fully denature the protein. SDS is a polar molecule with a hydrophobic chain on one end and a negatively charged sulfate group on the other end. As the amino acid side chains become exposed during protein unfolding, the hydrophobic ends of the SDS molecules bind to the hydrophobic portions of the denatured proteins, coating them with negative charges. The intrinsic charges on the proteins are thus masked by the large number of SDS molecules bound, and all proteins become net negatively charged.

The denatured proteins are then put through a highly cross-linked gel made from an inert substance, such as polyacrylamide, and an electrical field is applied (Fig. 9.1b). The negatively charged proteins then migrate through the gel toward the positive electrode. Larger proteins will be exposed to a larger electrical force, but due to the nature of the polyacrylamide matrix, they will have more drag and move slower through the gel. A solution of thousands of proteins can thus be separated into a series of discrete bands according to their molecular weight alone.

This process tends to be a more reliable way to separate proteins than those discussed previously, as it removes the variables of net ionic charge and protein shape to fractionate the proteins purely based on their size. Unlike chromatography, this process is effective for all types of proteins, including insoluble proteins or those which are part of large complex aggregates [3]. The disadvantage is that only small quantities of a solution can be used in electrophoresis, as opposed to the large sample volumes that column chromatography allows.

Capillary gel electrophoresis (CE-SDS) is another technique for the separation of proteins that can provide some advantage over SDS-PAGE. In this technique, the separation occurs in a capillary filled with cross-linked gel matrix or a sieving medium with the charged analytes being separated based on their sizes and shapes in the high electric field [26]. This technique has been shown to have higher throughput, improved ease of automation, and a shorter analysis time when compared to SDS-PAGE [27].

Two-Dimensional Gel Electrophoresis

As one can imagine, proteins that vary by only a few kilobases are very difficult to separate by SDS-PAGE. Even when the starting solution contains thousands of proteins, both column chromatography and SDS-PAGE are only capable of segregating about 50 different proteins at once. To rectify this, the method of two-dimensional (2D) gel electrophoresis was developed, which is capable of resolving around 2,000 different proteins [3]. In the first part of this technique, the individual charges on the proteins are exploited by a process known as *isoelectric focusing* [28, 29] (Fig. 9.1c). The theory behind this process involves the principle that, since a number of amino acids act as a weak acid or base, proteins can behave as zwitterions (or molecules which can contain both positive and negative charges). Changes in the pH of a protein-containing solution can therefore make acidic or basic amino acid side chains negative, positive, or neutral based on the loss or gain of H^+ ions. As a result, all proteins have a characteristic isoelectric point, which is the pH at which the net charge on the entire protein is zero. In other words, proteins will not move within an electrical field when at their isoelectric point. In isoelectric focusing, a pH gradient is first created across a narrow strip of polyacrylamide gel matrix. Proteins are then allowed to move through this gradient in the presence of an electrical field. Each protein will move down the gel until it reaches the pH corresponding to its isoelectric point, then stop as it is no longer charged.

The second dimension of 2D gel electrophoresis relies on the same concepts as SDS-PAGE. After isoelectric focusing, the entire gel is subjected to SDS, which will again bind to and confer a net negative charge on all proteins. The narrow piece of gel is then placed along the edge of a new, standard piece of polyacrylamide gel at a 90° angle, and an electrical field is then applied. As with SDS-PAGE, the proteins will then migrate through the gel according to their size alone. The resulting gel thus contains proteins separated by isoelectric point along its width and by size along its length. As it is extremely unusual for two proteins to possess a similar size

and isoelectric point, one may assume that the individual spots on a 2D gel likely contain one unique protein [30].

Historically, criticisms of this technique mostly involved the ability to reliably duplicate each experiment. However, refinements on the products themselves have significantly improved the accuracy of 2D gel electrophoresis. In addition, newer modifications of the procedure, such as the use of fluorescent-tagged proteins to differentially label samples from separate treatment groups which can be run on the same gel (called 2D differential in-gel electrophoresis, or 2D-DIGE) [30–32], have significantly expanded the value of this method.

Protein Identification and Quantification

The methods discussed in this section are not only useful to identify and quantify the protein once it has been purified, but they may also be employed as the initial step to one's study. For example, measuring carcinoembryonic antigen (CEA) in the blood of patients undergoing surveillance for recurrent cancer [33] or measuring other biomarkers of disease such as lipase for pancreatitis [34] and troponin I during a myocardial infarction [35] does not necessarily require protein extraction or purification. Determining which technique to employ to identify the protein of study depends not only on the scientific question but also on the source of the protein and what reagents are available.

Mass Spectrometry and MALDI-TOF

One of the most accurate methods of determining the identity and elemental composition of a protein is through mass spectrometry (MS). In this process, the sample is first loaded into the machine, where it is vaporized then split into separate ions by bombarding them with electrons. The ions are then passed through an electromagnetic field, which bends the fragments into a beam. The speed at which the ions travel depends on both their mass and their charge. The ions are then analyzed and quantified as they pass through the detector. The resulting output is a spectrum of ionic masses unique to each protein [29, 31, 36].

There are a wide variety of methods that use permutations on traditional MS to improve the accuracy of the test. Most commonly used is called *matrix-assisted laser desorption ionization time-of-flight spectrometry* (or MALDI-TOF) [31, 37]. With this technique, proteins are first cleaved into separate peptide fragments and dried onto a metal or ceramic slide. A laser is then used to ionize the fragments—which is a gentler process than bombardment with electrons—and the fragments accelerate through the electromagnetic field. With this method, the ionized fragments are analyzed not only by their mass and charge but also by the time it takes to reach the detector, generating a more precise and unique mass spectrum. Tandem mass spectrometers—which sequentially fragment the proteins into peptides, then into individual amino acids—are also used to determine protein sequences.

Furthermore, chromatographic methods may be linked to the mass spectrometers to purify and separate the analyzed proteins before they pass through the ionizer.

The main use of MS is for protein identification. Direct sequencing can be performed but it is more cumbersome. More recently, as the field of proteomics is thriving, many are gaining interest in MS again, as this has shown to be useful in determining all posttranslational modifications of a single protein. While MS is perhaps one of the most accurate ways to identify proteins, the process often requires complex analyses using expensive machinery and requires highly purified protein [36].

One of the most significant advances in MS is its use for single cell proteomics. Single cell proteomics is a rapidly advancing field that looks at the diversity of protein expression in individual cells [38–40]. Although other methods are being used to look at single cell proteomics, a technique quickly advancing the field is the use of ultra-high sensitivity MS [41]. Ultra-high sensitivity LC-MS is able to be performed due to the use of miniaturized sample preparation, very low flow-rate chromatography, and a trapped ion mobility MS. This technique allows for over 10 times improved sample sensitivity and for the quantification of proteomes at a single cell level [41, 42]. This single-cell resolution of MS has also led to the ability to analyze protein-protein interactions spatially in three-dimensional intact specimens with single cell resolution in tissues [43].

Western Blotting

As discussed above, SDS-PAGE and 2D gel electrophoresis are reliable methods to separate proteins for further analysis. However, these procedures require additional steps to identify the protein in question. One method involves cutting out the band in question, dissolving the gel, and analyzing the protein by MS [37]. The accuracy of this is quite low, however, as the exact margins of the bands are not always clear and some may contain more than one protein. One of the most reliable ways of detecting and quantifying the protein of study after SDS-PAGE or 2D gel electrophoresis is by Western blotting. Clinical uses of Western blotting include testing for HIV and Lyme diseased [44, 45].

With this technique, all the proteins within the gel are transferred to a membrane, typically made from nitrocellulose or polyvinylidene fluoride (PVDF), both of which nonspecifically bind proteins [3]. Again, all proteins are all negatively charged from the SDS so that, when an electrical field is applied, the proteins will move toward the positive electrode. If a membrane is placed between the gel and the positive electrode (as a "sandwich"), the proteins will transfer to the membrane in a process called *electroblotting* [3] (Fig. 9.2a, left panel).

The desired protein is then detected and quantified on the membrane using antibodies tagged with a reporter system, typically by a color change or fluorescence (Fig. 9.2a, right panel). Since antibodies are proteins themselves, the membrane must first be blocked to minimize nonspecific binding of the antibody. The blocking step is typically performed with a solution containing a small amount of protein,

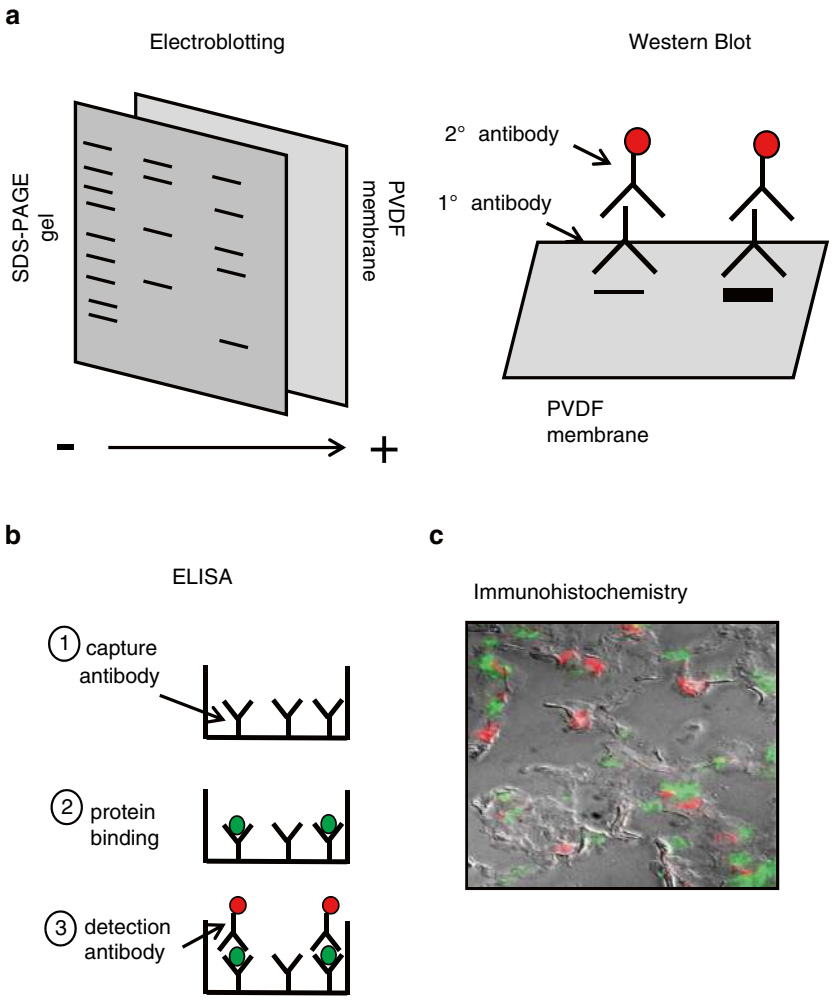

Fig. 9.2 Western blotting and immunoassays. After running protein samples on SDS-PAGE, *Western blotting* can be used to detect and quantify the protein of interest. As shown in (**a**), the SDS-PAGE gel is placed on top of a PVDF membrane that nonspecifically binds proteins. Electricity is then applied. The proteins are still negative from the SDS, so they will move toward the positive electrode. Once the proteins are transferred, the membrane is blocked to avoid nonspecific binding of antibodies and then incubated with a primary antibody against the protein being studied. The membrane is then washed and incubated with a labeled secondary antibody. When analyzed, the amount of protein from each unknown sample can be quantified by assessing the thickness of the band on the membrane. (**b**) To quantify a protein directly in solution, an *ELISA* can be performed. First, a 96-well plate is coated with a capture antibody that is specific against the protein being studied (1). Next, an unknown sample solution is incubated in the 96-well plate and allowed to bind with the capture antibody (2). The samples are then washed and the detection antibody is added (3). Typically, this antibody is linked to a fluorescent tag or an enzyme that generates a colorimetric change. The amount of the protein of interest in the unknown samples is therefore determined by the intensity of the color or fluorescence, as measured by a spectropho

Fig. 9.2 (continued) tometer or fluorometer. To determine the concentration of a protein in situ within tissue sections, *immunohistochemistry* can be applied. This method employs the same methods as other immunoassays and either direct or indirect antibody detection methods can be used. An example of an immunofluorescence image is shown in (**c**). Here, a section of lung tissue is incubated with a fluorescent-labeled antibody to a neutrophil-specific antibody (*green cells*). The tissue section is then washed and incubated with a fluorescent-labeled, macrophage-specific antibody (*red cells*). The total number of neutrophils and macrophages within the lung tissue can then be quantified. To better define the alveolar structure, this image is shown with an overlay of delay interference contrast microscopy

such as bovine serum albumin or evaporated milk. The antibody detection step can be performed in either one step (direct detection) or two steps (indirect detection). If a primary antibody directly linked to a colorimetric or fluorescent tag is not commercially available, the detection must be performed in two steps. For the first step, the membrane is incubated with a solution containing a primary antibody specific to the desired protein derived from an animal other than human. After rinsing off any excess primary antibody, the membrane is then incubated with a solution containing a secondary antibody, typically against the fragment crystallizable (F_c) portion of the primary antibody. For example, if the primary antibody was made in a goat against a human antigen, the secondary antibody would have to be against the F_c portion of goat antibodies. There are a wide variety of secondary antibodies available that are tagged with either a fluorescent label or a reporter enzyme that generates a colorimetric or chemiluminescent by-product when combined with its substrate. A commonly used enzyme reporter system is horseradish peroxidase, which has a number of substrates and can generate either colored or fluorescent by-products. Another system utilizes the strong affinity between biotin and streptavidin. If the antibody is constructed with a biotin molecule attached, this can be incubated with streptavidin molecules coated with a fluorescent dye. With the appropriate device, such as a spectrophotometer or a fluorometer, the intensity of the color or fluorescent signal can be detected and quantified relative to a standard curve with known concentrations of protein.

As with other techniques, improvements in western blotting are related to the ability for single cell western blotting (scWB). This has been done in situ on adherent cell culture and also through the use of separation encoded microparticles [46, 47]. The stages of scWB include 1) the settling of cells into microwells, 2) chemical lysis of cells in microwells, 3) PAGE of the single cell lysate, 4) UV light immobilization of proteins into the gel matrix, and 5) in-gel immunoprobing of proteins [48]. scWB allows for analysis of individual cells allowing the measurement of cell-to-cell protein expression variation.

Although Western blotting has a high degree of sensitivity and specificity, there are a number of limitations to this method. First, the electroblotting aspect of this technique does not guarantee that 100 % of all proteins within the SDS-PAGE gel will be transferred to the membrane or that all transferred proteins will actually bind to the membrane. In terms of using one- or two-step antibody binding, there are a number of advantages and disadvantages. In the direct detection method, the

process is quicker since only one antibody step is required. Indirect methods require the use of more than one antibody, which not only increases the time of the procedure but also increases the risk of cross-reactivity of the protein with other proteins on the membrane. In the direct method, however, the antibody itself is labeled, potentially affecting the binding affinity with its target, which is not the case with indirect detection methods. Furthermore, antibodies can only be constructed with a limited number of enzyme or fluorescent tags, making little opportunity for signal amplification. With the indirect method, multiple tagged secondary antibodies can bind to the primary antibody, which allows for signal amplification and detection of very low concentrations of proteins [49].

Immunoassay

The principles of immunoassays are similar to those used for Western blots. The main difference is that the procedure does not require the separation of a protein solution by SDS-PAGE. There are several immunoassays currently in use, including enzyme-linked immunosorbent assay (*ELISA*), multiplex assays, and enzyme-linked immunospot assays (ELISPOT).

As opposed to immobilizing proteins on a membrane for antibody detection, the ELISA (Fig. 9.2b) allows for a protein-containing solution to be measured directly in solution within a 96-well plate. Most uncoated, plastic, 96-well plates will non-specifically bind a majority of proteins, including either the protein of interest or antibodies. If the starting solution is a purified protein, an ELISA may be performed via a direct method whereby the protein is directly adsorbed onto the wells and quantified using labeled antibodies. If starting with an unpurified protein mixture, such as that from a tissue homogenate or cell lysate, an indirect (or "sandwich") method is typically employed. Here, the primary antibody itself (called the capture antibody) is first adsorbed onto the wells of the plate. The antibodies are then exposed to the protein mixture and selectively bind the targeted protein. Ideally, there are more capture antibodies coating the plate than the amount of the target protein in the unknown solution. If this is not the case, the protein solution can be diluted until one can ensure that all the protein in the sample will bind to the capture antibody and not over-saturate the system. The concentration of the protein is then determined by a subsequent antibody system (the detection antibody), either using another primary antibody directly tagged with an enzyme or an unlabeled primary and tagged secondary antibody as described above. The amount of protein present is then determined by adding the enzyme substrate and quantifying the resulting signal [50, 51]. For some common proteins, pre-made plates can be purchased which are already coated with the capture antibody of interest, making the process even quicker.

The traditional ELISA system only allows for the detection of one protein within a solution. More recent technology has allowed for the detection of as many as 100 proteins within a very small sample volume, termed *Multiplex Assays*. This has proven to be quite useful in the field of transplantation, where all human leukocyte

antigen (HLA) class I and II antigens of the recipient can be measured in one sample [52]. With this technique, a number of polystyrene or magnetic beads tagged with a specific fluorophore are coated with a particular capture antibody. A sample is then incubated with the beads and the proteins bind to their respective antibody. A detection antibody with a fluorescent tag (either directly or indirectly) is then incubated with the sample. The typical detection apparatus for the multiplex system functions in the same manner as a flow cytometer, detecting, sorting, and quantifying each fluorescent bead as it passes through the detector. The first laser detects the fluorescent tag of the detection antibody and discards all beads not bound to a detection antibody (and therefore not bound to a sample protein). The second laser then distinguishes the different fluorophores from the beads themselves, separating the various proteins being analyzed. This laser detects very minute differences in wavelengths between different fluorophores, which is the key to being able to analyze dozens of proteins at once. The strength of the resulting fluorophore signal is thus directly proportional to the concentration of the protein in solution, using a standard curve for comparison.

The ability to measure multiple different proteins in the same sample is cost effective, less labor intensive, and utilizes much smaller sample volumes. In addition, the reliability of the data is much stronger, as there is a certain degree of error that one must accept with each 96-well plate [53]. Since the detector relies on very minute differences between fluorophores in each sample, it is imperative that the assay be carried out with extreme preciseness.

Both the ELISA and multiplex immunoassay require the use of a protein-containing solution for analysis. This limits the protein source to secreted proteins, cell lysates, and tissue homogenates. There are times, however, when the degree of secreted protein at the single cell level is desired, such as with cytokine release or antibody production by B cells [54, 55]. The ELISA system can be exploited in cell culture systems in a process called ELISPOT [56]. First, a 96-well plate is coated with the desired capture antibody and cells are added with or without pre-stimulation. The cells are then allowed to incubate. As they generate the desired secreted protein, it can bind to the nearest capture antibodies. The cells are then washed away and the detection antibody is added (again in a direct or indirect method). At the end of the experiment, the bottoms of the wells appear as either colorimetric or fluorescent "spots" corresponding to the areas where cells were secreting the protein. The spots can then be counted to determine the relative proportion of a cell population that is stimulated to secrete the desired protein. This method is particularly useful for detection of very low quantities of secreted protein that may otherwise be undetectable by ELISA [56].

Multiplex immunofluorescence has continued to advance in the last 10 years through improved techniques and its ability to be used in 3D tissues [57]. 3D multiplex staining requires uniform immunostaining throughout tissues. Recent advances allow this through increased antibody diffusion rates, either using centrifugal forces or an electric field, and through decreasing antibody reaction rates so stains can travel farther into tissues [58]. This full sample technique allows for large scale morphological and molecular gradients in tissues.

Immunohistochemistry

Both the Western blot and the various immunoassays described above require either a protein-containing solution or a cell culture system. The basic concepts used in these systems can also be exploited to quantify proteins in situ using either frozen or paraffin-embedded tissue sections (Fig. 9.2c). The tissue sections are first blocked to reduce nonspecific binding and then incubated with antibodies via either a direct or indirect method. This is the method of choice for the clinical diagnosis of most tissue-based cancers but can also be used to quantify different cell types within a tissue [50] or to measure levels of protein expression by individual cells [16]. The term, immunohistochemistry, is the broad expression for any antibody-based detection system for tissue sections. When the resulting signal is a colorimetric change, this is still referred to as immunohistochemistry. Immunofluorescence is specifically related to the use of fluorescent-tagged antibodies to label tissue sections.

Flow Cytometry

The methods described thus far in this section are not capable of measuring cell surface proteins alone. Certain structural features of proteins embedded within the cell membrane include sugar moieties on the extracellular domain as well as a hydrophobic domain that incorporates into the cell membrane. Some techniques involved in purification of cell membrane proteins therefore rely on targeting these regions of the proteins. The purity of these procedures, however, is quite variable. One may also use immunofluorescence with confocal microscopy or immunohistochemistry, although the quantification with these methods is also often not precise. One of the most accurate methods to quantifying cell surface proteins is via flow cytometry [16, 51].

This method is similar to that described in the section on multiplex assays. With flow cytometry, however, only a few antigens on the surface of cells can be quantified at one time. The process is similar to other antibody-based detection methods whereby a cell culture is incubated with an antibody specific for the surface antigen being studied. The key is not to use any detergents that will permeabilize the membrane so that only cell surface antigens are exposed. If using fluorophores of different wavelengths, up to three or four antigens can typically be detected. The cells are then passed through a series of detectors that can differentiate between cell types and fluorophores.

The main use of this method is with immune cells, which are distinguished by both their size and granularity. When the cells pass through a laser, the relative sizes are determined by the amount of forward scatter and the granularity (which is directly related to the distribution and density of intracellular organelles) by the amount of side scatter. The subpopulations of cells are then analyzed according to their fluorescence to determine the relative amount of protein expression on the surface of the different cell types. The applications of flow cytometry are numerous and include cell cycle analysis, cell death analysis, functional assays, and intracellular expression. Its sensitivity is quite high and can therefore detect very small quantities of proteins. One new technique for flow cytometry is the integration of flow cytometry

and spectral analysis in what is termed spectral flow cytometry. As opposed to traditional flow cytometry, spectral flow cytometry uses multiple detectors to measure the full spectral emission of each fluorophore [59]. This allows for a detailed spectral signature for each fluorophore. It then uses a mathematical algorithm to unmix and be able to identify each fluorophore [60]. Again, this method requires expensive machinery but allows for the analysis of proteins that other methods cannot, such as changes in cell surface receptors with stimulation or ligand binding.

Immunoassays in Live Animals

With recent advances in imaging technology, certain immunoassays can also be performed in vivo for real-time analysis of protein distribution [61]. There are a few different ways in which a protein can be studied with this technique. One way is to tag cells with a fluorescent-labeled antibody and inject them into a small animal to determine the specific cell distribution. Another is to genetically engineer a strain of animal in which the protein in question is transcribed with a fluorescent tag. The native protein can then be detected with a specialized camera that can detect the fluorescent tag through living tissue. These studies have been particularly useful in tumor biology and in stem cell analysis [62, 63].

Identifying Protein Function by Analyzing Protein-Protein Interaction

For some scientific questions, it is enough to simply be able to quantify the amount of protein produced at baseline or after stimulation. To further clarify a protein's function, however, additional tests must be utilized. Clues about protein function can be determined by simply knowing the three-dimensional protein structure, which can be approximated by X-ray crystallography or predicted by the amino acid sequence [3]. In addition, localization within the cell by flow cytometry, immunohistochemistry, or even simple confocal microscopy can be an indication of a protein's function [64]. For example, movement of a protein from the cell surface to the nucleus in the presence of some stimulus suggests that the protein is related to gene expression. Assessing how the protein interacts with other molecules within the cell or on the cell surface, however, can even further define the protein's role in cellular function. The following are tests to determine protein-protein interactions, which can help determine cellular functions.

Protein Microarray

The microarray is another high-throughput assay that involves immobilizing a large number of target proteins on a solid surface and incubating them with potential binding partners labeled with fluorescent markers. After washing, the array chip is

then analyzed. Only those proteins that find their binding partner will have a fluorescent signal. Given the simplicity and reliability, this has widely replaced 2D gel electrophoresis and yeast two-hybrid. The main benefit of this system is that large numbers of proteins can be screened at once with minimal labor. Protein microarray technology has advanced through the use of approaches such as DNA-based protein arrays, micro-contact printing, label-free protein detection, and algorithms for data analysis [65, 66]. Other advancements come from gain-scanning to reduce variability of the array data leading to more sensitive differential analysis [67]. One caveat is trying to find an adsorption surface to which the proteins can bind without altering their tertiary structure. In addition, the microarray chips are often expensive and require separate machinery for analysis [37].

Co-Immunoprecipitation

This technique uses the principles of immunoassays but relies on antibody-mediated capture of a known protein within a large complex. By targeting the known protein with a specific antibody, the rest of the complex can be pulled down as well. Once the protein complex is isolated with an antibody pulldown, the proteins can be dissociated and denatured for analysis via SDS-PAGE and Western blotting or MS. An advantage of this technique is that it allows for the proteins to be pulled out of a solution in their native conformations. Recent improvements in co-immunoprecipitation come from the use of mass spectrometry, specifically affinity purification-mass spectroscopy (AP-MS), and its ability to enrich low-abundant proteins leading to better co-immunoprecipitation [68, 69]. The main limitation is that it cannot detect transient interactions between proteins, which is often the mechanism of many intracellular signaling pathways. In addition, this method does not define the protein(s) that directly bind the target protein within a large complex. Therefore, further studies are required after co-immunoprecipitation is performed [23].

Immunoaffinity Chromatography

As discussed above, one form of protein purification is affinity chromatography [23]. Here, antibodies specific to the protein of study are adsorbed onto the column beads. By using a cell lysate or tissue homogenate without any denaturing agents, the desired protein will likely be bound to its native binding partner(s). Thus, when placed through a column, the protein along with its partner will be eluted out. The proteins can then be denatured and analyzed through gel electrophoresis and Western blotting or MS. Again, this method can only detect stable interactions between proteins and cannot detect transient interactions.

A related method uses affinity tags, such as His or GST, which work by the same mechanism. This alternative process is typically used when an antibody specific to the protein in question is not commercially available, but a fusion protein can be made.

Fluorescence Resonance Energy Transfer

Introducing a gene constructed with a fluorescence tag, such as green fluorescent protein (GFP), into a cell for incorporation into the DNA will allow for the transcription of a GFP-tagged protein. This process alone can help to localize the protein within cells using confocal microscopy. Immunofluorescence can also be used to determine whether two proteins colocalize within a tissue section, but it does not verify that they interact. Confirmation of protein interaction can be determined by a process called fluorescence resonance energy transfer, or *FRET* (Fig. 9.3a) [70]. Here, the two proteins are labeled with different fluorescent tags, each with a distinct wavelength. If the two proteins come together spatially within a cell, the energy of one fluorophore will transfer to the other fluorophore, changing the emission spectrum. If the proteins do not come within 10 nm, the emission spectrum reveals two distinct proteins. New

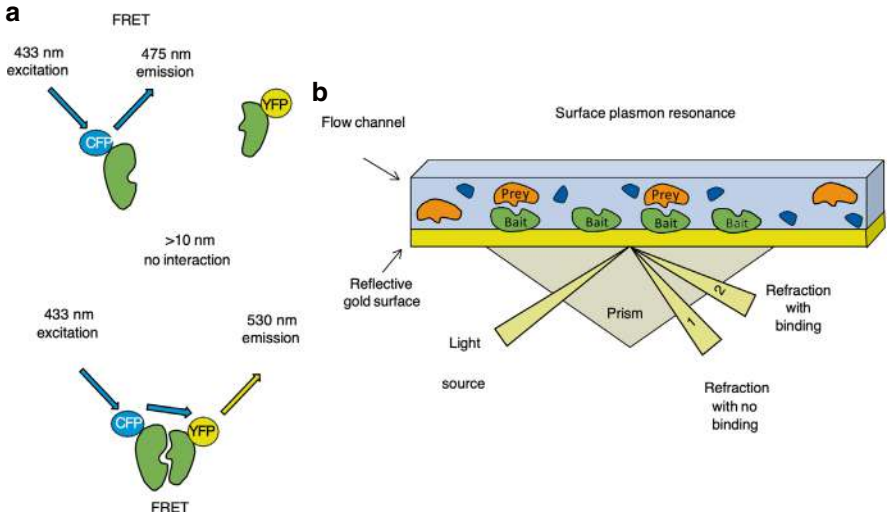

Fig. 9.3 Protein-protein interactions. (**a**) With any fluorescent-based technology, light emitted from the fluorophore is detected only after it is excited by a particular wavelength of light. *FRET* exploits the principle that, when two fluorophores are in close proximity to each other, the energy emitted from one can pass to the other and result in an emission wavelength corresponding to the second fluorophore. Fusion proteins can thus be generated in which fluorescent proteins are fused to the protein of interest and to a library of potential binding partners. In the *upper panel* of (**a**), the study protein is fused to cyan fluorescent protein (*CFP*) and a potential binding partner is fused to yellow fluorescent protein (*YFP*). If the two proteins do not interact, excitation of CFP at 433 nm will emit light at its typical wavelength of 475 nm. If the two proteins bind as shown in the *lower panel* of (**a**), excitation of CFP at 433 nm will result in energy transfer to the YFP, which emits light at a wavelength of 530 nm. This process is sensitive to protein interaction of <10 nm. A second technique is *surface plasmon resonance* as shown in (**b**). Here, the bait protein is adsorbed onto a reflective surface such as gold. When light is passed through a prism that sits on top of the reflective surface, the angle of refraction will be affected by the presence of the bait protein (beam 1). When the angle of refraction through the prism is altered, this signifies that a second protein or protein complex has bound to the bait protein, indicating a specific interaction (beam 2)

advancements in FRET stem from improved ultrasensitive technology using nitrogen-doped carbon quantum dots and gold nanoparticles [71]. This method is advantageous as it can detect protein-protein interactions in vitro or in vivo and does not require anything more than a fluorescence microscope [70]. The main disadvantage is that there are a number of factors involved in the two proteins interacting and a negative result does not necessarily mean that the two proteins do not interact. Consequently, troubleshooting with FRET is often more complicated than with other methods. Some, however, utilize this method for in vivo analyses [72–74].

Surface Plasmon Resonance

Another definitive way to assess whether two proteins interact is through surface plasmon resonance (Fig. 9.3b) [75]. In the most basic sense, this method utilizes the principles of light refraction through a prism. Here, the bait protein is first adsorbed onto a reflected surface. The refractive index is then measured with the bait protein alone. A purified solution of the target protein is then incubated with the bait protein. After the sample is washed, a change in the refraction of light will indicate that the two proteins bind to one another. An advantage is that small quantities of protein can be used and no tagged or fusion proteins are needed. This method is typically used as a confirmatory test and not as a high-throughput analysis, as only a few proteins can be tested at one time. Improvements in surface plasmon resonance stem from superior resonance biosensors, the use of nobel metal-assisted immunosensors, and the use of surface plasmon resonance microscopy in single-cell imaging [76–78].

Mass Cytometry

Another new addition in protein analysis is the use of mass cytometry. Mass cytometry allows for dozens of protein markers to be tracked in single cells. Instead of the use of fluorophores as used in traditional flow cytometry, mass cytometry uses heavy metal isotopes coupled to antibody probes [79]. The cells are then nebulized and metal ions provide a quantitative read-out of the marker's distribution within a sample [80]. Mass cytometry has many advantages over flow cytometry, which include the ability to detect over 50 markers at a time, cell barcoding, sample multiplexing, no concern for spectral overlap or autofluorescence, easier calibration, and stable metal tags. Current disadvantages of mass cytometry are slower acquisition times, lower throughput, and fewer available pre-conjugated antibodies.

Artificial Intelligence in Proteomics

As technologies continue to advance at an unprecedented rate, the amount of data that needs to be processed efficiently and carefully has increased exponentially. In order to process the immense amounts of data, artificial intelligence, specifically

deep learning, has led to significant advancements in proteomics. Deep learning has shown the ability to predict experimental peptide measurements, amino acid sequences, and biomarker discovery from mass spectrometry data [81, 82]. These deep learning abilities are also improving our ability to uncover protein-protein interactions, retention time prediction, major histocompatibility complex-peptide binding prediction, and de novo peptide sequencing [83].

Summary

Since the Human Genome Project has been completed, the field of proteomics has become highly dynamic. Historically, many of the above procedures could not be performed for a number of proteins, as the genomic or complementary DNA (cDNA) libraries, bacterial plasmids, and fusion proteins could not be made. At present, the options for deciding on how to analyze a particular protein are only limited by the nature of the methods themselves and by the ease of acquiring the necessary detection machinery.

This chapter provides a brief summary on how to choose a protein source, techniques for purifying and quantifying protein, and methods to help determine protein function. Again, setting up an experiment for protein analysis depends not only on the source and starting material but also on the nature and properties of the protein itself. In addition, knowing whether the protein incorporates into the cell membrane, remains in the cytoplasm, translocates to the nucleus, or becomes secreted is one of the most important aspects of protein analysis and can guide the investigator to determine the appropriate method to apply.

Taken together, these techniques will help us better understand basic human physiology and ultimately the mechanisms of human disease. The addition of the academic surgeon to the community of protein researchers is an important one and can only help to further our ability to adequately diagnose and treat a large population of patients.

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Considerations for Immunohistochemistry

10

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Abstract

Although immunohistochemistry (IHC) has been known and applied for decades, major advances such as robotic sample processing, digital slide image capture, and innovative computerized data analysis uphold the relevance of the technology in current laboratory practice. In keeping with this notion, the expression of a protein by a given tissue or cell can be readily defined using methods like immunoblotting or mass spectrometry. However, when it comes to assessing the microenvironmental location and potential cellular connections, only IHC can provide the pathophysiological spatial context. Thus, IHC enables the selective localization and visualization of protein antigens in tissue sections by means of antigen-specific antibodies that are conjugated to selective fluorescent or enzymatic tags, which can be readily revealed by fluorescence microscopy or chemically pigmented reactions. Since IHC relies on the highly specific antigen and antibody interactions, the technology can be used to identify cell or tissue antigens that range from amino acids and proteins to infectious agents and specific cellular populations and their relevant functional properties, thus underscoring its critical application as a key research/diagnostic tool for investigators studying animal or human tissues. With well-developed tests and controls, correct procedure, and equipment, IHC can be used to analyze a wide variety of cell and tissue

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structures, processes, and functions, such as cell cycle analysis and tissue protein binding in health and disease. Notably, these applications were previously inaccessible by traditional histochemistry staining techniques, which only identified a limited number of proteins and tissue structures. In the past, this process was semiquantitative at best, but new advances are making it possible to obtain quantitative results using IHC. The present chapter reviews the history and applications of IHC, basic principles, techniques, troubleshooting, analysis, and data interpretation. This chapter will further cover the latest innovations in IHC slide preparation and labeling for advanced diagnostic and prognostic applications, such as CyTOF and laser capture microdissection, which allows high-throughput quantitative methods of proteomics, real-time polymerase chain reaction (qRT-PCR), and genomics on selective cells and tissue regions.

Keywords

Immunohistochemistry · Sample preparation · Fixation · Embedding · Sectioning Antigen retrieval · Antibody · Staining · Counter staining · Microdissection Pathology

Introduction

Immunohistochemistry (IHC) is defined as the localization of specific antigens in tissues by staining with fluorophore-labeled or enzyme-labeled antibodies. The advantage of IHC is that it combines anatomical, immunological, and biochemical techniques to identify in situ discrete cell and tissue antigens. There are a number of methods that may be used to localize tissue antigens, which fall under the IHC umbrella. It is called immunocytochemistry when the methods entail the immunological search of antigens in cells cultured in a monolayer preparation or cell suspensions carefully placed on a glass slide. However, the method is usually referred to as immunofluorescence or immunoperoxidase staining when the technique, respectively, utilizes fluorescent-labeled or enzyme-labeled antibodies to detect cell- and tissue-specific antigens. The selection of the method is based on type of specimen under investigation and the sensitivity required. The IHC technique comprises mainly three phases: (1) sample preparation, which includes specimen fixation, processing, embedding, and slide preparation; (2) sample labeling, which comprises different steps involved in the reaction including antigen retrieval, nonspecific site block, endogenous peroxidase block, primary antibody incubation, and the employment of systems of antibody detection, counterstaining, slide mounting, and storage; and (3) sample visualization, interpretation, and quantification of antigen expression. IHC is widely used in diagnostic surgical pathology. The importance of IHC detection methods is growing, and it is, in fact, considered as one of the mainstays of biomedical research in broad range of studies, whether it be in cellular pathway analysis, biomedical device versus host interactions, or physiological and pathophysiological studies in animal models and clinical studies. In

addition to IHC, morphologic criteria are always used in research and diagnostic applications. While IHC is used as a descriptive or, at best, as a semiquantitative tool in a lot of biomedical applications, new advances are making it possible to obtain quantitative results using IHC, thus increasing its relevance in the search and validation of biomarkers for predictive and prognostic studies within the realms of diagnostic surgical pathology and in cancer treatment. Well-developed tests and controls, correct normalization, evidence-based methods, and establishment of minimum criteria for diagnostic accuracy will strengthen the use of IHC in future biomedical research and diagnostic pathology practice.

History

A study published by Marrack in 1934 described the production of specific reagents against typhus and cholera microorganisms using a red stain conjugated to benzidin tetraedro, which marked the beginning of immunostaining techniques [1]. However, the first recorded instance of IHC was described by Albert Coons in 1941, who demonstrated the use of a fluorescent-tagged antibody to identify and localize pneumococci in frozen sections, which could then be visualized by ultraviolet light [2]. The technique was limited and did not enter widespread use until a number of different modifications increased its power and sensitivity. The discovery of enzyme-labeled antibodies in the 1960s [3–5] allowed the detection of antigen-antibody interactions with an optical microscope and increased its utility to a broad group of researchers and pathologists. Subsequent discovery of the unlabeled antibody peroxidase-anti-peroxidase and the alkaline phosphatase-anti-alkaline phosphatase method significantly expanded the application of IHC technique [6–8]. The introduction of diaminobenzidine molecule (DAB) conjugation to antibodies [9, 10] and gold colloidal particles as pigmented materials led to an important method of subcellular immunostaining [11, 12]. Antigen retrieval methods (exposure of antigen epitopes present in tissue sections) significantly improved staining of formalin-fixed, paraffin-embedded tissue sections [13, 14], allowing widespread clinical use of IHC and more reliable immunostaining in specimens with better preserved architecture. The advent of secondary antibody detection using the avidin-biotin-peroxidase complex (ABC), labeled streptavidin-biotin methods, or other labeling systems including the tyramide signal amplification, and polymer-based labeling allowed the specific detection of tissue antigens in formalin-fixed and paraffin-embedded sections, making IHC a diagnostic routine of pathological anatomy [15–19].

Applications of IHC

The immunohistochemical reactions can be used in different settings within basic research or clinical and pathological anatomy laboratories. Through use of highly specific antibodies, sensitive and accurate detection systems, and wide selection of molecular targets and antigens, IHC has become an essential tool that is intimately

integrated into clinical medicine as well as basic science investigations for diagnosis and targeted therapy. As previously mentioned, the versatility of IHC lies in the spectrum of molecular targets, such as cytoplasmic, nuclear, and membrane proteins and lipids found in cells and tissues that can be detected with specific antibodies raised against those antigens. This enormously expands the specificity of staining beyond what is available by using special histochemical stains. However, IHC is best used to complement the accepted and standardized morphologic criteria provided by histochemical staining rather than replace it. Knowledge of special stains is useful to both the basic science and clinical researcher and may obviate the need to purchase expensive antibodies and develop an IHC protocol. A review of routine hematoxylin and eosin (H&E)-stained slides is often helpful as an anatomic reference, but depending on the tissue, other stains may give significantly more information regarding normal structure and pathology present. For example, Periodic acid-Schiff staining of kidney tissue under pathological and diseased states is advantageous over H&E, as it more effectively demonstrates the pathologic changes in tubular and glomeruli structure (Fig. 10.1). Similarly, heart valves under pathologic and progressive disease states are better visualized with pentachrome staining because they are comprised of highly organized extracellular matrix components including collagen, elastin, and glycosaminoglycans in different regions of the valve, which have different functions [20]. Table 10.1 highlights some of the commonly used special stains in both basic science and clinical research.

In basic science research and diagnostic surgical pathology, immunoperoxidase methods are widely used to extract additional information that is not available by H&E staining. IHC can be used to visualize the processes in both physiologic and

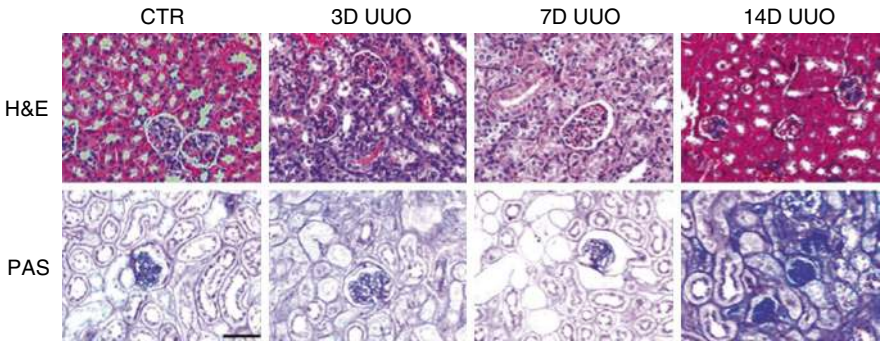

Fig. 10.1 Top row—hematoxylin and eosin-stained murine kidney cortex sections from control mice (uninjured mice—CTR) and mice after unilateral ureteral obstruction (UUO surgery) at day 3, 7, and 14 post injury. H&E staining demonstrates the overall kidney parenchyma with the tubules and glomeruli. However, the limitation of this staining is that it does not capture the change in the tubular structure under the progressive disease state. At day 14, we observe less intact tubular cell nuclei due to injury-induced cell necrosis, but we cannot see the tubules. Bottom row—Periodic acid-Schiff staining (PAS) allows the researcher to quantify the intertubular space, wherein increased space correlates with progressive pathophysiology of the disease, which is not adequately demonstrated by H&E. Scale bar is 75 μm

Table 10.1 Common special histology stains

Name	Components	Color	Tissue uptake
Acid-fast bacteria (AFB)/Ziehl-Neelsen	Carbol fuchsin	Bright red	Mycolic acids (lipids) mainly found in mycobacteria
Alcian Blue		Blue	Glycosaminoglycans
Alcian yellow/toluidine blue	Alcian yellow cation	Mucin—yellow *H. pylori*—blue	Gastric mucus, *H. pylori*
Alizarin red	1,2-dihydroxyanthraquinone	Red/light purple	Calcium
Bielschowsky silver	Silver nitrate	Red/brown	Nerve fibers
Chloroacetate esterase (Leder)	Naphthol AS-D chloroacetate, dimethyl formamide, New fuchsin, sodium nitrite	Leukocyte esterase—red Mast cells—red Nuclei—blue	Enzyme of neutrophil leukocytes, granulocytes
Congo red	Bis (diazonium) derivative of benzidine and naphthionic acid	Red	Amyloid
Cresyl violet	Cresyl violet acetate	Dark blue	Nissl substance in neurons
Dieterle	Silver nitrate	Gray/black; background: yellow	Whole organisms
Diff-Quik	Triarylmethane dye, xanthene dye, thiazine dye	*Helicobacter*—dark blue Background—light blue Platelets—violet to purple	*H. pylori* and *Pneumocystis jirovecii*
Elastin van Gieson	Fuchsin/picric acid	Black	Elastin fibers
Methyl green-pyronine		Blue-green (DNA) Pink-red (RNA)	DNA and RNA
Feulgen		Red	Nucleic acid (DNA)
Fontana massa	Ammoniacal silver nitrate	Black	Melanin, argentaffin granules
	Gold chloride	Pink-red	Nuclei
		Pale pink	Cytoplasm
Giemsa	Methylene blue, eosin, azure B	Blue (polyanions) Pink (polycations)	Polyanions and polycations

(continued)

Table 10.1 (continued)

Name	Components	Color	Tissue uptake
Gram	Crystal violet—Iodine	Violet (gram-positive bacteria)	Peptidoglycan
Gomori's one-step trichrome	Chromotrope 2R Fast green FCF Phosphotungstic acid	Nuclei—black Cytoplasm, keratin, muscle fibers—red Collagen and mucus—green or blue	Collagen and smooth muscle fibers
Grocott's methenamine silver (GMS)	Chromic acid, sodium metabisulfite, borax, methenamine, silver nitrate	Brown to black	Polysaccharide components of fungal cell wall
Hall	Fouchet's reagent	Green	Bilirubin
Herovici	Acid fuchsin, picric acid	Blue (young collagen and reticulum) Red (mature collagen) Yellow (cytoplasm)	Collagen, reticulum, cytoplasm
H&E	Hematoxylin	Blue	Nuclei
	Eosin	Red	Cytoplasm, collagen, muscle fibers
Jones	Methenamine	Black	Carbohydrates in membranes
	Periodic acid-Schiff	Red	Glycoproteins, polysaccharides
Leder	Chloroacetate esterase	Red	Mast cells
Lendrum's method (Picro-Mallory)	Phloxin, tartrazine	Nuclei—blue Acidophil virus inclusion bodies—red Paneth cell granules—red Background—yellow	Fibrin
Luxol fast blue/cresyl violet	Luxol fast blue	Blue	Myelin
	Cresyl violet	Turquoise	Erythrocytes
		Purple	Nuclei and Nissl substance

Masson-Fontana	Ammoniacal silver solution	Melanin, argentaffin cells—black Nuclei—red	Serotonin, melanin, and other silver-reducing (argentaffin) substances
Masson's Trichrome (TRI)	Iron-hematoxylin	Black	Nuclei
	Biebrich scarlet-acid fuchsin	Blue-green	Collagen fibers
	Fast Green FCF	Pink/red/brown	Cytoplasm
		Scarlet	Erythrocytes, keratin, myelin
		Light green	Mucin
Mayer's mucicarmine	Carmine, aluminum chloride	Red	Polysaccharides on cell wall
Movat's pentachrome	Iron-hematoxylin	Black	Nuclei, elastic fibers
	Alcian Blue	Yellow	Collagen, reticular fibers
	Resorcin-Fuchsin	Blue	Ground substance, mucin
	Scarlet-acid fuchsin	Scarlet	Fibrin
	Safran	Red	Muscle
Mucicarmine		Red	Mucin, polysaccharide
Oil Red O	Sudan Red 5B, $C_{26}H_{24}N_4O$	Red	Lipids (frozen section)
Orcein	Potassium Permanganate, oxalic acid, periodic acid, orcein solution	Dark brown	Elastic fibers
p-Dimethylaminobenzylidene rhodanine		Red/orange	Copper in tissues
Page's Eriochrome Cyanine R		Pink to red	Myelin
Periodic acid-Schiff (PAS)		Purple/magenta	Polysaccharide components of fungal cell wall
Phosphotungstic acid-hematoxylin (PTAH)	Hematoxylin, phosphotungstic acid	Deep blue	Abnormal neuroglia (reactive astrocytosis)
Picrosirius red		Nuclei, cytoplasm—yellow Collagen—red	Nuclei, cytoplasm Collagen

(continued)

Table 10.1 (continued)

Name	Components	Color	Tissue uptake
Prussian blue	Ferric hexacyanoferrate	Blue	Iron/hemosiderin
Periodic acid-Schiff		Red/magenta	Polysaccharides, glycoproteins, glycolipids
Schmorl	Ferric ferrocyanide	Blue	Melanin
Steiner & Steiner	Gum mastic solution, Hydroquinone, Oxidizer solution Silver nitrate solution	Black to brown	Whole organisms
Sudan Black B		Blue/black	Lipids
Toluidine blue O	Tolonium chloride	Blue-green	Nuclei
Sudan Black B	$C_{15}H_{16}N_3S$	Red	Mast cell, cartilage
Van Gieson	Picric acid and acid fuchsin	Red	Collagen, muscle
Verhoeff	Hematoxylin, iron (III) chloride, Lugol's iodine, Van Gieson's stain, acid fuchsin, acid fuchsin, sodium thiosulfate	Black	Elastic fibers
Villanueva Osteochrome Bone	Fast Green FCF, methylene blue, basic fuchsin, sodium acetate	Osteoid seams—green to red Osteocytes—Red Cytoplasm—Green	Mineralized or undecalcified bone
Van Gieson	Picric acid	Pink/deep red	Collagen
Villanueva Osteochrome Bone	Acid fuchsin	Yellow	Muscle, cytoplasm, erythrocytes, fibrin
Von Kossa	Silver nitrate	Black	Calcium salts
	Nuclear fast red	Yellow	Muscle, cytoplasm, erythrocytes, fibrin
Warthin-Starry	Silver nitrate	Dark brown/black	Whole organisms
Wright/Giemsa	Eosin	Red	Erythrocytes, eosinophils
Warthin-Starry	Methylene blue	Blue	Basophils, lymphocytes
Wright/Giemsa	Eosin	Violet/purple	Neutrophils, platelets

pathologic conditions. For example, murine skin has a complex structure with several layers (i.e., epidermis, dermis, adipose layer, and panniculus carnosus) which can be clearly visualized by H&E staining. However, the cellular composition is not homogenous in the skin. Several cell types, including a variety of epidermal cells, mesenchymal cells, immune cells, and stem and progenitor cells, reside in the normal skin, which cannot be accurately identified with H&E, and therefore one must rely on IHC techniques for the localization of these different cell types in the skin. When there is an injury in the skin tissue, the healing process spans several overlapping stages with contributions by different cell types at different stages of healing, which is again best evaluated by combination of histology and cell-specific IHC methods (Fig. 10.2) [21, 22]. Similarly, IHC in combination with basic histology can be used to identify the differences in physiologic and disease states. For example, as shown in Fig. 10.3, autopsy specimens from healthy control patient lungs versus those from congenital diaphragmatic hernia (CDH) patients demonstrate a significant thickening of the pulmonary artery as shown in H&E staining. Further special staining with Alcian Blue indicates a significant presence of collagen in the thickened artery vessel wall as expected. Interestingly though, staining with antibodies against hyaluronan (HA)-binding proteins shows significant accumulation of hyaluronan in the perivascular matrix in CDH lungs which suggests a role for hyaluronan in CDH-associated phenotype (Fig. 10.3). This level of deeper understating afforded by the combination of histochemistry and IHC techniques can propel new investigations and advances in biomedical sciences.

The specific three-dimensional configuration of molecules recognized by an individual antibody is called an antigenic determinant or epitope. Antibody binding to a cell-specific marker allows specific cell types to be recognized within a tissue. While routine H&E staining can potentially point to the presence of certain cell types based on their morphology, i.e., progenitor cells may have eosinophilic cell body with eccentric large nuclei (like boiled egg shape), or the polymorphic segmented nucleus of the neutrophils, IHC can define these cell types accurately (Fig. 10.4). While IHC can be used to recognize specific cells within a tissue, the actual specificity of the marker may need to be confirmed and optimized by an individual user for different tissue types. For example, CD31 staining is widely used as a marker for angiogenesis and new blood vessel formation in basic science research. However, CD31 is a broad-spectrum marker for several cells, including endothelial cells as well as circulating platelets, monocytes, and macrophages/pericytes localized in the blood vessel walls [23]. The use of a secondary marker, such as MECA-32 or VE cadherin, is therefore necessary to identify neo-angiogenesis under conditions such as active wound healing process, where the other cell types that can potentially express CD31 may also be present in abundance in the granulation tissue [22, 24]. It is not only sufficient to show the formation of new blood vessels in the granulation tissue, but it is also important to demonstrate the functional anastomoses of the newly formed vasculature with the host vasculature. Furthermore, the steps involved in IHC can also be used for functional evaluation by injecting fluorescent-labeled or biotinylated lectin into the animals and being allowed to circulate for a short time before euthanizing the mice. The wound sections from these

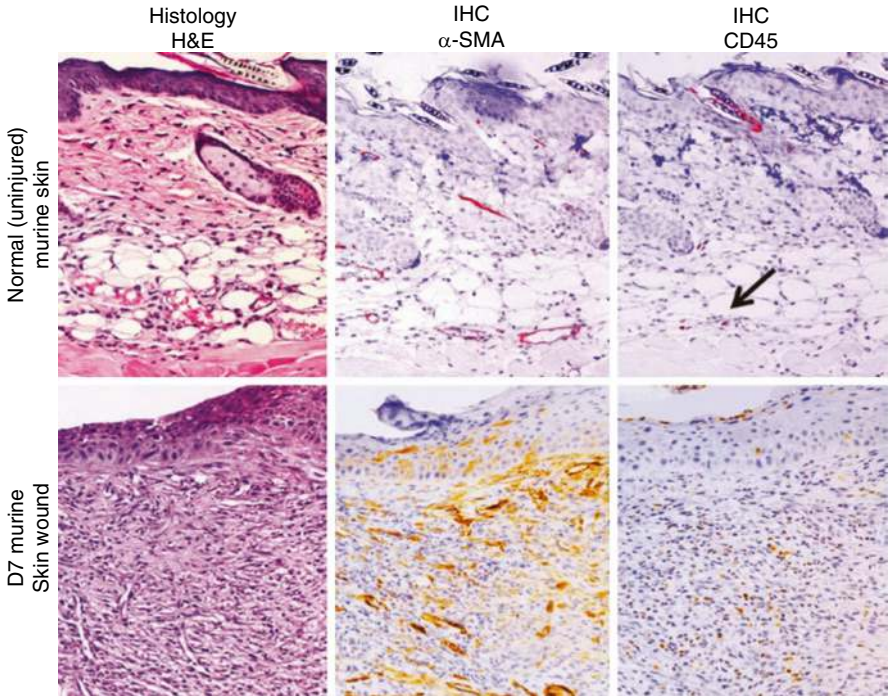

Fig. 10.2 Top row–murine normal (uninjured) skin. Bottom row—murine skin wound tissue sections at day 7 post wounding. Parallel sections were cut about 50 μm apart from each other from a block of FFPE skin and wound tissue. The first panel shows hematoxylin and eosin staining of the skin and wound, which allows the identification of the epidermis, dermis, hair follicles, adipose, and panniculus carnosus (underlying muscle) layers of the skin, in comparison to loss of the hair follicles, adipose, and panniculus muscle in the wound sections. The second panel shows IHC with antibody against alpha-smooth muscle actin (a-SMA) which marks myofibroblasts and the pericytes of the mature blood vessels. As noted, there are very few a-SMA-positive cells in uninjured skin under homeostasis, and most of the staining was associated with vessels. In contrast, wound tissue had significant staining in both the active myofibroblast cells and the newly forming vessels in the wound. The third panel is IHC for antibody against CD45, which is a common leucocyte marker. Again, very few positive cells are noted in the uninjured skin, as compared to an abundance of these cells in the wound tissue

animals can then be processed and stained with anti-GFP antibody or ABC-DAB to visualize the presence of positive staining of lectin in neovessels. Another example of the need for secondary markers is the use of CD68 (Cluster of Differentiation 68), a 110-kDa transmembrane glycoprotein, to identify human monocytes and tissue macrophages [25]. The CD68 antibody can be used to identify tissue macrophages by targeting the CD68 antigen. However, recent literature suggests subsets of tissue macrophages exist with very distinct functional attributes and can rapidly change behavior in response to microenvironmental stimuli to promote inflammation (M1), tissue deposition (M2a), or remodeling (M2c) [26]. IHC with dual labeling of subset cell-specific antigen along with a functional protein-specific antigen

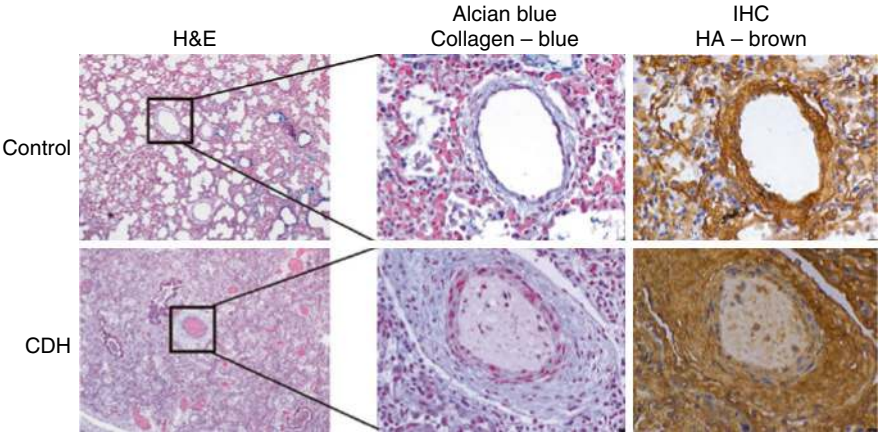

Fig. 10.3 Autopsy specimens from control patients (top row—human lung) and CDH patients (bottom row—human lungs from CDH-related pathology). Parallel sections were cut about 50 μm apart from blocks of FFPE lung tissues. The first panels with H&E staining demonstrate normal architecture of lung parenchyma with the gas exchange spaces in control lungs. CDH patient lung tissue appeared more dense and fibrotic with increased eosin staining. CDH patients also demonstrate thickening of pulmonary artery vessel wall, which was confirmed with Alcian Blue staining, which stains collagen. There was a significant HA accumulation and abnormal HA deposition in CDH patients as identified with hyaluronan-binding protein (raised against versican G1 domain)

will allow tracking macrophage phenotype changes in the healing (or lack thereof) of different conditions. For example, M1 macrophages can be stained with CCR7 and TNF-α, and M2 can be dual stained with CD163 or CD206 and arginase. In conditions such as human chronic diabetic foot ulcers, macrophages function as major regulators of healing through their dynamic phenotypic changes [27], and IHC can serve both as a detection tool of macrophage phenotypes, which holds potential to allow a personalized medicine approach to wound care and as a prognosis tool for tissue repair [28–30].

IHC can also be used to identify certain cellular processes such as proliferation, which can be recognized by antibodies to proliferation-specific markers Ki67, proliferating cell nuclear antigen (PCNA), or by injection of a synthetic nucleotide bromodeoxyuridine (BrdU) prior to euthanasia, followed by staining using an anti-BrdU antibody [31]. In addition, dual labeling with cell-specific and proliferation-specific markers allows the identification of proliferation within subsets of cells in a tissue [32]. Other important cellular processes, such as apoptosis, can also be studied in the context of their spatial and temporal regulation using IHC. Apoptotic cells may be recognized by terminal deoxynucleotidyl transferase dUTP [2′-deoxyuridine, 5′-triphosphate] nick end labeling (TUNEL) staining or by antibody detection of cleaved caspase-3 or other apoptosis markers. The TUNEL assay relies on the presence of DNA fragments generated by apoptotic cells that can be identified by terminal deoxynucleotidyl transferase (TdT), an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. The cleaved caspase

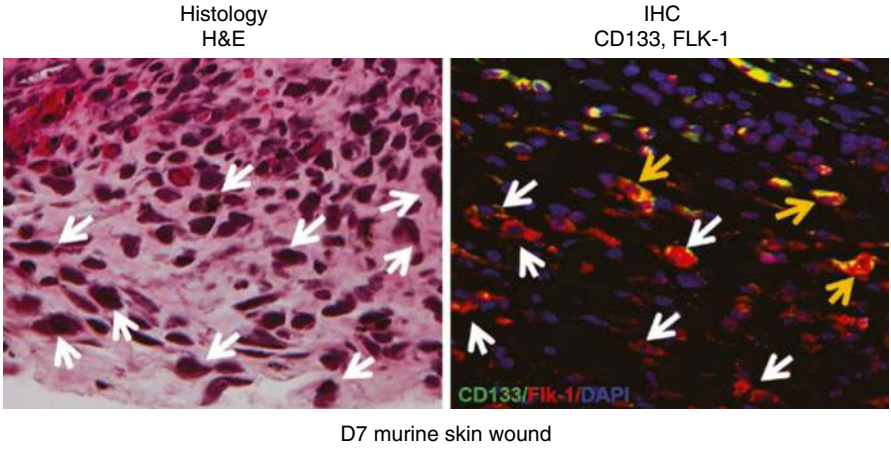

Fig. 10.4 Parallel sections were cut about 50 μm apart from blocks of FFPE murine skin wound tissue. The first panel with H&E staining demonstrates the wound bed with several large cell bodies with eosinophilic cell and large eccentric nuclei (black arrows). These characteristics broadly define the endothelial progenitor cells. However, when dual labeling with CD133 (a stemness marker present on several hematopoietic progenitor cells) and Flk-1 (committed endothelial lineage-specific marker) was performed, only few cells with the characteristic morphology had dual positive labeling for CD133 and Flk-1 as shown (yellow arrows in right panel)

assay, on the other hand, relies on caspase cleavage of protein substrates being a pivotal cascade that is unique to apoptotic cells [33, 34]. This process is commonly implemented in the field of wound healing and cancer research, as wound healing involves cascades of cell infiltration, necessitating programmed cell death of early invading cells to make way for new cells, and cancer research frequently focuses on inducing cell apoptosis in malignant tumors. In cancer specifically, differentiation between normal and rogue cells may be the deciding factor between life and death, and IHC plays a large role in the identification process. Similarly, changes in the cellular oxidative stress can be detected either within the nucleus (anti-8-hydroxy-2′-deoxyguanosine or anti-thymidineglycol staining) [35], lipid membranes of cells (multiple oxidized lipid markers), or by protein oxidation (anti-tyrosine dimer, anti-nitrotyrosine, or anti-halogenated tyrosine) [36].

Further, IHC localization of antibody staining within the nucleus, cytoplasm, or cell membrane can be used to determine cell-signaling and protein activation states [37]. While protein synthesis in itself is highly regulated, the proteins further undergo many modifications before complete maturation and functional activation [38]. For example, the functional modification associated with phosphorylation occurs on serine and threonine amino acids, and these changes may affect their structure [39], and potentially their interaction with antibodies produced against the inactivated state. Phospho-specific antibodies are now available for immunohistochemical use to determine the functional status of the protein and improve the results of immunohistochemical staining; however the success of IHC may rely on optimization of the sample preparation and staining protocols [40, 41]. One of the

well-studied transcription factors, signal transducer and activator of transcription 3 (STAT3), is implicated in several pathways downstream of wound healing and regeneration and is known to be activated by several cytokines. STAT3, when inactive, can be detected in the cytoplasm using an antibody to the py705 component of the STAT3 protein complex. After activation, STAT3 moves to the nucleus, and, therefore, nuclear immunostaining of STAT3 indicates that it has been activated (Fig. 10.5) [42]. Alternatively, a specific antibody that targets phosphorylated STAT3 (phosphor STAT3 Tyr 705) can be used to ascertain the findings. Another example is the transcription factor protein complex, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), that when inactive can be detected in the cytoplasm using an antibody to the p65 component of the NF-κB protein complex. After activation, NF-κB moves to the nucleus, and hence, nuclear immunostaining of p65 indicates that NF-κB has been activated. Likewise, antibodies can be developed against a phosphorylated form of protein such as phospho-extracellular signal-regulated kinase (pERK) or phosphorylated isoform of protein kinase C (PKC) epsilon, which do not recognize the non-phosphorylated form, allowing activation of the extracellular signal-regulated kinase (ERK) or PKC-epsilon pathway to be identified [43].

The cytoskeleton and extracellular matrix (ECM) can also be studied using IHC. Antibodies to microtubules, actin, and intermediate filament components vimentin, keratin, and laminin are widely available, as are antibodies to collagen, elastin, and proteoglycan species [44]. Specific structural components such as focal adhesions can also be identified by antibodies to components of the focal adhesion complex such as vinculin or focal adhesion kinase [45]. In addition, the proteoglycans found in the ECM which are associated with development and disease can also

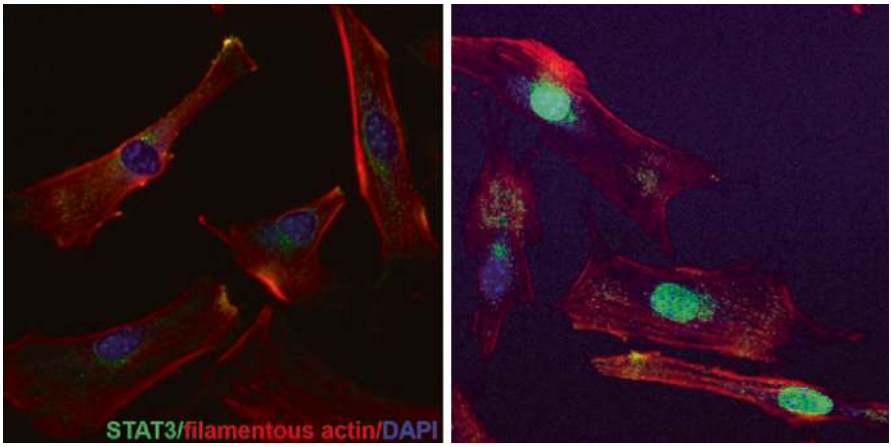

Fig. 10.5 Cell culture monolayers of murine skin-derived primary fibroblasts are labeled with antibody against STAT3. The left panel shows perinuclear and cytoplasmic STAT3-positive staining (GFP). When the cell culture was activated with an immunologic cytokine that is known to signal through STAT3, nuclear translocation of positive signal is observed within 45 min after activation, suggesting activation of STAT3

be studied with IHC [46, 47]. In basic science research, IHC can help immensely in the understanding and discovery of cellular cross talk with the ECM. With the availability of novel transgenic mouse strains, special compartments, such as the bone marrow, or specific cell types can be tagged with constitutive expression of a fluorophore. For example, in Tie-2 GFP mice, where all endothelial cells are GFP-positive [48], or collagen 1 and collagen 2 dual labeled mice [49], IHC can be used to visualize the tagged protein expression along with other cells of interest and ECM molecules. IHC can also be used to track and identify tissue-specific localization of treatments administered. For example, if GFP-tagged viral vector particles are injected in murine models for targeted delivery to a specific organ (e.g., heart, placenta, or kidney), IHC of sectioned tissue will allow determination of the viral particle-associated GFP tag along with determining the cell-specific uptake (Fig. 10.6). In addition, cell fate decisions and plasticity can also be studied using IHC for the localization of specific markers [50]. Using such techniques, it was recently shown that two-thirds of all granulation tissue fibroblasts, otherwise known to be of mesenchymal origin, are derived from myeloid cells, which are likely to be wound macrophages. This work was accomplished by using a novel animal model where only the cells of myeloid lineage express membrane-localized GFP and then using IHC to co-localize GFP with fibroblast-specific FSP-1 antibody [51]. Immunohistochemistry is now used in surgical pathology to determine cancer cell types, cancer subtype classifications, and possible cell of origin in metastatic cancer of unknown primary site.

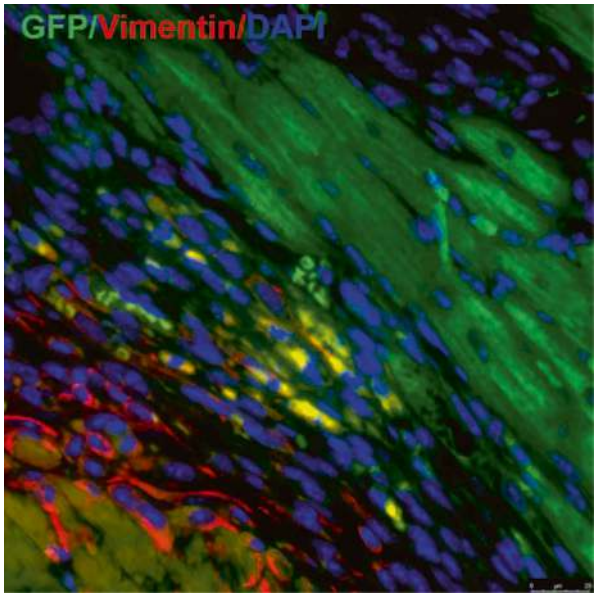

Fig. 10.6 Infarcted region of a mouse heart tissue that was treated with adeno-associated viral vector particles that express GFP label. Frozen section of the tissue was obtained and stained with an antibody against vimentin (T-red). Co-localization of green and red fluorescent signaling along with DAPI counterstaining demonstrates uptake of the vector by vimentin-positive fibroblast cells within the cardiac tissue

Basic Principles

The antibodies utilized in IHC are subdivisions of the immunoglobulin G (IgG) isotype, which can be separated into four isotypes. These distinctions should be considered when setting up controls, discussed further in this chapter. Consisting of two pairs of chains, one light and one heavy, the antibody is arranged in a Y configuration, the ends of which form the variable region. It is this structure that propagates the binding properties between antigens and antibodies, as the Y structure provides two antigen-binding domains on a single antibody.

The fragment crystallizable (Fc) portion of the antibody is species specific and contains domains for binding complement, the Fc receptor, staph protein A, and inflammatory cells [52]. The different IgG isotypes vary in terms of these binding capabilities. Antibodies in one species can be raised to match the Fc region of another species, allowing for the amplification of signal. The first antigen-specific antibody is known as the primary antibody, as opposed to a species-specific secondary antibody. To remove an antibody, a smaller single antigen-binding domain (fragment antigen-binding or Fab fragment) can be generated by using papain to digest the antibody.

When an animal is immunized with an antigen, it generates serum with multiple antibodies capable of recognizing different epitopes at varying affinities, also known as polyclonal antibodies. To increase the specificity of the process, a myeloma cell line fused with an antibody producing cell (hybridoma) can give rise to a monoclonal cell line after antigen-specific selection, which will secrete antibodies of a unique specificity. Monoclonal antibodies are generated by immortalized cell lines, which results in greater consistency between different batches. It is vital to maintain accuracy when acquiring commercial monoclonal antibodies by verifying the manufacturer specified clone with those used in previous research and related literature. Polyclonal antibodies can also be used and are generally more sensitive, though they lack the specificity of their engineered counterparts and may result in consistency issues from batch to batch. Outside of common antigens, antibodies may be raised to recognize whole molecules or against the N terminus, C terminus, specific amino acids, or phosphorylated states.

Technical Procedures

There are numerous methodological approaches to IHC, but all involve three basic steps: preparation, labeling, and visualization. The process can be further broken down into individual procedural steps, ordered by their chronological necessity. Automated machines that can perform the vast majority of immunohistochemical protocols with the exception of antigen retrieval are available. In fact, the equipment and kits that contain required solutions for each procedural step are optimized and prepackaged in order to maintain consistent histologic quality in routine diagnostic and clinical pathology labs. However, the costs associated with the equipment, the required maintenance, and nonavailability of special kits (i.e., nonroutine stains and

nonhuman research with animal model antigen-antibody interactions) for basic research means the equipment is usually limited to histological core and diagnostic pathology laboratories.

Sample Preparation

Fixation

IHC builds on histochemistry by localizing target components within cells and tissue through the use of immunological processes. This is only possible if the cells and tissues are appropriately processed and fixed such that the preserved tissue architecture and cell morphology are as close to lifelike state as possible, and they are recognizable under the microscope. Different fixation and tissue processing methods may affect antibody recognition, such that strong staining is possible with one method, but not with another. In general, for research purposes, antibody staining is easier in frozen sections; however, tissue architecture is better preserved in paraffin sections.

As devitalized tissues undergo bacterial decomposition and autolysis from release of lysosomal proteolytic enzymes, preservation of antigenicity requires either rapid fixation, which deactivates enzymes, or thermal suspension, which involves lowering the temperature to a point where the enzymes are no longer active. Fixation serves two additional purposes: to immobilize molecules within the cell and prevent diffusion and to increase tissue rigidity for more successful sectioning.

In general, all fixatives should be made fresh each time, and care should be taken to avoid prolonged fixation which can chemically mask antigens and prevent antibody binding, which may or may not be reversed through antigen retrieval. Specifically, a 10:1 ratio of volume of fixative to volume of tissue being fixed is recommended. Since fixation takes place via diffusion, size and density of tissue specimen are important, and in general tissue should be no larger than 5 mm thick; otherwise perfusion or vacuum-assisted fixation steps should be employed.

Fixation Through Freezing

In this process, tissue is extracted and directly frozen. Due to the formation of ice crystals, freezing specimens generally leads to loss of tissue architecture. Two strategies are commonly used for preservation of antigenicity while minimizing loss of cell structure. The first is to freeze the sample as rapidly as possible. This can be achieved in multiple ways, such as immersion in isopentane at its freezing point, contact with liquid nitrogen on a metallic surface, or placement in some combination of dry ice and either acetone or ethanol. The other strategy is to use cryoprotection and dehydration. Sucrose is commonly used as a cryoprotectant [53]. For research purposes, specimens are generally embedded and frozen in optimal cutting temperature compound (OCT, mixture of polyvinyl alcohol and polyethylene glycol) to allow frozen sectioning. Cell membrane antigens and cytokines are frequently more successfully identified using IHC on frozen sections rather than with paraffin-fixed tissue.

Chemical Fixation

Chemical fixatives can be roughly divided between those that cause protein cross-linking and those that cause protein coagulation or precipitation. The most commonly used cross-linking fixatives are aldehydes. Formalin solution (37–40% formaldehyde existing as low polymers and 60–63% water) is routinely used in basic research utilizing animal models. The low polymers of formaldehyde must be broken down to monomeric formaldehyde for active fixation by protein cross-linking through the aldehyde group, also known as a methylene bridge. This can be done by dilution with a buffer solution at physiological pH, generally to 4% or 10%. Methanol (10%) is frequently added to formalin by the manufacturer to slow down polymerization to paraformaldehyde.

Paraformaldehyde, which is also commonly used for research purposes, consists of higher polymers of formaldehyde and requires heating in buffered solution to 60 °C within a fume hood for solubilization [54]. Currently, both methanol-free formalin and aliquots of frozen formaldehyde prepared from paraformaldehyde are also commercially available. Although formaldehyde generally penetrates the tissue after 12–24 h, significant cross-linking may require up to 7 days to occur. As increased cross-linking tends to decrease antigenicity, over-fixation may render specimens unusable for immunohistochemical analysis.

Glutaraldehyde has two active aldehyde groups that allow more thorough cross-linking, making it ideal for electron microscopy, but less ideal for IHC, as the free aldehyde groups must be removed or blocked to prevent nonspecific binding and the thorough cross-linking frequently destroys antigenicity. The more thorough cross-linking also impedes paraffin penetration. From a practical standpoint, the more expensive "electron microscopy (EM) grade" glutaraldehyde that contains the monomer and low polymer forms required for adequate cell and tissue penetration rather than the "technical grade" glutaraldehyde that has larger polymers should be used for research purposes.

Coagulating Fixatives

Coagulating fixatives include acetone, ethanol, methanol, trichloroacetic acid, and zinc-based fixatives. Acetone, ethanol, and methanol when used alone can cause significant shrinkage and hardening artifacts but do allow better preservation of DNA and RNA as well as preservation of antigenicity without the need for antigen retrieval. Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) or methyl Carnoy is a commonly used non-formalin fixative and may preserve carbohydrate and cytosolic membrane antigens more successfully than aldehyde-based fixatives [53].

Perfusion Fixation

Perfusion fixation is fixation via blood flood. For basic science research involving murine models, it is advantageous in terms of vessel morphology, as the fluid perfectly preserves the tissue structure. Small animals can be easily perfused at physiologic pressures through either the heart or the aorta using a sphygmomanometer or gravity-based perfusion fixation apparatus. This is particularly effective for antigen

localization, which is optimized by rapid fixation, due the improved delivery of fixative fluid. The method is less effective for larger specimens and can only be used once, as the specimen dies in the process.

However, the initial process of perfusion with phosphate buffered saline (PBS) can be very important to flush out blood (and clots) from tissues such as heart, kidney, liver, and lungs to achieve better staining and avoid too much background noise. The kidneys and liver can be perfused via left ventricular perfusion with PBS, and the investigator can observe the change in color of the organs with perfusion (typically 20 ml PBS is used in perfusion of adult mouse models). The lungs, on the other hand, can be perfused with right ventricular perfusion with PBS until the lungs flush to a white color.

In fact, the most commonly used method for lungs is called the perfusion and inflation fixation method, where in the animal is placed under deep anesthesia, the descending aorta is nicked, and the chest cavity is opened to expose the lungs and the heart, followed by right ventricular perfusion of PBS. The death of the animal is confirmed by secondary method of cervical dislocation. The throat area is then exposed, and the trachea is cannulated with an appropriate gauge needle attached to the end of a tubing with a three-way stopcock that is attached to a 20 ml syringe filled with fixative (typically neutral buffered formalin/PFA) placed at a height of 25 cm to allow the flow fixative under gravity while the lung lobes are inflated. The trachea is sutured after inflation, and the inflated lungs and trachea are dissected en bloc by gently lifting the trachea and releasing them from the chest cavity connective tissue. This process preserves the morphology of the alveolar spaces for further investigation of IHC.

Embedding and Sectioning

Sectioning and Mounting Procedures

Whole mounts can be prepared for IHC without sectioning. They provide an immediate and three-dimensional view of stained antigens, which is useful for rapid surveillance. In general, the technique is limited to tissues that can be prepared as small blocks (<5 mm thick) such as embryos. Antibody penetration can be limited, however, resulting in patchy or false-negative staining.

Frozen tissue embedded in OCT may be cut immediately using a cryostat. Different tissues required cutting at different temperatures, which generally range between −20 and −30 °C. Slice thickness varies depending on the tissue and the skill of the operator but generally ranges between 5 and 10 μm. Thinner sections provide fewer instances of cellular overlap and improved ultrastructural resolution.

Certain applications, such as confocal microscopy, may call for considerably thicker sections. Cutting may introduce folds or wrinkles in the tissue, degrading its stainability and architecture. A newer method of tape transfer for frozen sections has been developed to improve tissue architecture. Essentially, tape is applied to the block prior to sectioning. After the section is cut, it is transferred tissue side down to a polymer-coated slide. The tissue is bonded to the slide using a flash of ultraviolet light, at which point the tape is removed, and the slides can then be processed as

regular frozen sections for staining [55]. Tape transfer systems are commercially available for purchase, but the added expense may or may not be justifiable depending on the goals of the experiment. Slides should be maintained at −20 or −80 °C after cutting to maintain antigenicity.

Embedding Procedures

Paraffin wax remains the most commonly used embedding agent. Specimens that contain bone require special processing or decalcification to allow appropriate sectioning. Decalcification can be carried out using acids or chelating agents. Immunocal (formic acid) is commonly used in basic research. Tissues should be immersed in volume equal to at least ten times the size of the specimen to be decalcified. It may take up to 2–3 days for decalcification, which is deemed finished when the specimen is flexible to the touch or floating in the solution. Specimens must be adequately dehydrated to support infiltration of paraffin. The tissue is placed within plastic cassettes or molds in a specific orientation to facilitate sectioning. After a paraffin block has been created, a microtome is used to create sections down to 4–5 μm thick, which are floated within a 37 °C water bath onto the surface of glass slides. The tissue is bonded to the slides by warming to 60 °C. Although theoretically antigenicity can be maintained in paraffin sections for long periods of time, certain antigens will stain better if IHC is carried out within a few days of tissue harvest and cutting. An outline of standard procedure for formalin-fixed paraffin-embedded (FFPE) sections is listed in Table 10.2.

Prior to staining, the paraffin must be removed, generally using xylene as a solvent, and the tissue rehydrated to allow the water-soluble staining solutions to

Table 10.2 Sectioning of FFPE specimens

- Cut the tissue into consistent thin pieces (4–6 μm) to allow for examination on a slide and staining for specific proteins
- The most important requirement for good sectioning is a *very sharp blade*
- Blocks should be cooled in ice slushie before sectioning
- After section is cut, place paraffin ribbon in a water bath
  - About 42 °C
  - Allowed to spread (helps remove wrinkles in the tissue)
- If chipping or crunching of boney specimens occurs during sectioning, paraffin-embedded sections may be surface decalcified by placing the block face down in a dish of Immunocal solution for 5–10 min
- Pick up sections on a glass slide
  - Slide must be coated or + charged
  - Careful to prevent bubbles from coming between the tissue and the slide
  - Prevent wrinkles from forming
- Dry overnight
  - Can be placed on a warming Table 37–40 °C for 5–10 min after drying/draining to help tissue spreading
- Slides are stored with paraffin intact to help protect tissue
- Fresh slides are suggested to get the best IHC stain outcome
- Bake at 60 °C prior to staining to help tissue adhere to slide and to start deparaffinization process

penetrate. Once the tissue has been rehydrated, it is critical that it remains wet, or staining will not occur. Use of a PAP pen to lay down a hydrophobic barrier to keep small volumes of buffer or incubating solutions within a confined location on the slide may assist with this process as does using a humidified chamber for all incubation steps. Plastic resins (methacrylates and epoxy) are occasionally used because of their hardness to facilitate thinner sections for electron microscopy.

Histology core laboratories and diagnostic pathology services are frequently available to assist in tissue preparation, embedding, and sectioning for set fees. Routine immunohistochemical stains may also be available. If a laboratory does not frequently perform paraffin embedding, it may be more economical to outsource tissue processing and sectioning rather than investing in the necessary equipment. Furthermore, as sectioning requires some degree of skill and can be time-consuming, payment for sections cut by a professional technician may be a worthwhile investment.

Antigen Retrieval

Formalin fixation is notorious for loss of antigenicity. Two separate methods of antigen retrieval, enzymatic and heating, can significantly improve immunostaining in formalin-fixed tissue. Empiric testing of antigen retrieval methods may be required to determine the best method for a particular antibody. Trypsin, pepsin, pronase, and proteinase K are commonly used enzymes for antigen retrieval. A heat-based method involving boiling sections in heavy metal solution was first described in 1991 by Shi and colleagues [14]. Several molecular mechanisms for heat-based antigen retrieval have been proposed, including reversal of tyrosine-arginine binding to restore an epitope [56] and restoration of the negative electrostatic charge of tissue-bound antigens allowing them to better attract positively charged antibodies [57].

Heat-based antigen retrieval has been modified by numerous authors to use alternate buffers, most commonly citrate or ethylenediaminetetraacetic acid (EDTA), to avoid heavy metals and alternate heating methods including microwave, water bath, pressure cooker, steamer, and autoclave [58]. If the desired epitope is intracellular (either cytoplasmic, nuclear, or the cytoplasmic portion of a membrane component), further treatment to allow better penetration of antibody into the cell membrane may be helpful. Solvents such as acetone, ethanol, or methanol or detergents such as saponin, Triton-X™ (Dow Chemical Company, Midland, Michigan), Tween® (ICI Americas, Marlborough, Massachusetts), and sodium dodecyl sulfate (SDS) are frequently used for this purpose, although some authors argue that the purpose of detergents is merely to ensure continual hydration of the specimen. Commonly used enzyme and heat-induced epitope retrieval techniques are listed in Table 10.3.

Blocking

To improving visualization of specific target, most IHC protocols contain one or more blocking steps. Normal serum is used to prevent nonspecific antibody binding, either from nonspecific antibodies inherent in polyclonal antibodies or from attraction of the highly positively charged antibodies to negatively charged tissue

Table 10.3 Retrieving target antigens

Purpose: Unmask antigens that were cross-linked during fixation to allow for interaction with primary antibody
• Heat-induced epitope retrieval (HIER)—Uses heat and pH to uncross proteins – Tissue is heated to just under boiling temperature (95 °C) via microwave (10 min), water bath (20 min), or pressure cooker (set time) – Target retrieval solution Citrate buffer, pH = 6.0 EDTA, pH = 8.0 0.01 M Tris-HCl, pH = 1 or 10 (*) – After heating, tissue sections are gradually cooled in solution (20 min) – Rinse slides in PBS (5 min) • Enzyme-induced epitope retrieval (EIER)—protein are unmasked using an enzyme that digests tissue – Proteinase K (5–15 min at RT) – Pronase – Trypsin (0.1%, 5–15 min at 37 °C) – Pepsin – Rinse slides in PBS (5 min)

Note: The suggested times are only approximate. These have to be optimized for different applications and tissue types

components (frequently collagen). Normal serum from the same species as the bridge or secondary antibody is generally used.

Endogenous enzyme activity must also be blocked to prevent false-positive staining. Alkaline phosphatase is completely inactivated by fixation and paraffin embedding, but a short fixation step may be required for fresh frozen sections. Peroxidase activity is maintained through paraffin embedding and fixation and should be inactivated by incubation with either methanol and H_2O_2 or 0.075% hydrochloric acid in ethanol. If an avidin or streptavidin-biotin amplification system is used, endogenous biotin (high levels in kidney, liver, and spleen) may need to be blocked as well with serial incubation with avidin solution followed by biotin solution after the normal serum is applied and before incubation with the biotinylated antibody [58]. For immunofluorescence, autofluorescence from lipofuscins (by-product of red blood cells) and collagen and elastic can be masked by blocking in PBS with 0.2% picric acid. Fixative-induced autofluorescence from aldehydes can be masked with sodium borohydride or 50 mM ammonium chloride.

Sample Labeling

Primary Antibody

Primary antibody selection is an important determinant in the success or failure of IHC experiments. Although there are multiple manufacturers of commercially available antibodies, the quality may vary significantly between producers. As antibodies are relatively expensive, it is also important to consider several additional factors. The first factor is the species that produced the primary antibody. Mice are

the most common source of monoclonal antibodies. However, staining mouse tissue with mouse primary antibody may introduce nonspecific binding artifacts because of the need to use an anti-mouse secondary antibody for amplification. This may be overcome with additional blocking steps, but may not be necessary if a rat monoclonal antibody is available. The second factor is whether the antibody has previously been validated for use in IHC. Many antibodies will work for Western blotting, enzyme-linked immunosorbent assay (ELISA), or immunoprecipitation, but do not stain consistently for IHC. This information is available from the manufacturer. A third factor is the manufacturer recommended dilution. Antibodies which can be used at higher dilution suggest avid binding to the antigen. This allows both conservation of antibody and decreased nonspecific staining.

Amplification

Amplification is a critical step for improving sensitivity, which again allows higher dilutions (lower concentration) of antibody, improving specificity. Because each primary antibody has up to eight immunoglobulin-binding sites, secondary antibodies conjugated to fluorophores or enzymes were initially used for amplification. The ability of staph protein A to bind to certain immunoglobulin subtypes (human IgG1, IgG2, and IgG4 and mouse IgG2, IgG3, and IgG4) has also been exploited for amplification by conjugation of staph A to peroxidase. However, these two-step methods have, for the most part, been replaced by more sensitive three-step tyramide-based or dextran-polymer-based methods. It should be kept in mind that if the antigen is highly expressed within the tissue of interest, the density of primary antibody binding may actually interfere with some amplification methods.

The most commonly used three-step methods are peroxidase-antiperoxidase (PAP) and avidin-biotin-peroxidase complex (ABC). The PAP method was initially described in 1970 by Sternberger [6]. A primary antibody is used to bind the antigen of interest, for example, rat anti-mouse CD68. A secondary antibody is used as the bridge, e.g., a goat anti-rat antibody. Finally, a tertiary peroxidase-labeled antibody of the same species as the primary antibody is used to localize the enzyme complex to the antigen of interest, e.g., peroxidase-labeled rat antibody [52]. The ABC method exploits the strong binding affinity (K D = 10–15) of avidin for biotin, as this affinity is even stronger than that of antibodies for antigens. Again, a primary antibody is used to bind the antigen of interest. A biotinylated secondary antibody against the primary antibody species of origin is then applied. A preincubated complex of avidin and biotin conjugated to peroxidase is then used to localize the label to the secondary antibody. This method can be further refined by the use of streptavidin, which lacks carbohydrate side chains and has a near neutral isoelectric point, rather than avidin derived from egg white, to reduce background staining. Newer amplification systems exploit the use of pigments other than brown, including red and green pigments to get better contrast with hematoxylin counterstain (Fig. 10.7) and also for improved IHC quantification in specimens with more background, such as skin specimens with higher melanin.

Tyramide-based systems rely on the conversion of tyramide by horseradish peroxidase into a short-lived, extremely reactive intermediate. The intermediate

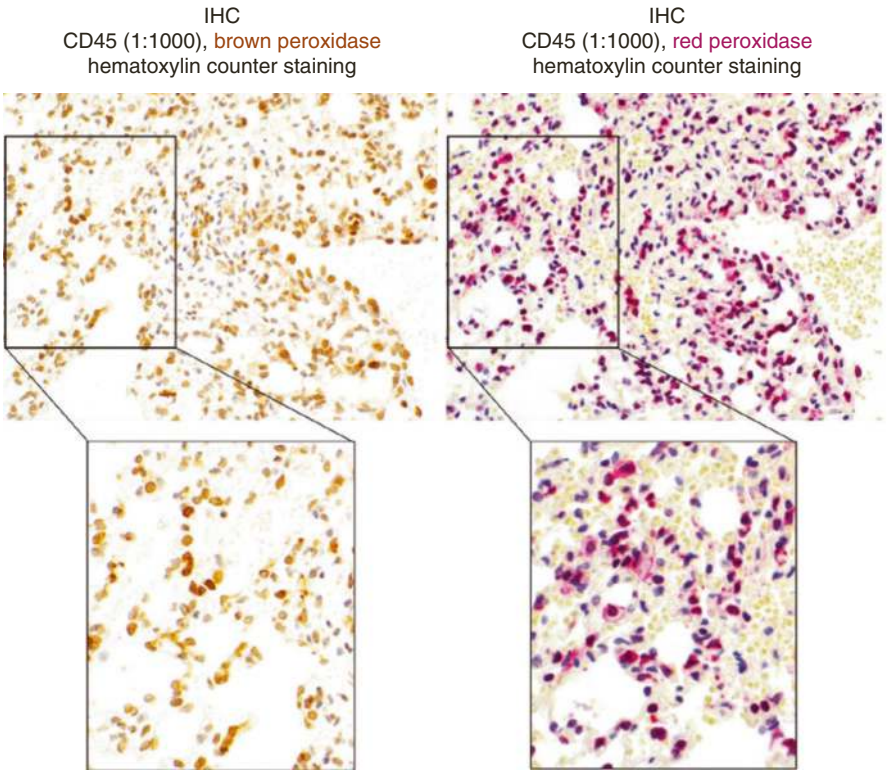

Fig. 10.7 Murine lung sections were perfused, formalin-fixed, and paraffin-embedded. Parallel sections 10–20 μm apart were obtained and stained with an antibody against CD45, a common leucocyte marker. Each of the parallel sections was developed with either PAP or ABC method to produce a brown pigment (left panel) or red pigment (right panel), respectively, and counterstained with hematoxylin. The staining on the right allows easier quantification of the positive cells

covalently binds electron-rich adjacent proteins (primarily tyrosine residues). Both biotinylated tyramide and fluorescently labeled tyramide are commercially available and provide very sensitive signal amplification [59, 60]. The system can be further refined to avoid cross-reactivity with endogenous biotin by the use of fluorescyl-labeled tyramide and a horseradish peroxidase anti-fluorescein antibody [61]. The dextran-polymer technique uses an unconjugated primary antibody followed by a secondary antibody conjugated to an enzyme (usually horseradish peroxidase)-linked dextran-polymer chain. The dextran chain may have as many as 70 molecules of enzyme and ten molecules of antibody attached, leading to strong amplification in the absence of reaction with endogenous biotin molecules [16]. This is likely the most sensitive but also the most expensive method of amplification.

Rolling circle amplification has also been described for IHC. Briefly, a primary or secondary antibody is conjugated to a short oligonucleotide sequence. After the antibody binds the antigen of interest, a complementarily sequenced circularized

probe is hybridized to the oligonucleotide. DNA polymerase is then used for linear extension. The extended DNA product can be hybridized to labeled (either biotin or fluorescent) oligonucleotide probes for detection [62, 63].

Sample Visualization

The primary methods of antibody detection are fluorescence and enzymatic creation of a colorigenic substrate. Fluorescence depends on compounds that, when excited by light of a specific wavelength, emit light at a separate wavelength. This necessitates the use of a fluorescence microscope. In general, fluorescence gives a higher signal-to-noise ratio and more precise localization of antigen than enzyme-based methods. With modern fluorophores, multiple labeling is also simplified when using fluorescence, allowing easier identification of double or higher-level labeled cells. Furthermore, because fluorescence emission is linearly related to the amount of probe present, fluorescent staining results can be assessed quantitatively more easily than enzyme-based methods, and the assessment also can be more easily automated. The basic fluorophores used for many years were fluorescein (greenish yellow) and rhodamine (red). Because they have different emission wavelengths, they can easily be used for double-labeling experiments. Currently, multiple derivatives are available with differing excitation and emission wavelengths allowing multiple-label experiments rather than simple double labeling [53]. Quantum dots, a newer technology, are photostable fluorescent semiconductor nanocrystals possessing wide excitation and narrow emission spectra. They can be conjugated to standard immunohistologic reagents, such as streptavidin, and then complexed with biotinylated primary antibodies in order to provide multiplexed staining of up to five colors [64].

There are several disadvantages to the use of fluorescent labels for research purposes. Labels tend to quench over time, giving a finite period within which documentation of staining must be obtained. The process of dehydration and mounting for cover slipping to optimize viewing resolution often destroys the fluorescent label unless a specific anti-fade solution is used for mounting, which can prolong signal to weeks to months. Fluorescence microscopy must be carried out in the dark because of light filtration requirements. Complex filtration is required to view the signal; this requirement increases with increased numbers of labels used. Lastly, many tissues have at least some degree of low-intensity autofluorescence, which may be enhanced by fixation and prolonged storage and lead to a false-positive signal [52].

Horseradish peroxidase is the most commonly used enzyme label. Diaminobenzidine (DAB) is the most commonly used substrate, because it creates a brown precipitate in the presence of H_2O_2 that is insoluble in the organic solvents used to clear and mount slides. DAB staining can be enhanced by including nickel, cobalt, or osmium to create a blue-black polymer. Alkaline phosphatase is occasionally used to facilitate double labeling, but careful attention needs to be paid to the pH of the substrate solution as the enzyme is only active in alkaline pH. Substrates for alkaline phosphatase include nitro blue tetrazolium-5-bromo-4-chloroindoxyl

phosphate (NBTBCIP) for a blue-purplish stain and diazonium salts. Glucose oxidase is another enzyme available to facilitate double labeling. Several commercially available substrate kits are available which have alternatively colored substrates for all three enzymes. Metals such as gold, mercury compounds, and ferritin can be used to directly label antibodies. Metals are primarily used for electron microscopy, as only gold is detectable with routine light microscopy. Double- or multiple-labeling experiments are possible using currently available IHC techniques. Whether incubations can take place simultaneously or serially depends on the availability of primary antibody from differing species, the amplification system used, and the method of detection.

Counterstaining is frequently used to provide contrast to the primary stain and to provide definition of individual cells. Hematoxylin is commonly used to stain nuclei but may interfere with recognition of a nuclear-based antigen. Eosin may be preferred as a general cytoplasmic stain in this instance. DAPI (4′,6-diamidino-2-phenylindole), Hoechst, and propidium iodide are other commonly used fluorescent nuclear stains. Fluorescence-conjugated phalloidin can be used to counterstain for actin filaments.

Controls

As with any experiment, appropriate controls are required to guarantee specific results from IHC. Controls can be further classified into primary antibody controls, secondary antibody controls, and label controls. Primary antibody controls demonstrate that the primary antibody binds specifically to the antigen of interest. The most common practice is the use of isotype-matched natural antibodies from non-immunized animal (mouse, rat, rabbit sheep, etc.) littermates of the original antibody source, which should not react to target antigen. Other procedures include binding sections from a wild-type animal, but not in a genetic knockout, assuming the knockout does not have a mutated or nonfunctional protein, which may still retain antigenicity. Tissue sections from multiple tissues of many knockout animals are commercially available for this usage. In the absence of an available knockout, Western blots can be used to show that the primary antibody labels a protein at the correct molecular weight. Alternate methods include using cells transfected with the antigen of interest, with non-transfected cells or cells treated with small interfering RNA (siRNA) to the antigen serving as a negative control. Co-localization of two separate antibodies to the same antigen or of the antibody with in situ hybridization for RNA of the antigen also can be used to demonstrate specificity. An absorption control involves preincubation of the primary antibody with the purified antigen, followed by immunostaining showing loss of staining [65].

Secondary antibody controls show that labeling is only due to secondary antibody binding to primary antibody, and not to nonspecific binding. Several variations of this control exist. The first is a no primary antibody control. When using monoclonal primary antibodies, an isotype control may be used instead, where the sample is incubated with a nonspecific immunoglobulin of the same isotype and

concentration as the primary antibody. No labeling should be seen with secondary antibody controls; if labeling is seen, additional blocking of negatively charged groups with bovine serum albumin or of Fc receptors using normal serum from the same species as the secondary antibody may be required. Rarely, autoantibodies may be present in sections of inflamed tissue, more commonly a problem when using a mouse primary on mouse tissues. This may require block using the Fab fragments derived from antibodies against the secondary antibody animal species.

Labeling or substrate controls determine whether endogenous fluorescence or enzymes are contributing to the staining seen. These involve processing tissue sections without added antibody or enzyme. Autofluorescence detected using this control can be a significant problem, especially in formalin-fixed or glutaraldehyde-fixed tissue, although not when paraformaldehyde is used. Detection of endogenous enzyme activity by staining when substrate is added in the absence of added enzyme mandates the usage of an endogenous enzyme-blocking step as described above.

From an experimental standpoint, for each batch of staining, a positive control internal to the slide or an included slide containing cells where the antigen is known to be highly expressed is valuable to demonstrate that no steps were omitted and all the necessary reagents retain activity. A negative control (general no primary antibody or isotype control) should also be included. Further controls are most important when developing a new IHC protocol or when troubleshooting or altering a known protocol.

Troubleshooting

Optimal antigen detection and identification in IHC is dependent on the tissue of interest, the method of processing, and antibody specificity. When analyzing an immunostained specimen, deposits of the colored chromogen indicate the presence of the antigen and represent specific positive staining. The most commonly encountered problems are absence/weak staining of the target of interest, excessive background staining, and artifacts. Having positive and negative controls help identify the problematic step(s).

Absence/weak staining of specimen with a reasonable staining on the positive control likely indicates that the antigen of interest is low or absent within the specimen. Adding an antigen retrieval step if working with formaldehyde-fixed paraffin sections, using an amplification procedure, or increasing the primary antibody concentration, incubation time, or temperature may increase the sensitivity of the detection. Under- or over-fixation can also mask or destroy the antigen. Excessive buffer on the slides can dilute primary antibody, causing weak staining. Although antibodies from different species have cross-reactivity and can be used as the primary antibody, validating the negative result with the same species control and test tissues is often necessary to confirm the absence of an antigen. Lack of staining on the positive control implies technical issues with possible sample preparation, sample volume, antibody titer, antibody compatibility, or improper counterstain [66, 67]. Often, simply using fresh solutions or substrate may solve the problem.

Excessive background staining can be caused by multiple factors. The most common cause is the attachment of protein to highly charged collagen and connective tissue elements of the specimen. Therefore, it is imperative to consistently include a negative control for background staining assessment. To reduce nonspecific binding of antibody protein to the specimen, a protein block using nonimmune serum from the same animal species as the secondary antibody can be performed to reduce nonspecific binding. Increasing concentration of the blocking agent or using a different type of blocking agent may be necessary to suppress endogenous enzyme activities or to overcome high endogenous biotin concentration.

Technical issues in sample preparation, such as thick tissue specimen section, excessive application of tissue adhesive, inadequate tissue fixation, and cellular processes compromising antigen integrity such as necrosis and autolysis, can lead to excessive background staining. Incomplete paraffin removal, inadequate buffer wash, or high incubation temperature can also result in excessive background staining.

Issues related to primary and secondary antibodies may affect the staining process. The use of concentrated antibody solutions or longer incubation times can cause high background staining. Using a primary antibody from a similar species as the test tissue or using a secondary antibody that recognizes endogenous immunoglobulins also can lead to high background staining. Additional blocking steps may be necessary to combat this problem. Furthermore, an accelerated substrate reaction secondary to a high antigen concentration or increased temperatures may contribute to excessive background staining. Polyclonal antibodies, in particular, should be monitored carefully during the substrate reaction.

Analysis

The most basic level of analysis for immunohistochemistry is based on the qualitative determination of the presence or absence of staining. Diagnostic pathologists frequently use a 3- to 4-point scale rating based on either the strength of staining or the percentage of cells expressing a specific antigen. However, these approaches have significant problems with reproducibility, as well as high intra-observer and interobserver variability [68]. For research purposes, therefore, it is better if the researcher quantitating the staining is blinded to the treatment groups. Sufficient parallel sections from the same block and biologic replicates from multiple animals should to be analyzed to avoid sampling error, as well as problems with staining inconsistency and biologic variability. Unfortunately, these methods do not allow true quantitative analysis of levels of protein expression. True quantitation requires that the entire IHC process be completely standardized starting from tissue collection and sample preparation. It also requires a quantifiable reference standard.

Several methods of creating quantifiable reference standards exist, including peptide deposits, cell lines (or a cell line block), or histoids (a faux tissue), where the amount of antigen present in the reference standard can be determined by the investigator [69]. Automated IHC systems also facilitate quantitation because of

improved consistency in staining. An alternate approach is to use tissue microarrays. These are created by embedding multiple core needle samples from paraffin-embedded tissues into a single recipient paraffin block in a precisely spaced array pattern. Sections cut from the recipient block by microtome may contain up to 1000 pieces of tissue within a single slide [70]. Because all the tissue sections on the slide are exposed to the same antigen retrieval and other staining conditions, a lot of the variability resulting from performing different batches of staining is eliminated.

Quantitation of staining has previously been limited by the inability of the human eye to resolve differences in pigmentation levels in a linear manner. The use of amplification strategies further complicates quantitation. However, both pigmentation and fluorescence can be captured by a digital camera allowing both better quantitation and the possibility of computerized automation of interpretation. A common strategy used in most basic science research applications is to count the number of positive cells to total cells in a standardized area (like a high-powered image), when the antigen of interest is a cell surface marker or a nuclear marker. When the antigen is a secreted cytokine or an ECM protein, the total positively stained area per standard field is measured. Image processing programs such as Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) have tools that can be adapted for use for quantitation of immunofluorescent signals [71]. The National Institutes of Health offers free software called ImageJ for image analysis. Several dedicated computer-based programs to allow automated quantitative analysis of IHC are commercially available, and most often, the microscope used for imaging comes with its own advanced image analysis software programs. They generally have algorithms to improve the resolution of the image and the signal-to-noise ratio. Again, the investigator must weigh the expense of investing in dedicated software against the amount of time, which can be quite significant, required for manual analysis and counting of immunohistochemical results.

Other Techniques That Utilize the IHC Slide Preparation and Labeling for Advanced Diagnostic and Prognostic Applications

Cytometry Time of Flight (CyTOF)

While better fluorochrome-conjugated antibodies and advanced imaging programs that enable low noise/signal rates, such as with multi-laser confocal imaging, are significantly advancing our ability to use immunofluorescence for complex investigation of multiple processes on a single histologic section, spectral overlap and the inherent autofluorescence of the histologic tissue limit the number of fluorochromes that could be simultaneously used. One of the recent inventions of this decade, cytometry time of flight (CyTOF) technology, which ingeniously uses mass reporters instead of fluorochromes is very promising for the research and diagnostic investigation and visualization of several (up to 40, but theoretically 100) different cell

surface receptor/transporters and intracellular and cell/ECM signaling molecules simultaneously on histologic sections [72–77].

In this technology, the antibodies are conjugated to metal-chelating polymers carrying stable isotopes from the lanthanide group of the periodic table of elements. Given the differential energy of the isotope elements, several of them can be detected simultaneously with higher detection sensitivity. The analysis/visualization technique is pretty neat as well and combines the principles of flow and mass cytometry. The initial process of sample preparation and immobilization of tissue section or cell smear on slides is the same as routine IHC. The sections are then stained with panels of labeled antibodies. Species-specific antibodies against the antigens of interest, same as those used in routine IHC, with additional antibody in conjugation with lanthanide polymers, are used in CyTOF. This process has to be optimized for each antibody of the panel and for different tissues of interest, which have to be done collaboratively with the research core facilities. The labeled slide is then placed into an ablation chamber where the tissue is scanned by a pulsed laser, and on each laser shot, the tissue from that small ablation spot is vaporized and carried by a stream of inert gas into the inductively coupled plasma ion source for analysis by the mass cytometer. The slide is moved under the fixed laser beam to scan the entire specimen spot-by-spot, with the metal isotopes associated with each spot simultaneously measured and indexed against the location of each spot, which ultimately yield an intensity map of all target antigens on the specimen under investigation [78].

Although this technology is currently expensive, the range of simultaneous verification of different cell and ECM antigens using CyTOF's multidimensional imaging analyses permits the researchers to undertake more challenging cell and molecular biology research and diagnostic tasks.

Laser Capture Microdissection (LCM)

Tissue is simply a composition of different cell types, distributed across three dimensions. A single pathological stimulus may elicit a wide variety of responses from each cell type, making visualization difficult and generalization impossible. It is for this reason that the ability to isolate cells is crucial to analyzing cells, especially on a molecular level. Laser capture microdissection (LCM) is a method for isolating target cells in microscopic regions, in order to overcome the heterogeneity of tissue. Also known as laser microdissection, LCM is a powerful research tool, where the sample can be used for further analytical techniques, such as polymerase chain reaction (PCR), real-time PCR, and proteomics [79, 80]. This is a brief introduction to LCM technology, principles, pros and cons, and further applications in various fields. Correct procedure and appropriate use have the potential to yield unprecedented insights into cell biology, especially those grown in their natural tissue habitats, in contrast to those raised in artificial environments.

IR LCM Versus UV LCM

Infrared and ultraviolet LCM (IR LCM and UV LCM) are two general classes of LCM systems. Both versions are based on the principle of inverted light microscopes, either with or without a fluorescent module, equipped with a laser device used to aid the visualization and procurement of target cells. All LCM instruments are mounted on either a manual or automated platform. These platforms usually include the inverted microscope, a solid-state near-infrared laser diode, a control unit, a microscope stage, a CCD camera, and a display output. Usually, the LCM microscopes are linked to computer systems, in order to improve laser control and image archiving. The laser beam of an LCM microscope has a minimum diameter of 7.5 μm and a maximum diameter of 30 μm. IR LCM and UV LCM make up all the commercially available LCM equipment, varying only in configuration and intended application.

Due to the photochemical effects of high-energy beams, the IR LCM systems are designed to allocate most of the heat during the cutting process to the membrane. This limits the exposure of the tissue to about 90 °C for several milliseconds, preserving the macromolecular structure of any subjects. The process begins with the placement of a thin piece of transparent thermoplastic upon a tissue section. After the subject is visualized microscopically, the cells of interest are adhered to the film with a fixed position, short duration, and focused IR laser pulse. The selective removal of the cells is based on the greater adhesion of the cells to the film instead of the slide. The sample is then detached through the lifting of the film and is ready for further analysis.

UV LCM is also performed by mounting tissue upon a membrane, 6 μm thick, and placed on glass slide for visualization. A UV laser beam is used to draw around the cells of interest, leaving the desired sample intact while ablating the unwanted tissue. The sample can then be catapulted against gravity into a receiving cap through the increase in the energy of the laser. Two major advantages exist in the UV LCM method: the process avoids any intricate operator dependent steps and increases specificity by eliminating the possibility of adherence by unwanted tissue on the adjacent edge of the sample.

Sample Preparation

The uses of LCM are widespread, including histological specimens, cell cultures, plant material, chromosome spreads, forensic preparations, and formalin-fixed paraffin-embedded (FFPE) tissues, fresh frozen tissues, stained or unstained tissues. Nowadays, the process of LCM is straightforward.

Numerous kits that aid in simplifying the procedures are commercially available. Optimal cutting temperature (OCT) compound-embedded frozen tissue and formalin-fixed paraffin-embedded (FFPE) tissue are two sample preparation processes that exist for capture. Both sections are achieved through sections cut at thicknesses of 5–15 μm. Any sections below 5 μm or greater than 15 μm may not microdissect properly.

Sections are placed onto membrane slides and are stained with hematoxylin and eosin and then dehydrated. The adequate dehydration of the sections is paramount

to minimizing the adhesive force between the slide tissues. The captured target cells can be kept in lysis buffer for DNA, RNA, and protein isolation. Prior to RNA capture, it is generally recommended that the RNA integrity numbers (RIN) are checked to make sure they are high enough to ensure success. Despite the lack of histological differentiation and handling inconvenience, frozen tissues offer much greater preservation of DNA, RNA, and protein integrity, which is essential to downstream analysis. A simplified process is shown in Figs. 10.8 and 10.9.

Quality and quantity of the sample, time and type of preservation, and fixation method and efficiency affect the yield of RNA from frozen tissues. The FFPE method has been the standard for tissue morphology preservation for decades.

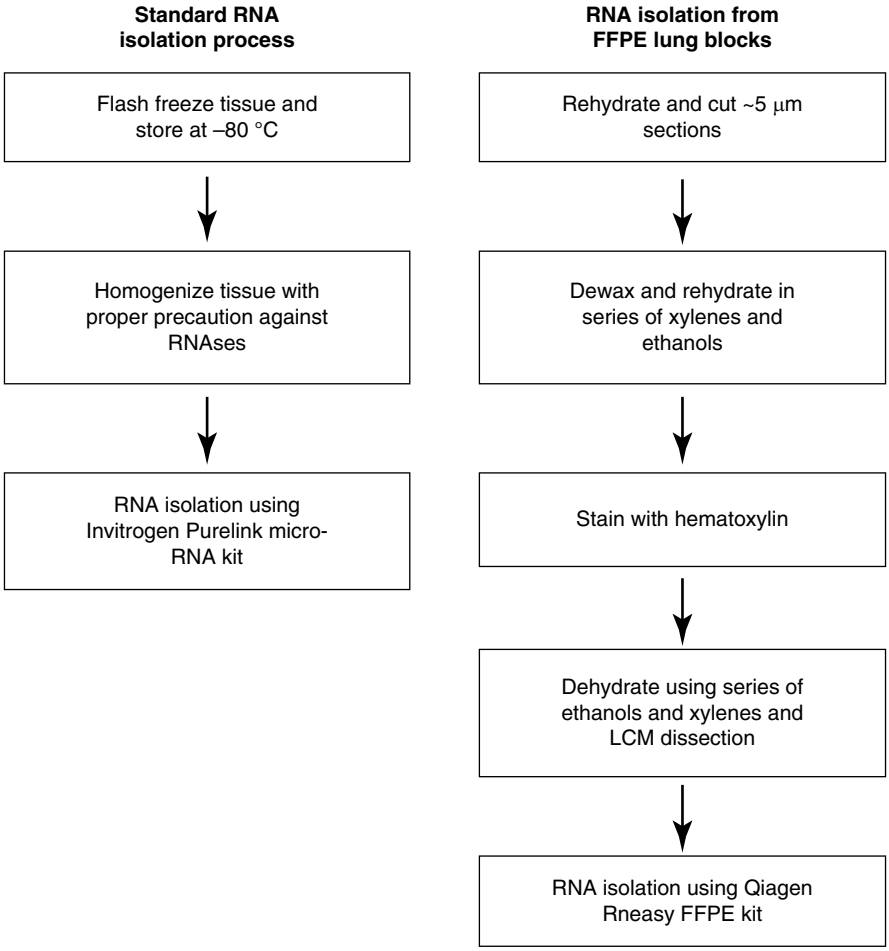

Fig. 10.8 A comparison of standard procedure for RNA isolation from frozen tissue specimens versus RNA isolation from formalin fixed paraffin embedded (FFPE) sections after selective tissue extraction with LCM

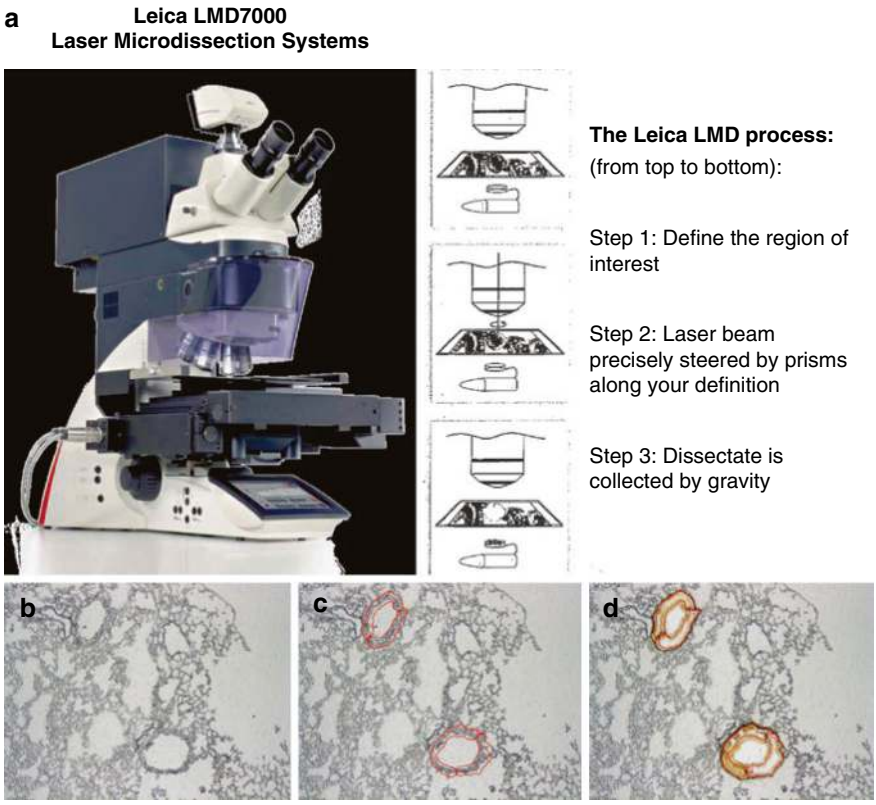

Fig. 10.9 Laser capture microdissection (LCM) system (Leica LMD7000) and section process. (**a**) Leica LMD7000 system and process steps. (**b**) 5 μm unstained section of murine lung prepared on Leica PEN membrane slides. (**c**) Defining the area for dissection. (**d**) Remaining tissue section on the slide after LCM dissection of the desired area

Though it creates cross-links between nucleic acids and proteins, DNA and RNA can be isolated for downstream applications such as PCR and RT-PCR.

The Advantages and Limitations of LCM

The advantages of LCM are speed, precision, simplicity, and versatility. Based on the beam diameter, the sample amount, and desired precision, thousands of cells can be captured within a short time frame. Stained tissue, such as tissues that have undergone the conventional hematoxylin and eosin staining process, can be successfully microdissected through the use of LCM, which allows for the identification of specific cells of interest.

LCM shares limitations with other microdissection procedures, including the lack of a coverslip on the glass slide. Without a coverslip and index matching between the tissue and mounting media, cellular details at high magnifications are obscured due to the refractile quality of dry tissue sections. The capture of particular cell types from complex tissues that exhibit few architectural features is

compromised without the placement of a coverslip. The issue can be resolved through the use of specialized staining techniques, in particular immunohistochemistry, which differentiates the cell population to be isolated. Additionally, small cells are often contaminated with fragments of adjacent cells. Certain dyes that are used to mark tissue during LCM may interfere with protein migration. Fortunately, hematoxylin and methyl green have little to no effect, indicating the potential to be used in the LCM procedure.

In summary, the speed, simplicity, and easy handling and documentation of dissected tissue make LCM a useful tool for the collection of specific tissue. LCM is a vital technology that enables researchers to study cells grown in their natural tissue habitat. The advancements in molecular analysis technologies improve upon the usefulness of LCM, allowing for single cell to subcellular structures to be identified, isolated, and analyzed. The development of LCM has allowed investigators to determine gene expression patterns from specific tissues of individual patients. LCM can provide samples from pure cell populations, which can be used for RNA extraction, cDNA library, and gene hybridization. Individualized molecular profiles can be obtained for histologically identified samples. Correlation of patterns of expressed genes with etiology and treatment response can be generated through multiplex analysis. In the future, personalized profiles can be created based on a patient's risk for disease and an appropriate treatment may be assigned. Results can be accumulated into a categorized database in order to create a minimal subset of key markers, which may be used to facilitate early detection and diagnosis of disease. The potential of LCM to isolate living cells for further cultivation. Without the unwanted cells in the captured sample, cultivation of a homogenous colony may lead to increased specificity in drug screening.

Mass Spectrometry (MS)

Mass spectrometry is used in many different fields and is applied to pure samples as well as complex mixtures, such as wound and bodily fluids and surgical resected tissues, etc. to measure the masses within a sample. MS is an analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio. The workflow of MS from fresh frozen tissues has been optimized. Briefly, the steps involve tissue or cell lysis and fractionation followed by in-solution digestion. The resulting purified peptide sample may be further modified based on the type of analysis chosen—LC-MS, LC-MS/MS, and/or MALDI-MS. All of these protocols may be institution- or individual-dependent and should be customized to specific samples and techniques for optimal results. There are several molecular biology textbooks and articles dedicated to the discussion of MS technology development, which is beyond the scope of this chapter. However, MS can be used for detection of proteins from FFPE tissues and, most recently, for the identification of specific cluster of cells or tissue areas from laser capture microdissected tissues.

Sample Preparation for Protein Extraction from FFPE

Formaldehyde fixation revolutionized tissue preservation in the late 1800s and has remained the gold standard since then, largely because samples can be stored for decades at room temperature. The challenge, however, lies in the fact that formaldehyde creates unpredictable intra- and inter-protein methylene cross-links in the tissue sample and current popular proteomic search engines and libraries cannot identify these cross-linked peptides. Nevertheless, careful preparation of formalin-fixed paraffin-embedded (FFPE) tissue samples can reverse these modifications, and both proteins (including posttranslational modifications and protein-protein interactions) and DNA/RNA have been successfully recovered from these samples for various downstream molecular applications.

As with any experiment, good sample preparation generally leads to good resultant data. First, prepare your workspace. Contamination of the sample with keratins from human skin should be avoided as much as possible. Work in a clean area of the lab (preferably in a laminar flow hood) wearing short sleeves and nitrile gloves—latex gloves can introduce latex proteins into one's sample—and use clean tubes and pipettes. Second, the sample should be deparaffinized with xylene or a xylene substitute (e.g., hexane), though some contend that certain solvents like xylene and DMSO may interfere with certain MS analyses, causing a high background signal and reducing the number of protein peaks observed. After deparaffinization, the sample must be heated to at least 95 °C, as heat is necessary for the hydrolysis of methylene cross-links and thus for effective protein identification. Most studies suggest that a stepwise heating program—a short duration of very high temperature followed by a longer duration of lower temperature—is the most effective in this regard. Following the heating step, some have proposed the addition of reducing agents or high pressure to the sample, but a lack of evidence for and the expense of these techniques limits enthusiasm and acceptance of these protocol modifications [81].

After this step, the basic workflow for sample preparation for MS is approximately the same as that of frozen or fresh tissue specimens. It should be noted that some silver-based stains are not compatible with mass spectrometry. In addition, the utility of FFPE tissue in protein analysis can be limited by regional tissue heterogeneity [82] both in cellular composition and preservation. Consequently, various methods for harvesting only the areas of one's FFPE sample that are enriched in the features under study become essential to the preparation process and, ultimately, to results. Because FFPE tissue maintains its cellular morphology and architecture, focused physical modification of the sample itself with microtome sectioning, tissue coring, or laser capture microdissection (LCM) is often used to isolate regions of interest, though each method has its own benefits and drawbacks.

Similarly, the ionization techniques used in MS analysis works best with limited sample complexity, with moderate numbers of peptide constituents in roughly equal concentrations. Certain samples may benefit from the preemptive removal of known hyper-abundant protein species (e.g., albumin, immunoglobulin, hemoglobin, amylase) that could otherwise "overwhelm" the signals of lower-abundance ones. Commercial kits using immunoaffinity techniques (e.g., immunoprecipitation and

co-immunoprecipitation) and gel electrophoresis with or without in-gel protein digestion—all of these (and more) have been successfully used for peptide depletion and enrichment. The process chosen should be at the discretion of one's institutional core facilities.

DNA/RNA Extraction from FFPE

In addition to protein, nucleic acids can also be successfully recovered from formalin-fixed paraffin-embedded specimens. Unlike fresh frozen tissue, which is known to produce high-quality DNA/RNA, FFPE tissue contains cross-links between nucleic acids and other cellular components secondary to the formaldehyde fixation process. RNA is particularly sensitive to this damage [83]. These cross-linked DNA and RNA fragments are still recoverable, but in a highly degraded form (lower molecular weight) and often in low numbers [84]. Nevertheless, purified nucleic acid extracted from FFPE tissue appears to be amenable to genetic analysis, including whole genome, whole exome, and single-gene sequencing.

DNA/RNA extraction begins with tissue deparaffinization by a solvent (e.g., xylene, heptane/methanol), followed by ethanol rehydration. This is a critical step in successful nucleic acid recovery as undissolved paraffin inhibits PCR amplification. Next, proteins and enzymes are removed from the sample with proteinase digestion, often in combination with and facilitated by a denaturing agent such as sodium dodecyl sulfate (SDS). From this tissue lysate, nucleic acids are purified with phenol and centrifugation, ultimately generating an upper, aqueous phase containing DNA/RNA and a lower organic phase containing proteins, lipids, and polysaccharides. The retained aqueous sample is then cleaned of contaminating RNA and leftover salts/buffers from the extraction process and is often heated to further reverse formaldehyde cross-links. The resulting purified nucleic acid sample can then be used in the gene expression platform of one's choosing or stored at low temperatures for approximately 6 weeks. Several commercial kits, optimized to reverse as much formaldehyde modification as possible and broadly following the above steps, have shown excellent results validated by next-gen sequencing techniques [85].

The quality of the recovered DNA/RNA is dependent on a number of factors, beginning with the age and type of tissue, the details of tissue preservation (including the duration of fixation and storage conditions), and the thickness of the tissue section used, as this can affect lysis efficiency [86]. As with proteins, various enrichment strategies, like LCM, are often used in FFPE sample preparation for nucleic acid extraction such that analysis is only performed on regions of interest.

Overall, these recommendations for FFPE tissue preparation for downstream applications are purely that. Considering the diversity of tissue types, analytical methods used, and experimental goals involved, it should be self-evident that there is no single standard method for all investigators. One should consult with one's institution's core facilities to see if there is a preferred protocol or commercial kit that has been consistently efficacious in the past, as ultimately, this collaboration is

the greatest determinant of success and productivity with these strategies. In addition, certain facilities may require a certain threshold amount of post-preparation sample—ranging from micrograms to milligrams of tissue, which has to be planned and accounted for.

Limitations of IHC

Despite the popularity of IHC in basic science research and clinical pathology, there are well recognized limitations to the use of IHC. First, commercial antibody reliability has increasingly been questioned, with increasing calls for antibody identification, validation, usage, and cataloging [87]. Methods to appropriately validate an antibody for research can include: blocking with an immunizing peptide, performing a Western blot on the tissue of interest, or demonstrating specificity in a knockout model. Second, the rise in RNA in situ hybridization allows for identification of specific cell types and verification of changes in gene expression. In contrast to antibody generation, the probes utilized for RNA in situ hybridization are controlled in design and synthesis to specifically complement the sequence of a targeted RNA transcript. As technology evolves, so does specificity for histological targets, which may limit the future application of IHC.

Recent Advancements in Immunohistochemistry

IHC has undergone remarkable transformations, significantly impacting diagnostics, prognosis, and therapeutic selection. These advancements have introduced sophisticated techniques and cutting-edge technologies, enhancing our understanding of disease mechanisms and improving patient outcomes.

Advanced Detection of Genomic Abnormalities: Genogenic Immunohistochemistry

One of the groundbreaking advancements in IHC is recent technology that enhances the ability to detect genomic abnormalities through genogenic IHC, a term first put forth by Gown over 20 years ago. This method employs IHC to identify specific genetic mutations, chromosomal translocations, and gene amplifications that produce novel chimeric proteins. Genogenic IHC offers deeper insights into the pathophysiology of disease and identification by detecting these genetic alterations, enabling personalized treatment strategies. This approach is crucial for selecting the most effective therapeutic responses, making it an invaluable tool in the development of new diagnostics and therapeutics [88].

Multiplexed Imaging Techniques

Multiplexed imaging techniques represent a significant leap forward in IHC. Techniques like IBEX (Iterative Bleaching Extends Multiplexity) and MIBI (Multiplexed Ion Beam Imaging) enable simultaneous visualization of multiple markers, providing a comprehensive view of the tumor microenvironment. MIBI, in particular, uses secondary ion mass spectrometry to detect metal-tagged antibodies, allowing for highly multiplexed and quantitative imaging. These capabilities allow researchers and clinicians to better understand the interactions between different

cell types and the overall architecture of tissues, which is critical for accurate diagnosis and effective treatment planning [89].

Whole-Body Imaging: wildDISCO

Whole-body imaging techniques, such as wildDISCO (wild three-dimensional imaging of solvent-cleared organs), have revolutionized the scope of IHC. This method uses conventional antibodies for whole-body staining in mice, creating comprehensive biological atlases that reveal pathological changes often missed by traditional methods. WildDISCO is particularly valuable for mapping disease progression and understanding the systemic effects of diseases [90].

New and Evolving IHC Markers

The introduction of new IHC markers has expanded the toolkit available to investigators. For instance, TRPS-1 is a novel marker for breast origin, offering new avenues for diagnosis and research. Simultaneously, the diagnostic utility of some classical markers is being reevaluated. E-cadherin, traditionally used to differentiate invasive lobular and ductal carcinomas, is facing scrutiny due to limitations in its specificity. This ongoing reassessment ensures that IHC techniques are based on the most accurate and reliable markers available [91].

Artificial Intelligence and Deep Learning Integration

The integration of artificial intelligence (AI) into IHC has significantly enhanced its capabilities. AI models are being developed to improve digital pathology, setting new benchmarks in cancer diagnostics using IHC data. AI algorithms can quickly and accurately analyze large datasets, identifying patterns and anomalies that might be missed by the human eye. This integration not only improves diagnostic accuracy but also streamlines the workflow, making the diagnostic process faster and more reliable. In particular, the use of deep learning algorithms for the quantification of IHC-stained images represents a major advancement. These algorithms can enhance the accuracy and efficiency of assessing biomarkers of interest. This improvement is critical for both diagnostic and research purposes, and can better ensure that IHC data is reliable and reproducible [92].

Digital Spatial Profiling (DSP)

DSP combines the power of IHC with high-resolution digital imaging and spatial barcoding, allowing precise quantification of protein expression within specific regions of interest (ROI). Each ROI is imaged at high magnification, allowing for precise localization of proteins. This is particularly useful in heterogeneous tissues where protein expression may vary significantly across different areas. The ability to quantify protein expression with high accuracy is essential for identifying subtle changes that may be associated with disease progression or treatment response. In DSP, each ROI is assigned a unique spatial barcode, which is used to link the protein expression data to its specific location within the tissue. This spatial barcoding enables the analysis of multiple ROIs within a single tissue section, providing a comprehensive overview of protein distribution and expression pattern [93]. The

integration of DSP with next-generation sequencing (NGS) technologies has further amplified its potential, enabling simultaneous analysis of protein and RNA expression. The integration of protein expression data with RNA sequencing data allows researchers to gain insights into the transcriptional and translational regulation of proteins within specific regions of a tissue, offering a more complete picture of cellular processes [94]. Despite its advantages, DSP in its current form has some challenges, such as the need for extensive computational resources and expertise in data analysis. Ongoing developments in bioinformatics and machine learning are expected to address these challenges, making DSP more accessible and user-friendly. Future advancements may also include integrating additional omics data, such as metabolomics and proteomics, to enhance the depth of spatial profiling.

Conclusions

IHC is a powerful research tool essential in the research field. However, the accuracy is heavily dependent on multiple variables starting from tissue fixation, processing and cutting, antibody availability, and other extraneous factors involving the tissue of interest, such as the presence of high levels of autofluorescence or endogenous biotin. Working up new immunohistochemical protocols and appropriate controls especially for previously untested antibodies can be time-consuming and expensive, so thorough research into the tissue, the antigen of interest, and alternative previously described antibodies is worthwhile. The researcher must also decide on the method of analysis in advance of designing the IHC protocol, as the determination of whether qualitative or quantitative results are needed for any given experiment may dictate the amplification and visualization system to be used. Recent advances in IHC have greatly increases the ability of IHC to yield new insights in disease and is a necessary application in an investigator's skillset.

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Fundamentals of Flow Cytometry and Cytometry By Time of Flight (CyTOF)

11

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Abstract

Flow cytometry is a flexible and useful tool in the armamentarium of translational and basic researchers. Based on a microfluidic system that has roots in the 1930s, flow cytometry allows for the multi-parametric analysis of samples on a single cell basis in a high-throughput manner. Flow cytometry has paved the way for multiple advancements in the field of cytometry including the development of cytometry by time of flight (CyTOF). CyTOF allows for higher-dimensional analysis of samples on a single-cell basis with over 100 parameters. Clinically, flow and mass cytometry have been frequently used in oncology and immunology. Research applications include a wide variety of uses including phenotyping, cell death and proliferation, cell signalling, fluorescence-activated cell sorting, monitoring immune responses, pharmacokinetics, and evaluating the proteomics landscape of a cell. This chapter describes the fundamentals of flow and mass cytometry for the surgeon scientist.

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Keywords

Flow cytometry · Mass Cytometry · Time of Flight · FACS · Fluorescence-activated cell sorter · Fluorochrome · Analysis · Research · Phenotype Immunology · Cancer biology · Tumor immunology · Clustering · Imaging Mass Cytometry · Surgery

Background of Flow Cytometry

Flow cytometry (FC) is an advanced technique to study the characteristics of individual cells. The principles of flow cytometry were first described in 1934 by Andrew Moldavan, although it is unclear if he ever built a prototype [1, 2]. Cells were passed through a capillary tube so that they proceeded in single file and were exposed to light through an objective that was set up like a microscope with a photodetector in place of the eyepiece. Further work by Kamentsky et al. in the 1960s established a microscope-based cytometer system to detect abnormal cervical cells in human preparations [3]. Shortly after Kamentsky's prototype was built, a number of other groups made improvements that resulted in a system that looked very little like a microscope but closely resembled current flow cytometers [4–7]. Flow cytometry today is based on the same principles but primarily uses lasers that emit light, which excites fluorescent compounds conjugated to antibodies or taken up directly by cells. Early flow cytometers contained a single laser which limited the number of signals able to be detected. Current cytometers support as many as five different lasers, ranging from ultraviolet to far red, which significantly expands the number of parameters that can be detected and provides greater ability for cell characterization. This technology is indispensable today for many fields, both clinical and research-oriented, particularly immunology and cancer biology. In this chapter, we will describe the fundamental concepts needed to understand and utilize flow cytometry effectively.

Fluorochromes

Cells or particles can be characterized or separated by expression of specific markers, such as cell surface proteins. Fluorochromes or fluorescent dyes are used to "stain" for the target of interest. They are excited by specific wavelengths (absorption spectrum) of light and then emit light at specific wavelengths (emission spectrum). The emission spectrum can then be detected by a set of filters and sensors in the flow cytometer [1]. The filtered light is detected by a photomultiplier tube (PMT) or photodiode. Each PMT only collects fluorescent light at a certain wavelength. The precise arrangement of filters and PMTs allows the simultaneous detection of multiple signals. To obtain the most data from each sample, it is important to choose the right "colors" or fluorochromes. There are a wide range of fluorochromes available for use with different cytometers. Additionally, there are DNA-binding dyes

that fluoresce at specific wavelengths for detection of DNA content, cell death, and proliferation.

When approaching staining, it is important to first learn about available cytometers and understand experimental needs. Cytometers may have 1–5 lasers allowing the detection of up to 20 parameters. Understanding the configuration of the instrument (lasers and filters) is critical to correctly choose the number and type of fluorochromes for each experiment. Manufacturers typically provide a chart of fluorochromes suitable for use with different cytometers. These charts are available on the manufacturers' websites or through the core facility housing the cytometer. As mentioned above, each fluorochrome has a specific emission spectrum with a maximum emission wavelength. While having different maximum emission wavelengths, different fluorochromes often have overlapping emission spectra [8]. Therefore, when performing multicolor staining studies, the possible spectral overlap between fluorophores needs to be considered. To account for emission spectra overlap, fluorescence compensation was developed (discussed later). The farther apart two different fluorochromes' maximum emission spectra are and the less overlap of emission spectra, the easier and better data collection will be. Additionally, fluorochromes have different "brightness". Brighter fluorochromes are often used to detect low-abundance proteins, whereas dimmer dyes are used for staining more abundantly present targets. Most manufacturers provide detailed information regarding emission spectra overlap and brightness index for each fluorochrome, which is quite helpful when choosing a staining panel.

FC Sample Preparation

Flow cytometry, as described above, is a laser-based single cell or particle analysis. This is accomplished by a complex system of microfluidics where each cell is exposed individually to lasers of specific wavelengths to detect the characteristics of individual cells or particles. This can provide a variety of specific information with relatively small amount of sample. In order to achieve this, tissue or fluid for analysis must be adequately prepared. Poorly prepared samples will result in, at best, poor data collection and, at worst, can clog the machine, requiring costly maintenance.

Cytometers typically can analyze particles between 1 and 30 μm. This means that the prepared sample must contain cells and particles approximately no larger than 30 μm [1]. With specialized techniques, particles much smaller than 1 μm can be analyzed, such as microvesicles that are 100–1000 nm [9]. For reference, a normal circulating human monocyte is 10–15 μm in size with macrophages, dendritic cells, and cancer cells often being significantly larger [10]. Cells for flow cytometry analysis are typically derived from the following three main sources: suspension or adherent cell culture, whole blood or body fluid, and solid tissue. The final sample must be prepared as a single cell suspension. This is generally not particularly difficult for samples obtained from cell culture or body fluids such as blood, urine, or peritoneal fluid. Blood or bloody fluid analysis typically requires lysis of red blood

cells or density-based centrifugation to remove unwanted elements [11]. For tissue, however, this can be more difficult, requiring multiple steps to mechanically dissociate and enzymatically digest tissue to ensure creation of a single cell suspension [12]. This can be done manually or using kits and processors that are commercially available. It is important to thoughtfully approach dissociation and digestion such that cells are not overly damaged which can cause cell death and alter target expression. After creation of solution, mechanical filtration should be done to remove large particles. Filters of 70 μm and/or 40 μm are often used at this step depending on the sample source and level of concern for large particles. Final sample concentration should ideally be in the range of 1×10^6–10×10^6 cells/mL.

Typically, proper staining solution contains phosphate-buffered saline (PBS) and a low concentration of fetal bovine serum (FBS). Most commonly, cells are stained for surface antigens with an antibody directly conjugated to a fluorochrome or with DNA-binding dyes. Staining of intracellular proteins, like cytokines, requires fixation and permeabilization steps to allow antibodies access to intracellular proteins. Cells can often be "fixed" with paraformaldehyde-containing solutions which cross-link proteins, so that staining is not lost during permeabilization of the cell membrane. Depending on the cellular location of the antigen (cytoplasmic versus nuclear), different levels of permeabilization may be required. This step significantly alters the size and granularity of cells and so will change some analysis parameters (discussed later). There are a number of protocols and commercial kits available that provide reagents for different applications [8]. Additionally, most flow cytometry core laboratories will provide new users a primer on appropriate sample preparation based on the capabilities of available machines.

Data Collection

After staining, the samples are then run and data collected on the flow cytometer. Multiple parameters can be collected, and a large amount of data can be gleaned from each individual cell or particle. The first two parameters collected in nearly all cytometers are data on the size and granularity of the cells, called forward scatter (FSC) and side scatter (SSC). Forward scatter measures the amount of light scattered by the cell in the forward direction, and side scatter measures the amount of light bent by the cell at a 90° angle to the cell [1]. Forward scatter therefore gives us a measure of the cell's size as larger cells refract more light than smaller ones. Side scatter provides a measure of the cell's complexity or granularity (Fig. 11.1). FSC and SSC together can be used to differentiate certain cell types. As an example, lymphocytes which are relatively round, smaller, and non-granular can usually be differentiated from larger, more granular myeloid cells such as neutrophils (Fig. 11.1). In addition to the FSC and SSC area, on most newer cytometer models the width and height of both parameters can be collected which may help to exclude doublets (cells stuck together), as well as debris and dead cells.

When collecting data on the fluorescent parameters included in the experiment, there are two important considerations, the voltage applied to the laser and the

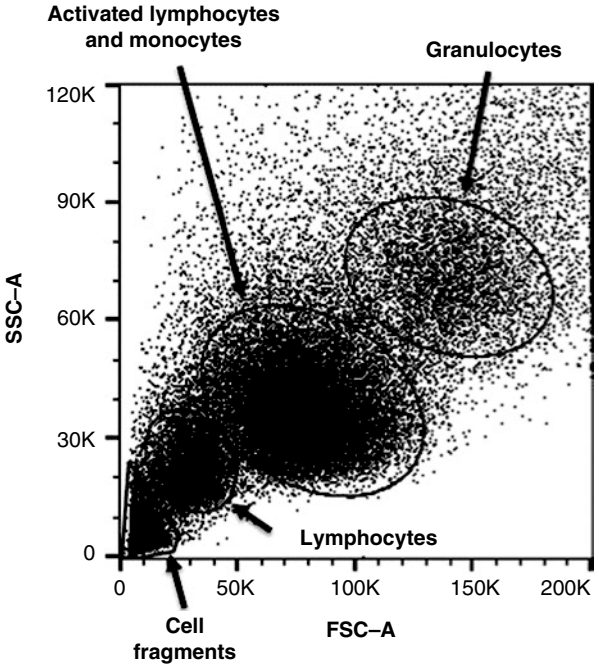

Fig. 11.1 Forward and side scatter. Forward scatter and side scatter provide information about cell size and texture. Different cell types can often be distinguished based on these characteristics alone as shown here for a normal murine lymph node preparation. Demonstrated here are cell fragments, activated and nonactivated lymphocytes, monocytes, and granulocytes. *SSC-A* side scatter area, *FSC-A* forward scatter area

overlap between the emission spectra of fluorochromes used [8]. During the first few times each experiment is collected, compensation controls, either sample or beads stained with only one fluorochrome, should be used. With this single-stained sample, the voltage applied to the laser can be set so that the signal is appropriately detected and a positive and a negative population are clearly defined. Once the voltage is selected for individual fluorochromes or fluorescent dyes, the compensation algorithm can be calculated manually or automatically to remove the overlap between different fluorescent parameters. Compensation subtracts the percent of overlap of one fluorochrome or fluorescent dye's emission spectrum from others' emission spectra and can only be done reliably using singly stained samples. When using a new antibody or protocol, it can often be helpful to include positive and negative controls, i.e., tissue (or fluid) that is known to contain the cells of interest, as well as the isotype control antibody (IgG) to determine background staining. After compensation and controls are set, the experimental data is collected for each sample and saved. The number of events collected typically ranges from 10^5 to 10^6 depending on the abundance of the cells of interest.

Data Analysis

Flow cytometry data is saved as an .fcs file. These files are then typically downloaded and transferred to another workstation for analysis. There are a number of programs designed to analyze data generated during flow cytometry. Each program

has its own strengths and limitations. After the data is collected, the rest of the process is essentially computer-based analysis.

Flow cytometry excels as an analysis method because it allows simultaneous collection of multiple data points about individual cells in a quantifiable manner. Additionally, without sacrificing other cells or particles within the sample, data can be collected about a diverse array of targets. Data from .fcs files can be visualized in a number of different ways. The most commonly used ways to visualize data are dot plots or histograms (Fig. 11.2). Histograms display a single parameter on the *x*-axis and cell counts on the *y*-axis. This allows visualization of the peak fluorescence and evaluation of populations that express a marker of interest (Fig. 11.2a). Dot plots allow the ability to visualize data based on two parameters. Those parameters can be FSC, SSC, or fluorescence (Fig. 11.2b). Additionally, dot plots can be shown in a number of different ways, including pseudocolor or zebra, which show different colors based on the number of data points, as well as density and contour plots (Fig. 11.2c–e), which display information about relative intensity of events. The fundamental analysis of flow cytometry data is the focused examination of specific cell populations using "gating" (Fig. 11.3). Gates are placed around populations of cells based on differential expression of cell surface or intracellular markers. Using these methods, the frequency of cells of interest can be determined in a given sample, which can then also be used to determine the density of those cells in the sample. Additionally, the mean fluorescent intensity (MFI) of a target antigen on a given group of cells or particles allows the user to determine the level of expression. This information can be useful in situations such as determining the activation of cytotoxic T lymphocytes in the tumor microenvironment. The frequency of target expression as well as the level of expression are complementary information. After the data values are generated using .fcs analysis software, any statistical package can be used for further analysis and comparison.

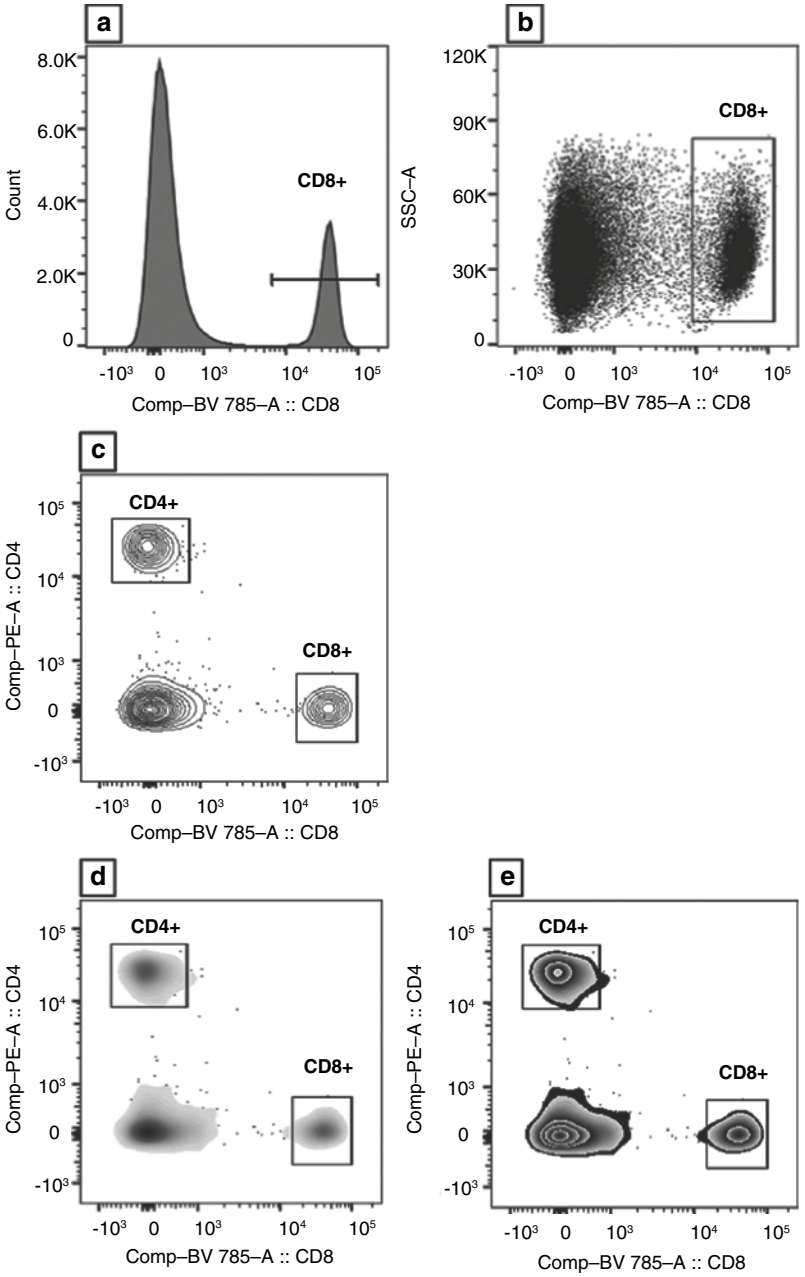

Fig. 11.2 Data visualization and analysis. Data gathered from flow cytometry can be visualized and analyzed in a number of ways. (**a**) Histogram demonstrating a single parameter (CD8 expression). (**b**) This demonstrates a dot plot of the same data showing two different parameters, side scatter (SSC-A) and CD8 expression. Two parameter data can also be used to show cells with expression of different specific markers such as CD4 for helper T lymphocytes and CD8 for cytotoxic T lymphocytes and can be shown in other ways such as contour (**c**), density (**d**), and zebra (**e**) plots

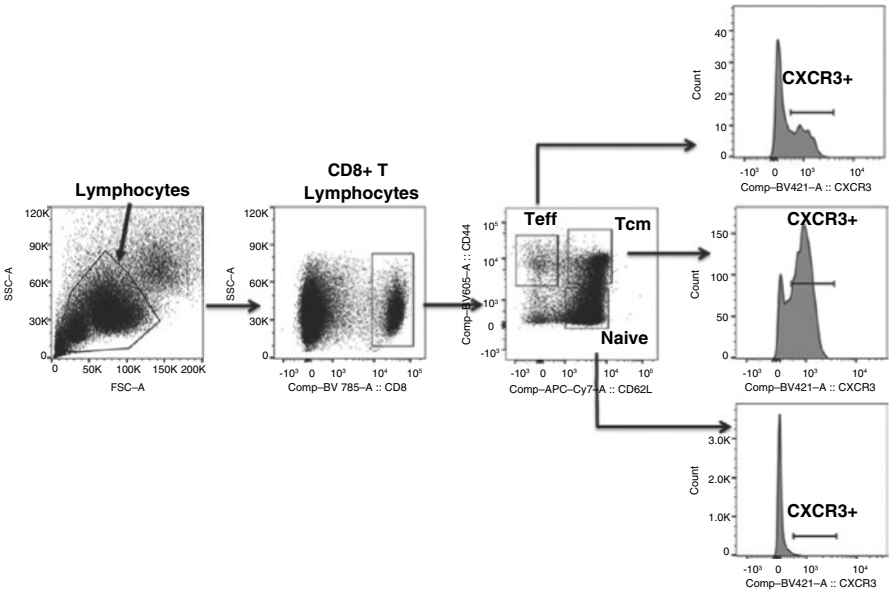

Fig. 11.3 Gating. One of the most useful aspects of flow cytometry is the ability to focus on specific groups without sacrificing other data. This is accomplished using "gating." Demonstrated here is an example of gating to determine the expression of a specific chemokine receptor (CXCR3) on different populations of cytotoxic T lymphocytes (CD8+). *SSC-A* side scatter area, *FSC-A* forward scatter area, *Teff* effector T lymphocyte, *Tcm* central memory T lymphocyte

Applications

As mentioned above, flow cytometry serves a large variety of uses, both clinical and in research. Clinically, flow cytometry is primarily utilized in the characterization and study of immune parameters associated with transplantation and hematologic malignancy. This has significantly enhanced the ability to detect and prevent rejection in the transplant patient, as well as more accurately assess hematologic malignancies for treatment and response [8]. Due to the high-throughput nature of flow cytometry, as well as the sensitivity, specificity, and diversity of collectable information, future potential in clinical applications abound. These range from identification of targets for immune-based cancer treatments and circulating tumor cells [13], defining platelet dysfunction in bleeding disorders [14], and accurate quantification of microbe populations in patients' urine [15].

Cell Viability, Proliferation, and Death

Research applications for flow cytometry are currently more widely varied than clinical applications. Flow cytometry was initially used to determine cellular DNA content using naturally fluorescing compounds [13]. These compounds, such as

propidium iodide (PI) and 7-amino-actinomycin D (7-AAD) are often used to determine cell viability as the cell membrane must be disrupted to allow entry. Cells taking up these dyes are "dead" and can be analyzed or excluded as such [16]. Cells undergoing apoptosis can also be detected using specific markers of apoptotic cell death, such as annexin V, which binds to inappropriately exposed membrane phospholipids, or intracellular staining for cleaved caspase-3 and Bcl-2, critical regulators of apoptosis [8]. Cell proliferation can be measured by flow cytometry through staining for proliferation markers. Ki-67 is a nuclear protein associated with proliferation and is expressed during all phases of the cell cycle [17]. In addition to antibodies, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) is taken up by cells and reliably halves with each cell division. CFSE is particularly useful as cells can be labeled and adoptively transferred for analysis at a later date due to long-term stability of the compound in cells [18]. There are additional methods focused on determining the specific phase of the cell cycle after treatment or stimulation [19].

Phenotypic Characterization

One of the most widely used applications of flow cytometry is the phenotypic characterization of cells of interest. As mentioned above, this aspect of flow cytometry is particularly useful for the clinical characterization and identification of hematologic malignancies due to its high-throughput, specific analysis that is able to identify rare cell populations [20]. Additionally, due to the multi-parametric nature of flow cytometry, multiple aspects of cells can be identified simultaneously. Experimental staining protocols often include both surface and intracellular staining allowing identification of properties of various cell subsets. As discussed above in the section on data analysis, cells can be grouped based on expression of multiple markers (Fig. 11.3). In this way, different groups of cells can be compared for frequency or expression of different markers. This type of analysis is particularly useful for applications such as determining T-cell activation or exhaustion [21] or identifying cancer stem cell populations [22]. Another commonly used function of flow cytometry is the detection of antigen-specific immunity using major histocompatibility complex I or II peptide complexes conjugated with fluorochromes in the setting of infection, transplantation, and neoplasia [23].

Intracellular Staining

While most commonly used to detect expression of specific markers on the surface of cells, flow cytometry can also be used to understand intracellular events. This includes signaling events downstream of specific targets or cytokine production. This can be very useful when investigating drug activity or targeting and immune function [21]. Additionally, some cell-specific markers can only be determined by using intracellular staining such as regulatory T cells (Foxp3) [24]. While the methodology is more complicated than cell surface staining, for many intracellular

applications, specific kits and protocols are commercially available [25]. This also includes assays to determine DNA methylation status [26] as well as mitochondrial function, damage, and reactive oxygen species [27].

Fluorescence-Activated Cell Sorting

It may seem that sorting cells for further analysis would be a natural outgrowth of the capability of flow cytometers to identify specific cell populations with a high degree of purity in a high-throughput manner. Interestingly, however, flow cytometry was initially developed with the aim to sort different cell types with high resolution [1, 5]. Flow cytometry is even often referred to as "FACS" which stands for fluorescence-activated cell sorting. Today, however, the majority of flow cytometry is not used for this purpose, and most cytometers are not equipped for cell sorting. Despite this, FACS can be a powerful method to acquire individual cell populations for subsequent downstream applications. After FACS, cells can be analyzed further using Western blot, RT-PCR, or RNA-seq. Additionally, specific cells can be selected for adoptive cell transfer or other functional in vitro or in vivo experiments.

Future Directions in FC

Due to the popularity and utility of flow cytometry, further expansion of this technology continues to develop. New techniques and clinical applications to improve the quality of patient care including better selection of hematopoietic stem cells for bone marrow transplant and enhanced detection of cancer and recurrence are constantly under development [13–15, 20, 28]. Additional advancements have also been made to build upon current cytometers. One such innovation is mass cytometry. This technology takes advantage of the single cell analysis of flow cytometry; however, instead of using fluorescence-based cell analysis, it uses mass spectrometry to differentiate targets. Current machines used for data collection are referred to as time-of-flight mass cytometers, or more commonly, CyTOF. Instead of fluorochrome-conjugated antibodies as in flow cytometry, mass cytometry labels samples with antibodies bound to heavy metals for analysis which eliminates problems in overlap of emission spectra. This allows the user to garner a much larger amount of data on a single cell basis, in most cases over 40 different targets can be analyzed on each cell [29]. One limitation of mass cytometry, however, is that cells cannot be saved after analysis as the cells are vaporized. This limits the ability of secondary analysis afforded by FACS. Another advancement founded on the principles of flow cytometry is imaging flow cytometry (IFC). This technology was originally described not long after flow cytometry; however, only recently have advancements come along to broaden its applicability. Imaging flow cytometry combines aspects of conventional microscopy and flow cytometry. Up to 12 images of an individual cell can be taken simultaneously including fluorescent data as well as dark- and bright-field microscopy, broadening the array of morphologic data that can be

garnered. Additionally, IFC has the ability to analyze up to 5000 cells/s and detect rare cells at or below 0.001%. This technology has important potential applications in the detection and study of rare cell populations such as cancer stem cells [28, 30].

Background of Cytometry by Time of Flight (CyTOF)

Cytometry by Time of Flight, or CyTOF, is a mass spectrometry-based technique that has revolutionized single-cell analysis of heterogenous cellular populations in a variety of fields including immunophenotyping, neuronal cell identification and describing tumor microenvironments [31–34]. Bandura et al. first described CyTOF in Analytical Chemistry in 2009 where they employed this technique to simultaneously study 20 different antigens in leukemia patient samples using metal isotope tagged antibodies [35]. Today, CyTOF has increasingly gained popularity for its ability to characterize complex cellular heterogeneity with minimal signal overlap and in small sample sizes [31, 36]. Prior to CyTOF, the previously described flow cytometry with fluorophore-tagged antibodies was the conventional methodology used to characterize and cluster cellular populations at the single-cell level without the use of gene sequencing [37]. However, flow cytometry is limited by the number of fluorophore signals that are compatible in a singular experiment due to fluorescence overlap and the maximum number of channels or parameters that can be used simultaneously [31, 36–38]. In contrast, the use of heavy metal isotope tagged antibodies in CyTOF enables detection of over 50 different parameters through detection of individual metal ions rather than fluorescence intensity [31].

CyTOF Sample Preparation

CyTOF protocols begin with cellular harvest prior to incubating cells with unique radioactive heavy metal isotope tagged antibodies or metal-tagged probes to allow for binding to appropriate targets [38, 39]. These antibodies or probes target a wide variety of antigens including cell surface markers, protein modifications, cytoplasmic or nuclear proteins, or even nucleic acids [39]. Due to the prior explanation of cell staining and fixation strategies for flow cytometry, which have applicability to CyTOF sample preparation, this section will primarily focus on the key discrepancies in sample preparation unique to CyTOF.

Barcoding

Barcoding is an important differentiating step in CyTOF protocols [38, 39]. Barcoding allows for combining multiple batches of experimental cells with unique labels to help identify sample origins. Barcoding precedes antibody incubation and should be added to samples after initial fixation. The two most common methods for barcoding samples include: monoisotopic cisplatin barcoding or palladium barcoding [40–42]. For both processes, ready to use kits contain barcode isotopes (for up to 20 unique barcodes in standard kits), that can be added to the samples after

fixation and permeabilization [42]. Post-data acquisition de-barcode software is then utilized to compile data from cells of a unique barcodes into individual FCS files.

Iridium Labeling of DNA

Unlike flow cytometry where each cell is automatically counted within the forward/side scatter plots, mass cytometry does not automatically account for a single cell as an event unless there is a detectable metal isotope present [38, 39]. Therefore, all cells must be labelled with one metal that can allow for initial detection. To overcome this, all cells are labeled with iridium-containing DNA intercalators [43]. This, in addition to the cell barcoding, allows for initial optimization of sample detection by ensuring each cell containing iridium is registered and with only one barcode.

Panel Selection

Panel selection refers to the process of pairing antibody probes with the detected molecules (the heavy metal conjugates) for mass cytometry [44]. The overall goal of panel design is to maximize the signal detection of proteins of interest while minimizing the background, especially for difficult to detect targets. There are online tools to help with experimental panel selection. For example, Fluidigm offers an interactive online panel design tool that allows users to select metal-conjugated antibodies (MCAs) for the Helios system [45]. There are several guiding principles for success in panel design: (1) Choose high sensitivity channels for the low-abundance targets (in general, for the Helios machine the greatest sensitivity for detection is in the 153–176 Da range). (2) When choosing channels for high abundance targets, avoid using mass tags within 1 Da of a channel assigned to a low abundance antigen to minimize crosstalk. (3) Finally, for antigens with variable or unknown expression, always titrate staining reagents and perform preparatory pilot experiments to estimate abundance and to choose the appropriate channels [44, 45]. Additionally, for those wanting to utilize pre-existing panel sets, there are readily available panels that can be modified to user specifications. Panel selection is often the most complex part of the experimental preparation and is personalized to each experiment. Thus, investing time in selecting the appropriate channels leads to optimal signal detection and allows for ease of data interpretation downstream.

Data Acquisition

Currently, the most used platform for mass cytometry experiments is the Helios CyTOF system through *Fluidigm*. After sample preparation is complete, cells of interest are placed in a liquid suspension which is injected into a nebulizer [31]. The nebulizer aerosolizes the cells into individual droplets and exposes them to an argon plasma torch, allowing for the ionization of the cellular atoms to take place [31, 38, 46]. Once the cell has been atomized, the predominant biological ions are removed (ex: Na, K, Cl ions for example) primarily by weight distribution (<75–100 Da).

The remaining heavy metal reporters (>100 Da) are then processed and detected accordingly [31, 38, 46].
These ions then enter the Time of Flight (TOF) chamber where the ions are separated by mass to charge ratio (m/z) [31]. in the time-of-flight chamber, an electric current accelerates generated ions through a field, and the time of flight to reach the detector is then measured [31, 38]. The time of flight is inversely proportional to the square root of the mass to charge ratio. Thus, the ions of interest are separated, and the read-out is converted to an electrical signal that is reported in a table or matrix format for how many isotope signals were detected for each cell [31, 38]. Each cell can theoretically occupy many detectors due to multiple signals. Therefore, this information is then multiplexed and projected as single-cell events onto a data plot to visualize cellular populations with similar signals.

Data Analysis

The largest discrepancy in cytometry techniques between CyTOF and flow is in the realm of data analysis and visualization. Unlike the Boolean-based gating strategies that are user-selected in flow gating schemes, CyTOF data analysis is more automated with identification of cell groups through parametric and non-parametric clustering approaches [46].

CyTOF data is initially reported similar to flow cytometry data in FCS files. However, for downstream analysis CyTOF data is converted to an expression matrix through the extraction of ion counts from the raw FCS files [46, 47]. For CyTOF experiments, the matrix usually contains cell events in rows, with corresponding target proteins in columns. Prior to analysis, data preprocessing methods first do the following: de-barcode, perform live/ dead cell gating, and normalize/ batch correct cells [36, 46–48]. Analysis itself consists of three overarching principles that guide the workflow: (1) Cell subset detection (Clustering). (2) Analysis of differential abundance of expression. (3) Visualization via dimensionality reduction [46]. At each step, there are multiple existing software packages with differential approaches to perform the analysis. For brevity, the most used methods will be discussed here.

Cell Subset Clustering

The primary goal of cell subset clustering is to first identify functionally distinct cell populations with the high-dimensional data provided by CyTOF [49–51]. With flow cytometry, cell subsets are identified manually through gating of cell groups that cluster together on dot plots where the x and y axis are two different proteins. This method depends on prior knowledge of the differential gating strategies of the cells to derive these clusters; i.e. the user must know the two gates to select to visualize the populations of interest [49–51]. In CyTOF, supervised or unsupervised clustering techniques automate this process and allow for detection of cell groups through segregation into phenotypically distinct populations, allowing for new cell populations that may have previously been gated out through standard strategies to be discovered [46, 52].

PhenoGraph is a CyTOF cell subset clustering technique that uses k-nearest neighbor classifier method (k-NN) to construct cell subsets based off of N closest neighbors for each cell [47, 50, 53]. Each node is one cell, and it is connected to its nearest neighbor by phenotypic similarity based on the marker expression for each cell and its closest neighbors [47]. By doing this for each cell, clusters of cells emerge from interconnectivity and therefore are part of distinct cellular populations. This tool automatically defines the number of clusters in the data, though the user must define the number of N neighbors for a cell [47, 53]. PhenoGraph does not provide visualization of clustering and requires PCA/ t-SNE or UMAP plots for visualization (see dimensionality reduction).

FlowSOM Cluster is a more complex clustering algorithm that utilizes two levels of clustering to visualize the number of clusters and the intensity of cell type markers across the population. It uses self-organizing maps (SOM) to group cells with similar features using all events (parameters) for a given cell in a dataset. Uniquely, FlowSOM clustering starts with a random initialization where clusters with similar events are first created. Next, a second round of clustering known as meta-clustering is used to group clusters that are most like each other. This method offers its own visualization package where users can also annotate the clusters.

Analysis of Differential Abundance

The next component of data analysis finds associations between different clusters and target expression once subset clustering has taken place [47, 48, 52]. Like differential gene expression analysis in RNA sequencing experiments, analysis of differential abundance seeks to identify protein expression changes in different clusters or between different sample sets. Two popular tools for this analysis for CyTOF include CITRUS and Statistical Scaffold [54].

CITRUS refers to cluster identification, characterization, and regression [54]. It works by first using unsupervised hierarchical clustering to cluster all events in a dataset [53, 54]. Then, it calculates the percentage of cells out of the total population present in each cluster and the median expression of different functional markers in each cluster [53, 54]. By doing this, it identifies biologically relevant features of each cluster.

The statistical scaffold method differs from CITRUS in that the initial cell populations are identified by manual gating in a known sample [55]. These populations are then used as scaffolds or nodes on a map to compare against other experimental samples [55]. If there is a high degree of similarity between the median marker values in the representative node vs. an experimental sample, the sample will map onto the same node on the scaffold map [55].

Dimensionality Reduction

Finally, dimensionality reduction is a key component of the workflow that allows the user to visualize the data. Conceptually, dimensionality reduction functions to reduce the total dimensions (features or parameters) in a dataset while retaining the key information that defines the dataset [47, 51, 56]. The reduction decreases complexity to help visualize the higher dimensional data in a lower dimensional 2D space. The most common methods for dimensional reduction include principal

component analysis (PCA), t-distributed stochastic neighbor embedding (t-SNE) or its CyTOF specific version named "viSNE," and uniform manifold approximation and projection (UMAP) [57]. As these three methods have been extensively used and described for visualization of sequencing datasets, a detailed description of these processes is outside this chapter's scope.

CyTOF Vs. Flow Cytometry

Advantages

Mass spectrometry offers several advantages in comparison to the current flow cytometry protocols for cell sorting and characterization. First, it reduces spectral overlap as time of flight accurately separates heavy metal isotopes using their mass to charge ratio [31, 32, 58]. Second, antibody conjugation with fluorophore probes in the current flow cytometry practices have broad emission spectra and in experiments with multiple fluorophores being utilized simultaneously this necessitates compensation experiments to correct for fluorescence background [31, 32, 59]. Moreover, autofluorescence of cells is no longer an impedance to cell detection and sorting as the cells injected into the argon flow chamber of a mass spectrometer are vaporized to form the ion clouds that are then detected [31, 32, 59]. Thus, minimal compensation and autofluorescence reduction are strong advantages of CyTOF.

Next, the ability to form elaborate panel designs to help isolate and study cells with the theoretical use of 135 parameters (currently ~50 parameters are widely used for a singular cell) allows for greater specificity of evaluation of cell populations enabling CyTOF to be ideal for testing large numbers of antigens in rare sample sizes [36]. Intriguingly, mass spectrometry has the unique ability to also investigate protein modifications at a high-throughput level [35, 36, 60]. When this capacity is multiplexed with single-cell RNA sequencing (scRNA Seq), this enables single-cell analysis of cellular transcriptomics and its functional relevance in cellular proteomics. CyTOF principles are also used in combination with imaging mass cytometry (IMC) where the metal-tagged antibodies can be used instead of fluorescently tagged antibodies for immunohistochemistry on tissue sections [61]. Instead of starting as a liquid suspension, the sample is a stained tissue slide that is then vaporized using an UV ablation device that specifically vaporizes 1 micrometer of surface area at a time, allowing for the position of the markers to be recreated in a post-analysis image of the tissue [61].

CyTOF also allows for detection of mRNAs as DNA probes can be conjugated to metal tags for mRNA identification which when multiplexed with scRNA seq can further confirm and validate gene expression data [62, 63]. When coupled with IMC, this can be spatially localized and provides an in-depth view into gene expression dynamics in tumor microenvironments [63]. Finally, compared to sc-RNA sequencing, CyTOF is comparatively much cheaper. It is estimated that sc-RNA sequencing costs $20 per cell analyzed (this considers the cost of reagents and data acquisition but not the down-stream data processing), whereas the per cell cost for

mass cytometry is less than 1c per cell [31, 62]. As a single-cell analytical modality, CyTOF is extremely cost favorable and reduces overall expenditure for high-throughput experiments.

Disadvantages

Unlike flow cytometry that allows for preservation of the sample through the process, CyTOF vaporizes the cells into an ion cloud and this process precludes any downstream cell sorting for further experiments or analysis. As such, CyTOF samples are terminal or static experiments that allow for evaluation of a group of cells at a moment in time [31, 32]. The same cells cannot be measured over time. It is also important to note that the heavy metal isotopes that are conjugated to the antibodies comprise of rare earth metals which can be costly and the conjugation itself is a laborious process. Metal isotope tagged antibodies (MitAbs) are the reporting probes in CyTOF and the elemental mass tags (EMTs) are usually comprised of either noble, post-transition, rare-earth metals, or halogens [31, 32]. This allows for minimal cross-reactivity with endogenous cellular elements. Ensuring that the MitAbs have minimal spillover and that the Mit-Ab conjugation is secure requires complex chelating agents and rigorous reagent testing [64]. This has therefore limited the commercial availability of the metal-isotope tagged antibodies and normalization of different CyTOF experiments.

The rate of acquisition between flow vs. mass cytometry differs significantly. Flow cytometry can acquire anywhere from 2000 to 20,000 cells per second [36, 40, 65]. In contrast, CyTOF acquisition rates are around 200–300 cells per second, requiring run times that are 10x longer for the instrument to collect the same total number of data points. The longer run times can lead to signal intensity decay for runs longer than 6 h or lead to a decline in overall instrument sensitivity. To overcome this, in longer CyTOF experiments it may be necessary to correct signal fluctuations by using polystyrene beads that have a known concentration of isotopes as "reference points" along with the cell samples [66]. This additional step is unnecessary in flow experiments.

Finally, while most metals used in CyTOF do not cross-react with endogenous cellular elements, in rare cases such as cancer patients with exposure to platinum-based chemotherapies or those exposed to radioactive isotopes such as Gadolinium can have some signal cross-reactivity [31, 66].

Future Directions of CyTOF

For surgeon scientists, the next frontier of *in vivo* real-time mass spectrometry analysis for surgical guidance is a particularly exciting future application of CyTOF [67, 68]. Surgical resection margins continue to pose an ongoing challenge for multiple aggressive tumor types and real-time feedback to help resect down to healthy tissue has long been a goal within surgery. The development of the iKnife has been based on rapid evaporative ionization mass spectrometry (REIMS) where aerosolized tissue is analyzed real-time during electrosurgical dissection. Here, by analyzing

differences in lipid composition and metabolism, the iKnife has been able to distinguish healthy breast tissue from a positive breast carcinoma margin [69]. It has also been applied in clinical practice with cervical cancer specimens in real-time [68]. The iKnife's applicability in other surgical fields are currently being explored including the field of cardiac aneurysmal repair by testing extracellular matrix stability post and pre-repair to determine rate of repair failure [67].

Another active area of CyTOF development is in the field of deep phenotyping of circulating tumor cells (CTCs) [65]. CTCs have been hailed as a biomarker for cancer recurrence; however, they are difficult to study due to their rare numbers and have often been detected through high sensitivity liquid or droplet polymerase chain reactions [65]. To enrich enough CTCs for deep phenotyping has so far been a considerable challenge and has resulted in bulk sequencing of CTCs or using conventional flow cytometry to characterize populations in which considerable data dimensionality is lost. However, with mass spectrometry which excels in phenotyping rare cells this is finally starting to become possible.

Finally, studies in drug bioavailability, pharmacokinetics, and nanomaterials are starting to utilize CyTOF in highly innovative experiments to determine drug response and activation states [60, 70]. Moreover, immune response to biomaterials leading to differentiation or activation is also an area of active study. One can envision how pre-clinical data testing of small molecules can be utilized in conjunction with CyTOF to assess therapeutic response across a wide variety of parameters [60, 70]. Additionally, the downstream analysis could even help determine if drug responses shift cells towards a non-diseased state or if they eliminate individual diseased cell clusters altogether.

Summary

In summary, FC and CyTOF are a powerful technique for single-cell analysis that allows integration of numerous parameters to help identify and classify cell populations. There are a variety of advantages that make CyTOF increasingly popular in the current landscape. While it is important to realize that conventional flow cytometry still allows for valuable data collection, sorting, and interpretation, the new integration of CyTOF based techniques can help surgeon scientists identify new clinical phenotypes and understand the proteomics landscape in increasingly complex experiments and rare samples.

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Effective Cell and Tissue Culture

12

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Abstract

Cell culture is a powerful tool for biological investigation and one of the most widely used approaches to mammalian biology. This technique allows rapid iteration with lower costs than other methods, particularly animal models. At the same time, it is sufficiently complex to model various intracellular processes reliably. An understanding of the technique is essential to a modern scientific career. This chapter introduces a basic framework for understanding key ideas in cell culture.

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Introduction to Cell Culture

History of Cell Culture

The cell theory, the idea that the basic unit of life is a cell, was first devised by Matthias Schleiden and Theodor Schwann on the basis of their observations of plant and animal tissues in the late 1830s [1, 2]. This idea was the culmination of two centuries of technological improvements in microscopy driven by many scientists, including such luminaries as Robert Hooke, Anton von Leeuwenhoek, and Robert Brown [1]. Subsequent key experimental work by Louis Pasteur, Joseph Lister and others later in the 1800s also played an important role by demonstrating that unicellular microbial life did not arise spontaneously from non-living matter, but rather from preexisting microbes [2–4]. Roughly contemporaneously Rudolf Virchow, among others, proposed that multicellular organisms generate new cells from the division of existing cells [1, 5]. This new framework for understanding life naturally led to redoubled efforts to study cells and the tissues they compose.

Modern cell culture was developed primarily in the first half of the twentieth century. A number of scientists, including Sydney Ringer, maintained and studied animal organs for short periods in various saline solutions (including the eponymous Ringer's solution) in the late 1800s [6]. The first *in vitro* culture technique with demonstration of replication was performed by Ross Harrison. Pieces of frog tissue were maintained in lymph in a depression on a microscope slide, enabling live imaging [7]. Early attempts resulted in bacterial contamination, resulting in an emphasis on sterile technique in cell culture which persists today. The surgeon Alexis Carrel, in addition to his positive work in vascular surgery (for which he won a 1912 Nobel prize) and organ perfusion, and his negative contributions to humanity through his ardent support of eugenics, made a number of key contributions to cell culture. He identified serum as an excellent culture medium, demonstrated that cells could grow on a 2D surface, and developed methods for passaging cells [8–10]. As the early 1900s gave way to the mid twentieth century, a number of scientists made significant contributions including identifying new types of media, the use of trypsin to create cell suspensions, and the identification of growth factors and other elements which allowed the development of defined media [8]. A new field of inquiry, virology, led to key advances in cell culture as scientists including John Enders, who won the 1954 Nobel prize for this work, pursued improved methods to mass-produce and replicate viruses using cell culture [8, 11]. One limitation in first half of the 1900s was that human cell culture lines were not able to replicate indefinitely; although primary cultures could be subcultured repeatedly, they eventually became senescent. The first immortal tumor cell line, the well-known HeLa cell, was established in 1951 from cervical adenocarcinoma cells from a young African

American woman, Helen Lacks [12]. Notably, this occurred in ethically fraught circumstances in which neither Ms. Lacks, who died within a year of diagnosis, nor her family were informed of or consented to the use her cells [13]. Immortalized cells are now the most commonly used tool in cell culture. Cell culture techniques and technologies continue to evolve, but the tools established largely in the last century continue to be the foundation of cell culture today.

Utility and Limitations of Cell Culture

Cell culture is a valuable tool in the hands of the surgeon-scientist. Cells are the simplest unit of life, and studying them in the controlled environment of cell culture is useful for almost any reductionist approach to understanding biological phenomena. Exploring mechanisms inside cells offers a convenient balance. It allows the isolation of function from the complex influences of other cells, tissues, and hormones inside an organism, but retains the intracellular machinery that influences the biochemical, molecular biological, or cell biological system under investigation. Cellular function and dysfunction is key to understanding many disease processes. Cell culture can also be used to produce biological tools for use in other experiments and to produce drugs.

As an example of how cell culture can be used to study human disease, inflammatory conditions and infections each involve expression of various cytokines from a number of different cell types. Release of inflammatory cytokines such as TNF-α is induced by bacterial signals including lipopolysaccharide (LPS) in the setting of bacterial infection [14]. This same pathway can be studied in cell culture by exposing a given cell type in culture to LPS and measuring cytokine production [15]. Similarly, TNF-α produced by cells can be purified and injected into a mouse or applied to another cell type *in vitro* to study the effects of TNF-α signalling [16, 17]. Studies using these techniques supported the idea that TNF-α signalling is important in inflammatory bowel disease [14]. These findings led to the development of monoclonal antibodies which are expressed in cell culture and purified to produce drugs such as infliximab, which is a treatment for refractory inflammatory bowel disease [18]. This example illustrates how cell culture techniques are at the core of much of modern mammalian biology and medicine.

Cell culture offers numerous advantages over other experimental approaches. The primary alternatives for most experiments are *in vitro* biochemical experiments and animal models. Since it actually uses cells, as noted above, unknown intracellular factors are incorporated into the experiment, unlike with purely *in vitro* biochemical approaches. When compared to animal models, in addition to removing the additional complexity of the whole organism, cell culture experiments are also less costly and often faster (typically an experiment will last days rather than weeks or months), allowing repeated rapid iterations.

Cell culture also has clear limitations. A human cell is not a human. Data from a given cell type can miss differing levels of complexity that happen in an organism, where multiple cell types or even the same cell type in different tissues may respond

uniquely to the same stimulus. Choice of cell line and also comparing different cell lines is important in most experiments. There are ways to circumvent some of these limitations. Additional levels of complexity, including cell-cell signalling can be added by co-culturing multiple cell types together or restoring three-dimensional tissue architecture in organoid or spheroid culture, as we discuss later.

The utility, limitations, and techniques of cell culture are fundamental knowledge for the surgeon-scientist. As probably the most widely-used tool in a modern biology laboratory, this technique offers unique advantages in biological investigation. Cell culture does not replace other experimental methods, but rather complements them. The following sections outline basic tools and considerations in using cell culture. This is not meant to be an all-encompassing text or laboratory manual, but an outline for the surgeon-scientist wishing to embark on a new scientific journey.

Elements of Cell Culture: Culture Media

Cell culture uses two main components: media and cells. The culture medium is the fluid in which cells are grown. Media has the key functions of providing pH balance with an appropriate salt content while supplying nutrients, allowing oxygen delivery and carbon dioxide removal, and serving as a sink for metabolic waste products [19]. Most cell types or lines also require growth factor signalling [20]. These factors differ by cell type and species. They were historically supplied by animal derived fluids, most prominently serum (the liquid component of blood after clotting), but the subsequent development of chemically defined media enabled an alternative for experiments that cannot be performed in serum [19, 20].

Most cell culture is performed with a basal media, which consists of electrolytes (usually with a composition mimicking extracellular fluid), essential amino acids, often glucose and various vitamins, and a buffering system [21]. A well-known example is Dulbecco's Modified Eagle's Medium (DMEM). These basal media require supplementation for growth of most cell types, either with serum or a defined mixture of other factors.

Serum-supplemented media continues to be widely used in cell culture. Serum generally contains a wide array of required nutrients, including carbohydrates, amino acids and proteins, and lipids and fatty acids, as well as vitamins and a number of vaguely defined growth factors (often signalling proteins that promote cell longevity, growth, and replication, and inhibit cell death) [19]. Different sera are slightly different in this regard. The most commonly used serum is fetal bovine serum, which supports the growth of a wide number of different cell types. However, serum suffers from variability in composition across batches [21]. It is important to always document which batch (LOT#) of serum was used in experiments in order to consider as a cause for inconsistencies in the results. Undefined factors in serum can also confound experiments. Serum can occasionally be a source of contamination from microbial organisms or viruses [20]. Finally, growing immune cells with serum can cause immune incompatibility issues, particularly if the goal is to return the cells to an organism with a functioning immune system [22].

The limitations of serum can be overcome with use of chemically defined media. While it is beyond the scope of this chapter to outline all chemically defined media, they consist of a basal media supplemented with additional factors from a synthetic source, rather than with serum or another mixture derived from an organism [19, 21]. Distinct cell types often require different media. The chemically defined additions to basal media typically include recombinant albumin, transferrin, and specific lipid mixtures, including cholesterol [19–21]. They often include other micronutrients such as various essential metals and vitamins. Protein growth factors are often added for specialized cell types or other experimental purposes, including organoid culture, stem cell culture, and more [20].

Elements of Cell Culture: The Cells

The other essential element of cell culture is the cells themselves. A key concept is that there are a plethora of cell types, origins, and growth methods, each of which have utility in distinct experimental applications. These ideas can be broken down into different categories which we expand upon here.

Culture Methods

Cells can be grown by distinct culture methods. The classic method is adherent culture on a 2D surface. In the modern era this is typically performed on plastic culture dishes or plates with wells of varying size [13, 23]. The growth surface is often functionalized with anchoring proteins or ECM components to enable cell attachment and proliferation. This remains the most common technique. Advantages of adherent cell culture include easy media exchange, since the cells stay fixed to the plate while media can be removed and replaced, ready visualization of cell density, and straightforward application of some microscopy techniques.

Many cell types, including some immortalized cell lines and most blood cell types, can be grown in suspension [13]. This has the primary advantage of allowing cells to reach higher numbers in a smaller space than adherent culture, which is particularly useful when producing or isolating compounds from mammalian cells [24].

In the past several decades significant technological advances have led to widespread use of 3D culture. 3D culture, unlike adherent culture, results in cells adhering to one another and adopting a 3D architecture [25]. Ideally this architecture is similar to that in the tissue or tumor of origin, and replicates other features of that tissue which are absent in 2D culture [26]. These features can include effects from cell-cell signalling, cell polarization, nutrient diffusion, and extracellular matrix contact [26, 27]. In this way, 3D culture restores some, but not all, of the complexity of an organ. It can be thought of as a way to more closely approximate the organismal environment without the cost, complexity, and time disadvantages of an animal model. The methods for achieving 3D culture vary significantly with the cell or

tumor type, and a detailed description is outside the scope of this chapter. Briefly, there are scaffolded and scaffold-free methods. Scaffolded methods utilize an artificial matrix, usually either a biopolymer hydrogel made of collagen or other biological material, or an inert mechanical scaffold made of polystyrene or other synthetic polymers [25, 27]. Scaffold-free methods include use of hydrophilic plates to discourage cells from adhesion and micropatterned surfaces to induce clustering [25, 27]. Each method encourages self-assembly of cells, but they often also require unique culture media constituents specific to the cell or organ type of interest. The resulting cell agglomerations are usually called spheroids if derived from a tumor, or organoids if derived from and assembling similar to a given organ tissue [25]. The methods of achieving 3D culture are more complicated than normal adherent 2D culture, but they offer advantages in more closely simulating an *in vivo* context.

A final modern culture technique is the use of co-culture. In this case two or more cell types or lines are cultured together in the same system [28]. As an example, tumor cells and various types of immune cells can be cultured together to investigate the response of each to the other. This technique restores some of the natural cell type-cell type crosstalk that occurs *in vivo*, enables culture of more fastidious cells that require factors secreted by adjacent cell types for growth, and allows investigation of more complicated questions than can be answered by single cell-type culture. It can also be combined with 3D culture techniques to replicate more of the *in vivo* cellular context [25, 28].

Primary Culture and Immortalized Cell Lines

Cell culture can be performed with either primary cells (cell lines derived from primary cells) or clonal immortalized cell lines (Fig. 12.1). Primary cell culture refers to cells derived directly from the organ or tissue of interest [29, 30]. This can be very useful as these cells reflect very closely the characteristics of those cells in the tissue of origin, they mirror the heterogeneity present in the cells in that tissue, and depending on the isolation method, there may actually be multiple cell types present, just as in the parent tissue. Unfortunately, most primary cells have a limited lifetime. Many primary cell lines can only undergo a certain number of cell divisions *in vitro*; they become senescent and the culture will die off [29]. This means that a primary culture has to be repeatedly re-isolated from fresh tissue. For some applications, this is acceptable, but for other experiments, having a consistent cell line that can be used for the same experiments repeatedly over time is valuable.

Cells of most types typically live longer than the media in which they grow is functional. They eventually fill the vessel (such as a culture dish) in which they are grown, which inhibits replication. Therefore, cells are typically passaged (or subcultured, in older terminology) by isolating a subset of the cells and replacing the media [29–31]. When primary cells are passaged, they are termed a cell line [31]. An important point is that growth in culture, particularly with serial passage, is a selective process. The

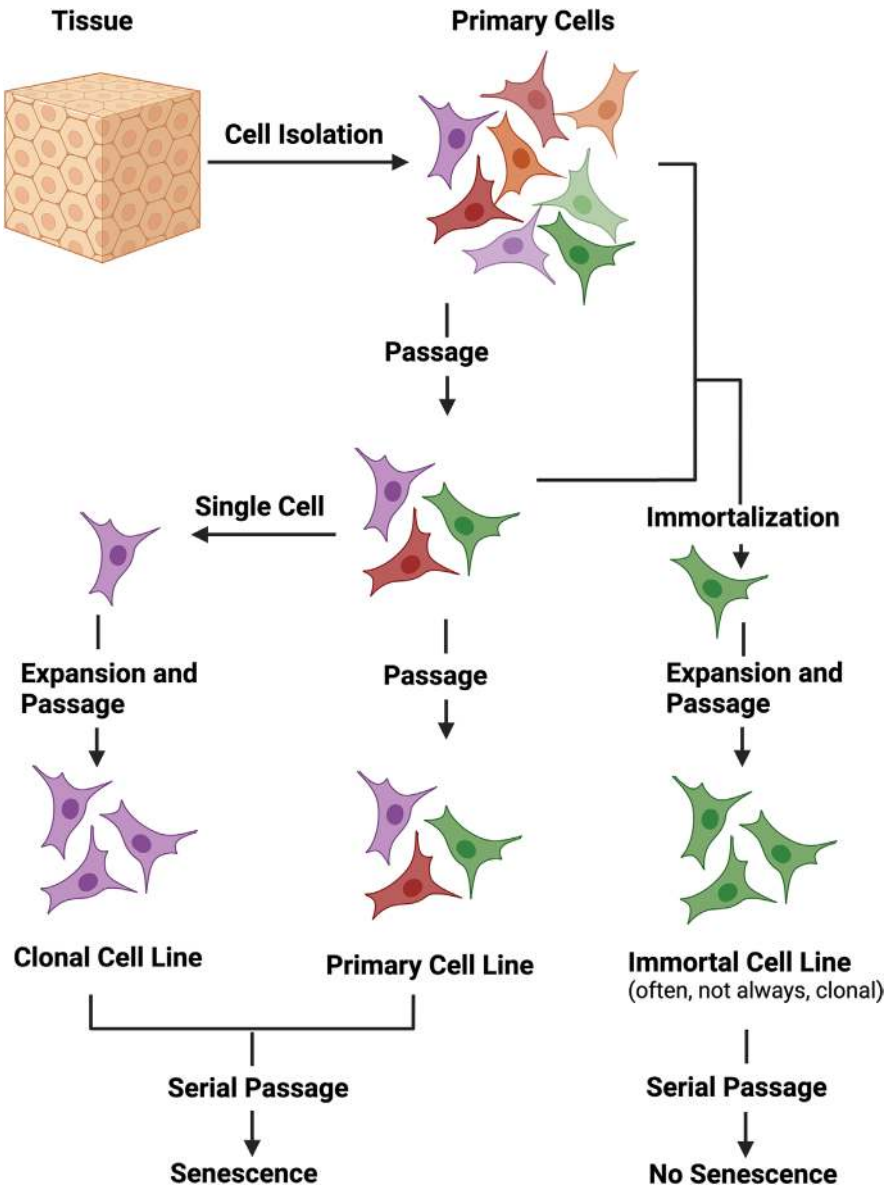

Fig. 12.1 Nomenclature of Cell Lines in Cell Culture. Shown is the relationship between primary cells derived from tissue and cell lines, including clonal, primary, and immortal cell lines. Created with Biorender.com.

variability in a cell population decreases with serial passage, and particular traits are selected for as a cell line is established. This can alter outcomes of experiments. Most primary cells can only be passaged on the order of tens of times, although the number of passages varies by cell type (*e.g.,* differentiated epithelial cells tend to allow fewer divisions than less differentiated fibroblasts [32]). Cells can become immortalized through the development of genetic changes that remove limits on replicative potential—leading to them becoming an immortal cell line, sometimes also called a continuous cell line [31]. There are a number of techniques for inducing immortalization. The most common methods in modern use involve viral expression of either human telomerase reverse transcriptase or SV40 large T antigen, but oncogene expression can also facilitate immortalization [33]. Because immortalization is a relatively rare event, the resulting population is often not fully representative of the original primary cells. However, these cells now have unlimited replicative potential.

Cell lines can become clonal [30, 31]. If only one cell is used to propagate after passage, the resulting cell line will be clonal and all descendants will be of the same parent cell. Clonal cell lines can be established from either primary culture using cell selection techniques or as immortal cell lines, but not all primary or immortal lines are clonal. It should be noted that clonal does not imply the complete absence of variation among cells within a cell line [31].

Cancer cells in tumors share many of the features of immortalized cells. They lose inhibitory influences on cell growth and division and acquire changes that promote cell survival [33]. Therefore, while tumor cells can be grown in primary culture like any other cell, tumor cells have a lower tendency to senescence, and occasionally become immortalized during serial passage [33]. This often does not require viral or chemical treatments as in other primary cells.

Immortal cell lines offer strengths but also have weaknesses as an experimental tool. Because they are immortal, they can be used in many experiments, theoretically without any changes in the characteristics of the cells. This means that experiments done even years apart or across the world can be directly comparable. Laborious cell isolation from tissue does not need to be done for every experiment, offering significant time savings. However, the features that result in immortal, clonal cell lines aren't all advantageous. These cells can have biological features that differ from the original primary cells or the original tumor [29]. The diversity of individual cells, even within a given type, is often lost. Response to various stimuli, genetic structure, transcription of individual genes, and composition of protein machinery can all differ. However, despite these limitations, immortal cell lines are still the most widely used tool in cell culture.

Techniques and Concepts in Cell Culture

Cell culture is not a particularly complex technique. However, certain aspects of the process are important for conducting effective experiments. Here we outline key laboratory principles in cell culture. For detailed protocols, refer to one of the cell culture laboratory manuals referenced below.

Essential Equipment

Cell culture requires relatively simple laboratory equipment (see [13, 23, 29]). Cells are grown in a sealed incubator with augmented carbon dioxide levels (usually 5%) and controlled humidity. A microscope, usually an inverted phase contrast model, to monitor cell density in plates or dishes should be available. A method for cell counting, such as hemocytometer slides for use with the microscope, or alternatively an automated counting device, is necessary for performing precise dilutions of cells in suspension. A laboratory centrifuge for collecting cells is important. One of the key challenges in cell culture is maintaining sterility. For this purpose a biosafety cabinet for use when uncovering plates or flasks and performing manipulation is necessary. Disposable filters for media or other additives as well as pipettes and other standard laboratory glassware are also required. Access to an autoclave is necessary. The cells themselves are contained and grown in culture plates or flasks, which are generally made of various plastics and are disposable. Finally, laboratory refrigeration equipment, including a −80 °C freezer and access to cryopreservation in liquid nitrogen are required for cell storage.

Sterility in the Cell Culture Lab

Sterility is vital to performing cell culture. In fact, recognizing the importance of sterility and then creating technology to minimize contamination was a turning point in the history of cell culture [2]. Cell culture media is conducive to bacterial and fungal growth. Viruses can also infect cells in culture and replicate unchecked. Techniques for maintaining sterility aim to limit introduction of microbes into cell culture and to provide antimicrobial therapy to eliminate bacteria or fungi if contamination occurs.

The mainstay of aseptic technique in cell culture is using sterilized equipment [13, 34]. Disposable equipment for cell culture is typically supplied sterile. For all other equipment, sterilization must be performed by the user or laboratory staff. There are three commonly used methods for sterilization in the lab: application of heat (wet or dry), chemicals, or radiation. Autoclaves or ovens are commonly used methods for sterilization of portable, heat-stable lab equipment. Autoclaves are also used for sterilizing liquids that can tolerate high temperatures. Chemical and radiation sterilization also both have a role. Laminar flow hoods contain a UV light that is kept on while the hood is not in use to help with decontamination of any organisms that have entered the hood. Further, the hood itself and, most working materials that enter the hood, and the scientist's gloves are usually treated with a generous application of 70% ethanol to help disinfect them. Ethanol is reasonably effective against many bacteria and some viruses; it is not particularly effective against spores or fungi. Other chemicals, including bleach (sodium hypochlorite), formaldehyde, and glutaraldehyde, also have applications in sterilizing surfaces. A final tool used for reducing microbial load is filtration—fluids that cannot be heat treated are filtered through very fine filters that remove most microbes.

The use of a biosafety cabinet is critical in cell culture. These hoods use a filtered, continuous flowing "curtain" of air across the entry to protect the internal contents from the outside environment. It should be noted that some hoods, termed laminar flow cabinets, do not offer protection to the user from exposure to organisms or fumes inside the cabinet. For this purpose, a biosafety cabinet is used instead. There are various classes of biosafety cabinets, some do not isolate the interior environment from outside organisms. The most common kind found in cell culture laboratories, the Class II biosafety cabinet, performs both functions: the particular type of hood is relevant to understanding what work can safely and effectively be performed in that hood [13]. In addition to sterilizing surfaces in the cabinet with UV light and chemicals as noted above, standard aseptic technique should be followed. This includes thoughtfulness regarding what enters and exits the hood, how items are handled within the hood, and always wearing clean PPE, including gloves washed with ethanol.

Contamination of cultures still happens despite all the above measures [35]. An additional mainstay is the utilization of antibiotic and antifungal compounds in cell culture. These drugs are often added to media to reduce the competitiveness off invasive organisms. However, antimicrobials can have cytotoxic effects on some cell lines. The full details of which antimicrobial drugs to use and how to address contamination is beyond the scope of this chapter. However, a special note should be made regarding *Mycoplasma*, which is both a common contaminant and relatively hard to clear due to high resistance to many antibiotics [36]. Mycoplasma is found in many cell lines, and can be transferred between cultures through improper technique. It is also good practice to quarantine new cells and screen them before exposing them to the remainder of the lab. Polymerase chain reaction (PCR) can and should be used to detect contamination [37]. It is recommended to screen cell lines in the lab for *Mycoplasma* on a regular schedule in addition to screening any cells that enter or leave the lab. Importantly, evidence of or an attestation of screening is required for publication of cell culture experiments in many journals. Any unexpected results or growth of cells in culture should also initiate efforts to rule out contamination.

Mammalian cells can also contaminate cell lines. This is surprisingly common. This is most commonly due to inadvertent transfer of one cell line into another during routine experimentation [38]. The same aseptic technique applied to preventing microbial infection of cells should prevent this unfortunate circumstance. Particular care should be taken to avoid working on two cell lines in the same hood at the same time.

Cell Passage and Counting Cells

The most common technique for cell growth remains adherent culture. An essential cell culture skill is evaluating the cells for density, or confluency, releasing them from the cell surface, and replacing them [29]. Confluent cells cover 100% of the surface of the plate. The cell density is described as the percent confluence,

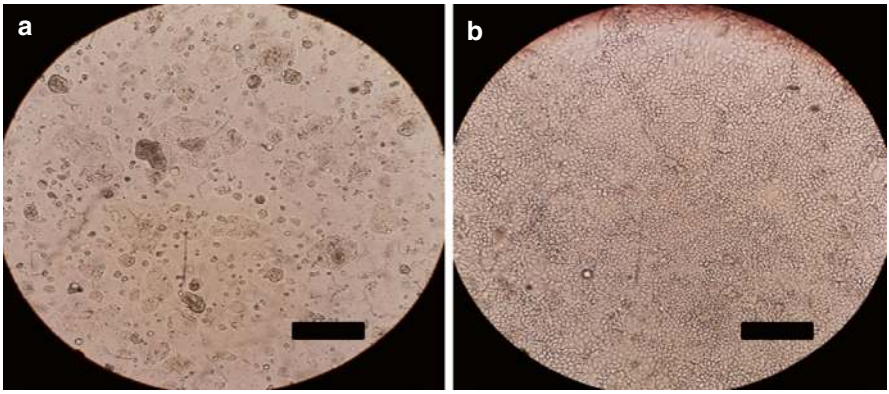

Fig. 12.2 Confluent versus non-confluent cells. (**a**) $CaCO_2$ cells, a commonly used line of cells derived from human colonocytes, that have just been passaged and are not yet confluent. The spaces between groups of cells can be visualized. (**b**) $CaCO_2$ cells that have been growing for 7 days. No spaces visualized between cells which are arranged in a lattice-type network. Scale bars = 50 μm

meaning the percent of the surface area covered by cells. This is estimated using phase contrast microscopy in the plate or dish the cells are grown in. The density of cells limits cell growth and replication, a phenomenon known as contact inhibition. It also impacts cellular function. Therefore, cells are passaged, a term referring to removal of the cells from the plate followed by dilution and return to a new plate in new media [13, 23]. Passage is done before cells are fully confluent to maintain healthy growth (often 70–90% confluence) (Fig. 12.2). For most cell lines this will be necessary every few days to a week. Plate removal is usually performed by the application of trypsin and EDTA. The former digest extracellular matrix components anchoring the cells to the plate, while EDTA chelates calcium, stimulating cadherin release. When used together in solution, EDTA makes it easier for trypsin access and break peptide bonds. Prewarming trypsin is critical for optimal enzyme functioning. Although being exposed too long to trypsin can cause damage to cells by removal of surface proteins, diligent use of this solution is key for efficient separation of cells for the purpose of passaging. They are then suspended in media or a buffered saline solution by gentle pipetting, diluted after being counted, and exchanged into new media conditions in a new plate. Further, immediately after passage is a poor time to perform experiments as the cells are anchoring to the new surface and recovering from the perturbation. It should be noted that various experiments work best at different degrees of confluency: experiments requiring cell-cell contact will not work well at low confluence, while experiments measuring cell growth are not ideally performed at high confluence.

Measurement of cell concentration or number is vital to performing cell culture passage and to replating with appropriate dilution [13, 29]. It is also necessary when quantifying almost any protein or small molecule in cell culture, since these values must generally be normalized to the number of cells present. Cells are most

commonly counted in suspension after release from the plate via trypsin and EDTA treatment. The classic historic method is the use of a hemocytometer, a special chambered microscope slide to which a known volume of cell suspension is added. The cells in a demarked area of known volume are then counted and a concentration is calculated. Various dyes can be used to distinguish viable cells from dead or dying cells and debris. More advanced equipment has also been developed to more quickly and accurately count cells. Many labs utilize these. Generally speaking, they rely on either detection of cells in suspension being passed in front of an electrical impedance detector or a light scattering device (Coulter counter and flow cytometer, respectively) or they utilize automated microscopy image counting [29]. Regardless of method, the scientist must understand and master one or more of these approaches to perform effective and reliable cell culture experiments.

Storage: Freezing and Thawing Cells

Banking or storage of cultured cells is an important topic in cell culture. Frozen cells stored in liquid nitrogen can remain viable indefinitely if properly frozen and thawed. This allows for the continuous use of cells from the same stock across experiments and for sharing cell lines with other labs. Banked frozen cells also provide insurance as a source to replenish stocks in case of infection, culture loss, and operator or equipment failure. Detailed protocols for freezing and thawing cells are widely available in cell culture manuals [39]. The main principles are outlined here. Cells should be frozen in suspension, usually in serum, at a known concentration. A cryoprotectant, usually either DMSO or glycerol, should be added to the solution. Combined with gradual freezing at −80 °C followed by transfer to liquid nitrogen, this usually maximize subsequent viability. A key step is firm but not overzealous tightening of vials, as damage to the gasket can allow liquid nitrogen to enter and cause explosive cap failure on thawing. Care should be taken to label each tube or receptacle with the necessary details to identify the cell stock so that the cells can be reliably identified even years later. Thawing is usually performed rapidly but with care to avoid overheating, followed by transfer to pre-warmed media in a plate and incubation. Early media exchange is important to remove residual cryoprotectant. Cell viability with careful attention to these points within standard protocols is typically greater than 90%.

Successful Experimental Design Using Cell Culture

Quality experimental design in cell culture is both an art and a science. The basic principles are the same as any other experiment. Strong controls are essential, as is a design that maximizes the probability of a meaningful result. The more complicated the experiment, the more ways it can go wrong: simple and elegant experiments often yield the best results.

In cell culture, just as with other techniques, a strong grasp of the literature is vital. Particular attention should be paid to experiments addressing similar questions and to what cell lines have been used. If a given cell type has been widely used in the past, that may be a good starting point. It can be challenging to compare results to prior experiments if a different cell model has been used.

Which cell line to use is typically the most important decision. Immortalized lines are less costly and time-consuming to work with and are easy to utilize in iterative experiments, but don't necessarily reflect the tissue of origin as closely as a primary cell line. However, they are often the first choice. If investigating interaction between two cell types, co-culture can be an excellent approach. When a better imitation of tissue or tumor context is required, 3D culture may be the best approach. If there is significant uncertainty, start with the least costly and most rapid approach. An important principle is that sometimes the initial experimental approach should not be aimed directly at addressing the primary question, but rather at establishing whether the chosen model is able to answer that primary question. The best cell line or method often takes repeat experimentation to determine.

Many cell culture experiments are designed to cross between cell culture and animal models, particularly mouse models. Compatibility between cells and animals must be considered in this setting. Human primary tumor cells or cell lines cannot be transplanted into a fully immunocompetent mouse. For some experiments, syngeneic tissue is the best model. In other cases, varying degrees of immunodeficiency may be necessary. Be aware that not all signalling pathways, for instance, use the same components in mice and humans. A negative result can easily be misinterpreted if immune compatibility and conservation of biology are forgotten in xenograft experiments.

Organ and Primary Tissue Culture

Cell culture is by far the most commonly used method of tissue culture. However, there are other methods of tissue culture that also provide a cheaper and quicker alternative to extensive *in vivo* animal experiments and can reveal important factors of pathophysiologic processes. Tissue culture is a process originally developed by zoologist, Ross Harrison, in 1907 and there are two main types: organ culture and primary explant culture.

Organ culture is a technique that involves harvesting of whole or partial organs from embryos or partial organs from adult organisms. These specimens main the endpoint differentiation and architecture of their original source, making them a viable option for studying macroscopic effects of external agents like pharmaceuticals [40]. One can also examine the effects of certain factors on the functionality of organ such as hormone production. This points to a particular benefit of organ culture being the ability to study the relationships between organs which are farther apart anatomically [40].

Primary explant culture uses fragments of animal tissue or organs. This technique has the benefit of maintaining an environment that more closely resembles the

in vivo conditions in which the primary tissue resides compared to established cell lines. Throughout the culture timeframe, cells migrate from the periphery of the tissue specimens creating outgrowths that be of particular interest in certain biological processes. This feature of primary explant culture allows for its application in the study of cancer cell and tumor proliferation patterns as well as cell morphology [40–42]. The use of immunofluorescence techniques in this process also allows for the observation of specific growth patterns as in neuron development and regeneration [43].

Many of the specific aspects of obtaining tissue and maintaining the culture are similar to that of cell culture. The tissue fragments are usually plated with adherence to a surface coated with extracellular matrix (ECM) before the addition of a basal culture media [43]. The ECM can be made from type 1 collagen, the most abundant *in vivo* collagen, to promote and enhance cell differentiation, adhesion, proliferation and migration [43]. The tenants of maintaining sterility throughout, using standard incubation conditions, and changing culture media on scheduled basis all also remain similar to that of cell culture.

The Role of Organoids

The use of organoids in tissue culture is an innovative technique that enables the study of cells in an environment closely resembling *in vivo* conditions. Organoids are three-dimensional (3D) miniature organs or tissues that are created from stem cells collected from the organ of interest. The cells self-organize into 3D structures and carry the unique advantage of conserving the gene expression and DNA mutation capabilities identical to that of the original tissue. Additionally, organoids can contain various cell types that carry out their specific functions [44].

Organoids can be derived from animal tissue or surgically resected human tissue. Once tissue is collected, it is broken down from its normal architecture and then dissociated into individual stem cells that are plated. Cells must be suspended in a matrix that allows for reorganizing into a 3D structure as the cells grow. Once cells are confluent and have been passaged to the desired number, the organoids can then be remain as stem cells or they can be differentiated using a tissue-specific differentiation media [44] (Fig. 12.3).

There are many beneficial applications of organoids such as in the study of disease processes, drug effects, and genetic mutations. An example of this is in the use of small intestine-derived organoids, termed enteroids, for the study of necrotizing enterocolitis [45]. Enteroids are polarized and contain the full diversity of cells within a normal intestinal epithelium (i.e. enterocytes, Paneth cells, goblet cells, enteroendocrine cells, etc.) [45]. Diseases like necrotizing enterocolitis (NEC) are multifactorial and involve many dysfunctional cellular processes [45]. Thus, the organoid model is a able to produce a more robust and physiologically accurate response to stressors such as LPS and hypoxia in the setting of NEC induction. This example among others point to organoids continuing to rise as a frequently used method of tissue and cell culture.

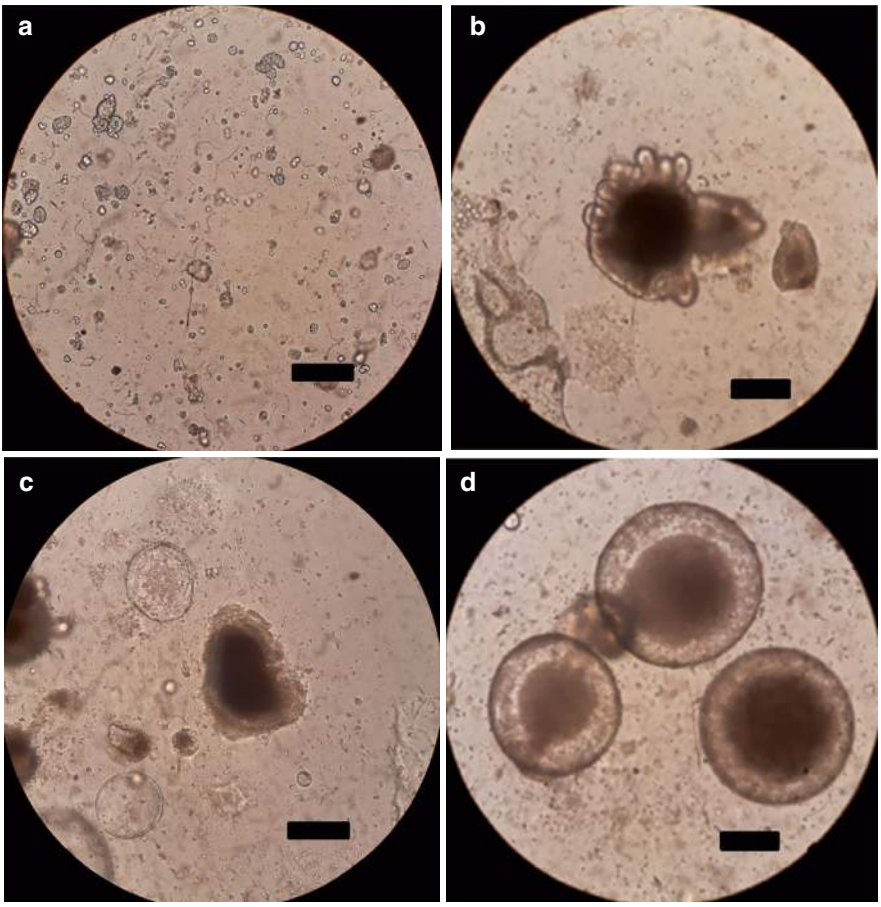

Fig. 12.3 Enteroids at different stages of life. These are examples of enteroids derived from the small bowel tissue of a wild type mouse. (**a**) Photograph of enteroids on day 1 after passaging, smaller in size. (**b**) Enteroid that has already started dying with loss of structure. (**c**) Enteroids that are ready for passaging on day 6 with darker lumens, budding, and larger in size. (**d**) Enteroids on day 3 after passaging, spherical in shape and darkening lumens. Scale bars = 50 μm

Conclusion

The beauty and excitement of science lies in creatively applying techniques to carefully and rationally design experiments to acquire knowledge. Cell culture is one of the most widely used techniques in biology. It is not only readily combined with other methods outlined throughout this book, but also serves as the foundation for entire fields of research. As such, cell culture offers a vast array of cell lines and methods which can be harnessed and combined to address almost any biological question. The equipment required for cell culture is

typically readily available in most laboratories, the skills necessary are readily acquired, and the experiments themselves are often cost-effective with relatively rapid results. Understanding and utilizing this tool is essential to the modern translational laboratory.

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Gene Therapy & Gene-Editing Techniques

13

Christopher J. LaRocca and Clifford J. Steer

Abstract

Gene therapy and genome editing have revolutionized our approach to the development of new therapeutics for previously untreatable medical conditions. Much of this progress has been due to designer nucleases (most notably CRISPR-Cas9) that have enabled targeted genomic modifications. The delivery mechanism(s) of these genome-modifying tools is equally important and can be accomplished in variety of ways, including *ex vivo* and *in vivo* approaches. As more investigational agents are progressing through the pre-clinical pipeline and arriving to clinical trials, it will become critically important to be mindful of how we regulate such products and ensure that there is equitable and affordable access to them.

Keywords

Gene therapy · Genome editing · CRISPR · Clinical trial

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Introduction

Molecular biology has advanced so rapidly over the last few decades that it has resulted in paradigm shifts in science and medicine. This is nowhere more evident than in the field of gene therapy. There are numerous diseases (many of which are rare, inheritable disorders) for which conventional medicine has not been able to find a cure. Therefore, there remains a need for novel approaches to not only ameliorate symptoms for afflicted patients, but also to eradicate the disease. It is for this reason that the field of gene therapy holds such promise and has drastically expanded its scope in recent years.

Historically, there have been a few overarching themes/approaches including, (i) replacing a pathogenic/mutated gene with a wild-type copy; (ii) inactivation of a disease-causing gene; and (iii) delivery of genetic material that will treat a disease or its symptoms [1]. Ideally, these goals can be accomplished successfully while limiting off target effects and adverse events [2]. Some of the earliest approaches for gene therapy were focused on recessive, monogenetic diseases whereby the disease of interest was treated via the addition of genetic material. Key examples of gene addition therapy include beta-thalassemia, hemophilia A & B, and Duchenne muscular dystrophy [3]. Challenges with this type of an approach include regulation of gene expression (as native promoters can be difficult to simultaneously deliver depending on cargo capacity of the delivery method), neighboring genetic sequences, and titrating levels of gene expression [3]. In addition, certain viral vectors are known to remain episomal in function, in contrast to integrating into the host genome.

To expand the potential range of diseases that can be treated, researchers have explored multiple genome editing techniques with much success. Recent years have seen the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approve multiple gene therapy products (Table 13.1). Furthermore, there are numerous ongoing clinical trials and many more investigational agents in preclinical development.

This chapter will provide an overview of gene therapy approaches including genome editing techniques and methods for delivery of genetic material.

Table 13.1 Key FDA-approved gene therapy drugs

Brand name	Generic name	Manufacturer	Year of approval	Disease	Gene target	Delivery method & vector
Beqvez	Fidanacogene elaparvovec-dzkt	Pfizer	2024	Hemophilia B	Factor IX	In Vivo—AAVrh74
Casgevy	Exagamglogene autotemcel (exa-cel)	Vertex Pharmaceuticals	2024	Beta Thalassemia	*BCL11A*	Ex Vivo—CRISPR/Cas-9, Electroporation
Casgevy	Exagamglogene autotemcel (exa-cel)	Vertex Pharmaceuticals	2023	Sickle Cell Disease	*BCL11A*	Ex Vivo—CRISPR/Cas-9, Electroporation
Elevidys	delandistrogene moxeparvovec-rokl	Sarepta Therapeutics	2023	Duchenne Muscular Dystrophy	*DMD*	In Vivo—AAVrh74
Lenmeldy	atidarsagene autotemcel	Orchard Therapeutics	2024	Metachromatic leukodystrophy	*ARSA*	Ex Vivo—CD34+ HSC, Lentivirus
Lyfgenia	lovotibeglogene autotemcel (lovo-cel)	Bluebird Bio	2023	Sickle Cell Disease	$\beta^{A\text{-}T87Q}$-globin	Ex Vivo—CD34+ HSC, Lentivirus
Roctavian	valoctocogene roxaparvovec-rvox	BioMarin Pharmaceutical	2023	Hemophilia A	Factor VIII	In Vivo—AAV5
Hemgenix	etranacogene dezaparvovec-drlb	CSL Behring	2022	Hemophilia B	Factor IX	In Vivo—AAV5
Zynteglo	betibeglogene autotemcel	Bluebird Bio	2022	Beta Thalassemia	$\beta^{A\text{-}T87Q}$-globin	Ex Vivo—CD34+ HSC, Lentivirus
Skysona	elivaldogene autotemcel	Bluebird Bio	2022	Cerebral adrenoleukodystrophy (CALD)	*ABCD1*	Ex Vivo—CD34+ HSC, Lentivirus
Zolgensma	onasemnogene abeparvovec-xioi	Novartis Gene Therapies	2019	Spinal muscular atrophy (SMA)	*SMN1*	In Vivo—AAV9
Luxturna	voretigene neparvovec-rzyl	Spark Therapeutics	2017	Retinal dystrophy	*RPE65*	In Vivo—AAV2

Genome Editing

Identification and targeting of the desired genomic sequence and generation of a double strand break (DSB) within DNA is traditionally the necessary first step for genome editing. These DSBs result in activation of cellular repair mechanisms which ultimately enable site-specific genome modifications [4].

One of the main pathways of DNA repair is non-homologous end joining (NHEJ) [5]. This typically results in small, random insertions/deletions (indels) that can ultimately lead to gene disruption and knock-out mutations [6].

When a donor DNA template is employed, homology-directed repair (HDR) can be utilized to facilitate more precise DNA integration. One caveat to HDR is that it occurs more efficiently during certain G2 and S phases of the cell cycle and it is known that cell cycle synchronization increases successful gene targeting and editing [7, 8].

Targeted Nucleases

Zinc Finger Nucleases (ZFNs) & Transcription Activator-Like Effector Nucleases (TALENs)

While there are many naturally occurring meganucleases, the main challenge which has prevented their widespread adoption in gene editing is their fixed DNA recognition sequences that severely limit options for targeting [9]. As the field advanced, the structure of a class of eukaryotic transcription factors known zinc finger proteins (ZFPs) and their capability of binding to specific regions of DNA was recognized (Fig. 13.1) [10]. The combination of the DNA-binding domain of the ZFPs to the nuclease domain of the *Fok*I restriction resulted in the development of Zinc finger nucleases (ZFNs) [11, 12]. One of the earliest applications for ZFNs was in the design and generation of genetically modified model organisms for research [11]. Similar to ZFNs, transcription activator-like effector nucleases (TALENs) incorporate the nuclease domain of the *Fok*I restriction enzyme; however, it is instead fused with the DNA recognition domain of the TALE protein, which is derived from the *Xanthomonas* bacterium (Fig. 13.1) [13, 14]. When compared to ZFNs, improved ease of generation and targeting ability led to TALENs increasing popularity and use in gene editing [15]. However, one of the main drawbacks of both ZFNs and TALENs is the need for protein engineering to adjust their target specificity, which can be a laborious, time consuming, and expensive process.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Within bacteria and archaea are clustered regularly interspaced short palindromic repeats (CRISPRs) (that are separated by non-repeating spacer sequences) and function in adaptive immune pathways for prevention of viral infection [16]. Furthermore,

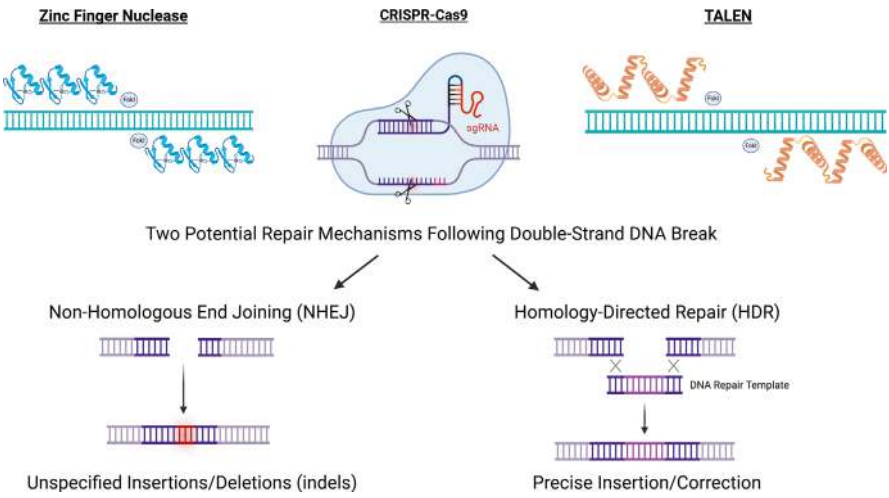

Fig. 13.1 Nucleases and mechanisms of DNA double strand break repair. Synthetic nucleases catalyze double strand breaks (DSB) within DNA and then rely on endogenous repair mechanisms to facilitate genome editing. Non-homologous end joining (NHEJ) results in insertions/deletions of varying length at the DSB site thereby resulting in gene disruption. Homology directed repair (HDR) utilizes a DNA repair template for precise DNA integration. (Figure created with BioRender.com)

these repeated sequences were associated with conserved genes encoding for CRISPR-associated (Cas) proteins, which function as naturally occurring RNA-guided DNA endonucleases (Fig. 13.1) [9]. There are multiple naturally occurring CRISPR-Cas systems and in characterizing them, they have been divided into two classes. Within CRISPR class 2 is the Type II system that utilizes the Cas9 effector protein, which is notable for being the most widely studied and used by the scientific community [17]. In the naturally occurring CRISPR/Cas9 system, the CRISPR loci are transcribed as CRISPR RNA (crRNA) and when combined with trans-activating crRNA (tracrRNA) are capable of sequence-specific DNA cleavage [18].

To facilitate its use as a gene-editing tool, the crRNA and tracrRNA were combined into a synthetically engineered single-guide RNA (sgRNA) [18]. Furthermore, a protospacer adjacent motif (PAM) was also required and consists of a three-nucleotide sequence on the target DNA (e.g., NGG for the originally described *Streptococcus pyogenes* Cas9 protein) to facilitate targeted DNA binding [18]. This becomes critically important as CRISPR-Cas enzymes will not bind to or cleave a DNA sequence unless located adjacent to the appropriate PAM [19].

Researchers have harnessed the power of RNA-mediated DNA sequence recognition and exploited it by swapping the guide RNA to target a wide variety of DNA sequences [20]. This represents such a monumental step forward in the gene editing field that the scientists who first described the use of CRISPR-Cas9 for programmable genome editing (namely Drs. Jennifer Doudna and Emmanuelle Charpentier) received the Nobel Prize in Chemistry in 2020. This technology has been transformative for its application to both clinical medicine and the basic sciences.

In certain situations, it may be desirable to generate a single nucleotide conversion or facilitate a targeted insertion or deletion into the genome while avoiding DSBs and the resulting HDR that could lead to undesired genetic recombination. To facilitate such efforts, further modifications to the Cas nuclease was necessary. In the case of Cas9, mutations in a single nuclease domain (rendering the enzyme only able to cut one of the DNA strands) results in a Cas9 nickase, while inactivating both of the nuclease domains generates a catalytically dead Cas9 (dCas9) [3].

Base editors (BEs) can introduce precise nucleotide exchanges, and they consist of a catalytically-impaired CRISPR-Cas nuclease that is combined with a single-stranded DNA deaminase enzyme [19, 21]. BEs were originally described as cytosine base editors, which consisted of a dCas9 that was fused to a cytosine deaminase (with later versions including a uracil glycosylase inhibitor) and converted a C•G base pair to a T•A base pair [22, 23]. Another approach named prime editing has the ability to facilitate a wider range of single-nucleotide conversions in addition to precise insertion or deletion of a DNA sequence. Prime editors are the result of a fusion between a Cas9 nickase domain that is fused to a reverse transcriptase and employ an engineered prime editing guide RNA (pegRNA) to facilitate targeted encoding [6].

The applications of CRISPR-Cas technologies have already generated an immediate impact in the fields of science, medicine, and agriculture. High throughput genetic screens have become more feasible due to the ease of generating CRISPR-induced gene knockouts. Similarly, multiplex editing has provided tools to simultaneously target multiple loci within a given genome. CRISPR has been utilized to generate genetically modified animals with much success across numerous model systems.

Delivery of Genome Editing Agents

With all the advances in genome editing technology, one key hurdle is ensuring that it can be delivered to the cells of interest. Importantly, the delivery mechanisms must be able to target and bind to cells of interest, effectively enter into the cell and nucleus, and deliver the payload.

Many of the ongoing gene editing trials involve *ex vivo* techniques whereby cells are removed from a patient, edited outside the patient, and later reintroduced. While these techniques are effective, they are limited to only certain cell types (e.g., hematopoietic stem cells) that can tolerate *ex vivo* manipulation and subsequent transplantation. Alternatively, *in vivo* gene editing techniques involve having the editing performed within a patient's body. In the context of the gene editing and gene therapy fields, this approach offers the most promise given the broad potential applicability; however, it is not without challenges. Successful *in vivo* gene editing will require the ability to target and enter cells of interest, deliver the relevant payload, and minimize off-target effects.

Lentivirus

Lentivirus is a genus of retrovirus, and the most commonly used lentiviral vectors are based upon the human immunodeficiency virus (HIV). They are enveloped viruses that have a single strand RNA genome and utilize a reverse transcriptase to generate DNA (Fig. 13.2). As with many types of viral vectors, the development of subsequent generations of lentiviral vectors has resulted in improvements in performance and safety. Importantly, lentiviral vectors can be rendered replication deficient by genome deletions resulting in a self-inactivating 3′ LTR [24]. Historically, retroviral vectors had the potential for genome integration and with that the concern for insertional mutagenesis and oncogenic activation via potential hot spots [25]. Naturally, researchers were interested in techniques to develop non-integrating lentiviral vectors. Using a modified package plasmid that affects the integrase coding sequence, lentiviral vectors can be rendered non-integrating without sacrificing gene transfer and expression [26]. Furthermore, one of the other attractive features of lentiviral vectors is the potential ability to stably transduce non-dividing cells, which can be especially attractive when cells such as hepatocytes need to be targeted [27]. While typically employed in *ex vivo* gene therapy approaches, there is some ongoing investigation for their use as *in vivo* delivery vehicles [6].

Adenovirus

Adenoviruses are non-enveloped vectors surrounded by an icosahedral capsid and contain a double-stranded DNA genome (Fig. 13.2). The genome is approximately 36 kb and lends itself well to modification and the insertion of transgenes [28]. There are 57 known serotypes of adenovirus and serotype 5 from group C is by far

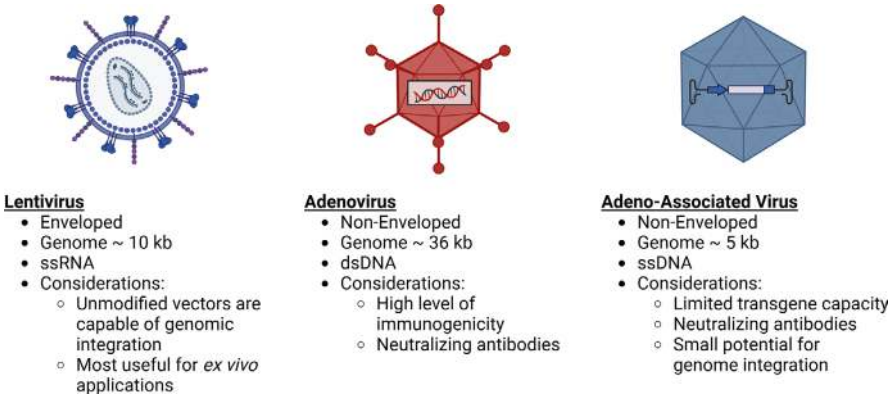

Fig. 13.2 Viral delivery vectors. Lentivirus, adenovirus, and adeno-associated virus are the three most commonly used vectors for delivery of gene modification agents. (Figure created with BioRender.com)

the most frequently utilized backbone structure in adenoviral vectors [29]. In fact, adenoviral vectors are the most popular and commonly utilized in virotherapy and gene therapy trials due to the large transgene capacity, transduction efficiency, and predictable biologic behavior [30].

Modifications to the adenovirus genome have been utilized to confer replication selectivity and to increase the transgene cargo capacity. For example, early generation adenoviral vectors had deletions in the *E1a/E1b* genes that resulted in replication-deficient vectors [30]. Furthermore, researchers identified that deletions in other areas of the adenovirus genome (e.g. *E2, E3, E4*) could increase the transgene carrying capacity [31].

Adenovirus vectors were some of the earliest-adopted delivery vehicles for gene therapy. This is evidenced by the use of an E1/E3-deleted adenovirus to deliver the normal human *CFTR* cDNA to the nasal epithelium in a patient with cystic fibrosis in 1993 [31, 32]. As enthusiasm for adenoviral vectors increased, there were increasing numbers of adenoviral vectors utilized in gene therapy studies. Unfortunately, in 1999, there was a tragic outcome in an 18 year old patient undergoing adenovirus-based gene therapy for ornithine transcarbamylase (OTC) deficiency whereby he had a severe immune response to the vector that resulted in cytokine storm, total organ failure, and ultimately death [33]. For the next decade or so, interest in adenovirus-mediated gene therapy waned as researchers were concerned about the immunogenicity of the virus [34]. While there has been some interest in using adenovirus vectors to delivery CRISPR-Cas9 gene editing tools [35], they have now found greater use in oncolytic therapies where harnessing the immune system effects can be advantageous when designing therapies for cancer.

Adeno Associated Virus

In recent years, adeno-associated virus (AAV) has become the predominant platform for *in vivo* viral vector gene therapy (Fig. 13.2). AAV is also a non-enveloped vector surrounded by an icosahedral capsid, but it differs from adenovirus in that it contains a single-stranded DNA genome which is only ~5 kb in size [36]. Its viral life cycle is dependent upon a helper virus (typically adenovirus) to facilitate replication, and it is not known to be pathogenic in humans. The genome of the wild type AAV2 vector was the first to be cloned. Research into other serotypes followed as investigators realized that each serotype's differing capsid proteins altered function and tropism that could be tailored to different applications [37]. Following viral infection, AAV DNA remains episomal and there is the potential for latent infection due to the ability of the wild type AAV genome to integrate into human cells at the *AAVS1* locus [38].

Wild-type AAVs are not typically utilized in gene therapy, but rather recombinant AAVs (rAAVs) have proven to be effective and relatively safe. These vectors employ the same capsid proteins but have had the viral protein coding sequences deleted from the genome such that transgene expression cassettes can be inserted [36]. Importantly, with just under 5 kb of genome space, the payload must be carefully designed such that all critical sequences can be incorporated. Furthermore, a large portion of the human

population has been found to have neutralizing antibodies against AAV [39]. Given this fact, researchers have employed naturally occurring capsid proteins with less seroprevalence or engineered capsid proteins to evade the effects of these antibodies [40].

In the United States, the FDA approved the first AAV-based gene therapy in 2017 which utilized an AAV2 vector to treat patients with inherited retinal dystrophy. Voretigene neparvovec (brand name Luxturna) is a rAAV vector that is delivered via subretinal injection and expresses the human *RPE65* gene. A randomized phase 3 trial demonstrated markedly improved functional vision in these patients with *RPE65*-mediated, inherited retinal dystrophy following gene therapy [41]. Additionally, there have been multiple other FDA approvals of AAV-based gene therapies including onasemnogene abeparvovec (Zolgensma) for spinal muscular atrophy in 2019 [42, 43], etranacogene dezaparvovec (Hemgenix) for hemophilia B in 2022 [44, 45], valoctocogene roxaparvovec (Roctavian) for hemophilia A in 2022 [46], delandistrogene moxeparvovec (Elevidys) for Duchenne muscular dystrophy in 2023 [47], and fidanacogene elaparvovec (Beqvez) for hemophilia B in 2024 [48].

Despite the initial successes of these agents, there are still challenges in the field that limit potential applications of AAV-based gene editing and therapies. One of the main challenges inherent to any AAV vector is the limited cargo capacity resulting from the small genome size. Potential solutions to this problem that are being explored include dual-AAV techniques whereby the gene-editing payload is split across two AAV vectors that are simultaneously delivered and the identification/generation of smaller Cas9 variants [6, 40]. Additionally, we have previously discussed the seroprevalence of neutralizing antibodies to AAV, which can limit the effectiveness of intravenous dosing as well as the potential for repeat dosing due to the robust humoral immune response [37]. Furthermore, there are many naturally occurring serotypes of human AAV which oftentimes have a particular tropism such as that of liver, muscle, or central nervous system tissues. It will be critical to identify capsid proteins (whether naturally occurring or chimeric) that can be optimized for systemic delivery of these AAV-based platforms and efficient transduction of the intended target site [36]. There is a theoretical risk of genotoxicity caused by insertional mutagenesis of wild type AAV, but there has been a strong track record of safety in ongoing clinical trials and a lack of data to suggest that rAAV can cause genotoxicity in humans [36]. Finally, the complexity of large-scale manufacturing techniques and high-cost of these agents may be a rate-limiting step to the continue evolution of the field, especially if interest extends beyond rare, monogenic diseases to chronic multigenic conditions that affect wider sections of the population [20].

Non-viral Vectors

Lipid Nanoparticle

In recent years, lipid nanoparticles (LNPs) have become increasingly utilized for the delivery of gene editing agents *in vivo* to the point where they are the most common non-viral based method. This is especially true for therapeutics targeting the

liver as LNPs can become coated with ApoE lipoproteins in the serum leading to hepatocyte uptake via LDL:ApoE interactions [49]. LNPs typically contain a few basic components including cholesterol, a cationic or ionizable lipid, a polyethylene glycol lipid, and a helper lipid [50]. They achieve the delivery of their payload by entering the cell via receptor-mediated endocytosis, disrupting endosomal membranes for release into the cytosol, and ultimately targeting the nucleus for uptake [6]. Given the relative ease with which these lipid components can be generated, it enables the creation of large libraries of lipid nanoparticle delivery systems that can be tailored to the scenario of interest. As the field of LNPs has continued to evolve, there have been multiple FDA approved uses for these agents including the delivery of siRNA to hepatocytes through intravenous administration and mRNA vaccines through intramuscular injections [49, 51]. Importantly, there is ongoing work in a Phase 3 clinical trial (discussed later in this chapter) to investigate the *in vivo* delivery of CRISPR-Cas9 mRNA to the liver [52].

There are potential advantages to LNPs as a delivery vehicle (when compared to viral vectors). These include (i) transient expression of the genetic material which can lessen the chance of off target effects; (ii) low levels of immunogenicity; (iii) lipid components that are relatively non-toxic and have shown good safety profile and limited adverse effects; and (iv) feasible production at a large scale [6]. Future directions will need to explore how these agents can be optimized to enable efficient delivery to non-hepatic sites.

Clinical Translation of CRISPR-Based Therapeutics

The advent of CRISPR-Cas9 has revolutionized gene editing and has served as a turning point for the field of gene therapy. It has the potential to drastically impact disease that up until now has had limited curative treatment options or cumbersome maintenance therapies. The last few years have witnessed the impact that CRISPR-Cas9 gene editing can have on two, heretofore, untreatable diseases, namely β-thalassemia and sickle cell disease (SCD).

Sickle cell disease is caused by a mutation in the hemoglobin β subunit (*HBB)* gene (which normally encodes β-globin) that results in production of hemoglobin S [53]. For these patients, the resulting clinical sequelae include chronic anemia, vaso-occlusive crises, and the potential for severe end organ damage. Similarly, β-thalassemia is also an autosomal recessive disease resulting from pathogenic mutations in the *HBB* gene leading to markedly lower production of β-chains in adult hemoglobin [54]. The result is a clinical spectrum of reduced erythropoiesis, chronic anemia, and changes in iron metabolism.

It has been observed that increased levels of fetal hemoglobin production after birth lessened the symptoms of both β-thalassemia and SCD [55, 56]. Typically, fetal hemoglobin is decreased secondary to the suppression of γ-globin in favor of increased expression of β-globin after birth. This process is regulated by the transcription factor BCL11a which is necessary for the aforementioned globin switching [56, 57].

With its discovery, the BCL11a transcription factor became a potentially attractive therapeutic target to improve outcomes of patients with β-thalassemia and SCD. Therefore, a team of researchers used a CRISPR-Cas9 system at the erythroid-specific enhancer region of *BCL11A* within hematopoietic stem and progenitor cells (HSPCs) to effectively reactivate production of fetal hemoglobin [58, 59].

CLIMB THAL-111 (NCT03655678) and CLIMB SCD-121 (NCT03745287) are the resulting phase III clinical trials testing the therapeutic agent known as exagamglogene autotemcel (exa-cel) [60, 61]. Autologous CD34+ HSPCs were obtained from patients and then a CRISPR-Cas9 system was utilized *ex vivo* to manufacture the product, which was later infused into patients following myeloablative therapy. Treatment with exa-cel eliminated vaso-occlusive crises in 97% of SCD patients for at least 12 months and resulted in transfusion independence in 91% of patients with transfusion-dependent β-thalassemia [60, 61]. The successes of these clinical trials set the stage for FDA approval of exa-cel, which was a historic moment for CRISPR-Cas9 gene editing approaches.

Another recent development for CRISPR-Cas9 systems is the start of a Phase III clinical trial for the drug NTLA-2001, which is the first *in vivo* CRISPR-based therapeutic to enter late-stage trials. Known as the MAGNITUDE study, it is a randomized, double-blinded, placebo-controlled study investigating the role of NTLA-2001 in patients with transthyretin amyloidosis with cardiomyopathy (ATTR-CM, NCT06128629). For patients with transthyretin amyloidosis, misfolded transthyretin (TTR) proteins form amyloid fibrils which accumulate in tissues such as in the cardiac system (leading to cardiomyopathy) and nervous system (resulting in neuropathy), which can lead to severe morbidity and even mortality [62]. NTLA-2001 is administered via an intravenous route and utilizes a lipid nanoparticle delivery platform with liver tropism to carry the CRISPR-Cas9 gene editing machinery to target the human *TTR* gene [52, 63]. In a phase I clinical trial (NCT04601051), NTLA-2001 demonstrated only mild adverse effects and marked decreases in serum TTR protein concentrations [52]. Targeted *TTR* knockout via an in vivo CRISPR therapeutic appears promising therapy for patients with advanced transthyretin amyloidosis.

Additional promising developments for the field of *in vivo* CRISPR-based therapeutics comes in the form of a recently completed Phase I/II trial for patients with retinal degeneration (Leber's congenital amaurosis) caused by mutations in the *CEP290* gene [64]. The Brilliance trial (NCT03872479.) was a single-ascending-dose study in which 14 participants received subretinal injections of the investigational agent (known as EDIT-101), which was based on CRISPR-Cas9, delivered via a rAAV5 vector, and designed to delete the pathogenic *CEP290* variant. Results of the trial demonstrated a good safety profile and over half of the patients had meaningful improvements in visual acuity [65]. Given the encouraging results, there is hope that future studies will bring a cure to this disease.

Regulatory Issues, Ethical Questions, and Accessibility

As the pace of genome editing and gene therapy research has increased in recent years, it has been necessary to have a regulatory framework develop in parallel to help govern the use of such agents [66]. One of the foundational meetings took place in Napa, California in 2015 when researchers and other key stakeholders met to discuss how best to move the field forward with respect to all of the potential scientific, legal, medical, and ethical concerns that came to light with the rapid advance of CRISPR and other gene editing technologies [67]. Since that time, there have been other meetings including the recent Third International Summit on Human Genome Editing in 2023 which was sponsored by the US National Academy of Sciences and Medicine, the UK Royal Society, and others. The organizing committee highlighted the need for international collaboration to guide ongoing scientific advances and innovation and emphasized an important point in that there is no role for heritable genome editing and that it is not ethically permissible at this time [68]. The US FDA has also provided guidance for developers of novel gene therapy products with special attention to what should be included as part of an Investigational New Drug (IND) application [69].

As with any developmental therapeutic, financial concerns are always a key consideration [70]. Manufacturing costs for gene editing agents and the delivery vehicle are costly and oftentimes are difficult to produce at scale; therefore, investments in infrastructure will be key to cost containment as the field moves forward [20]. With high costs incurred by pharmaceutical companies during production, there is the concern that the retail prices required to offset manufacturing costs are likely to be too high for many patients to afford. Some recently approved gene therapy agents can cost over $1 million dollars per dose. For example, Hemgenix is an AAV based gene therapy for adults with Hemophilia B and following FDA approval became the most expensive pharmaceutical on the market with a retail cost of nearly $3.5 million per dose [71]. While there may be some evidence to suggest that the high upfront price of these therapeutics may offset the cost of repeated treatments/infusions over a number of years, the initial cost of entry is likely too high for many patients and insurers. If cost is a barrier for affluent countries such as the United States and others, it is unclear what implications this will have for less affluent countries in terms of global access to these potentially life-changing therapeutics. To that end, the Global Gene Therapy Initiative (GGTI) is working on strategies to ensure that low- and middle-income countries are not excluded from roles in gene therapy development and delivery [72].

Conclusion and Future Directions

The development and optimization of genome modifying tools such as CRISPR-Cas9 have revolutionized the field of gene therapy. Recent years have seen unprecedented numbers of preclinical studies and clinical trials in gene therapy with increasing numbers of FDA approved agents. The hope for patients who suffer from

diseases (both mono- and polygenic) with limited therapeutic options will be gene therapy approaches that offer promise for both treatment and possible cures.

Despite all the future potential, there are going to be obstacles that will need to be addressed. Continued optimization of gene editing systems will be required to ensure targeted delivery to the cells of interest, minimization of off-target effects, and effective ways to deal with immunogenicity and neutralizing antibodies.

Important considerations will also be needed to ensure that these technologies will be appropriately and ethically regulated and available to those patients, far and wide, who might benefit from them at affordable prices.

Conflicts of Interest None declared.

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Single-Cell RNA Sequencing Technologies and Applications: A Brief Overview

14

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Abstract

Single cell RNA sequencing is a rapidly evolving technology that has enabled the determination of the gene expression patterns of tens of thousands of individual cells. The application of this technology has provided stunning new insights into the underlying mechanisms of organ development and disease. In this chapter, we discuss the basics of single cell RNA sequencing including experimental workflow, differences between single cell RNA sequencing and single nucleus RNA sequencing, and the potential for multi-omic analysis.

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Furthermore, we provide a high-level overview of the approach to data analysis, including quality control, dimensionality reduction, clustering, annotation, and a plethora of potential downstream applications.

Keywords

RNA sequencing · Transcriptomics · Single cell RNA sequencing · Single cell transcriptomics · Multiomics · Spatial transcriptomics

Introduction

During the last decade, high-throughput sequencing methods have revolutionized the field of biology. Essentially, all cells of the human body contain the same set of ~20,000 genes, but each cell expresses a different set of these genes, leading to between-cell differences in the expression of membrane components, ion transporters, cytoskeletal elements, growth factors, receptors and transcription factors. The gene expression profile therefore describes in exquisite detail the phenotype of a cell, which underlies its molecular functions.

Historically, gene expression studies have been limited to the analysis of pooled populations of cells, which was necessary to obtain sufficient RNA for analysis. For example, the combined expression pattern of all cells in a tumor would be examined in aggregate to identify perturbed molecular pathways. However, each tumor contains a heterogeneous population of cells, including vascular cells, fibroblasts, invading immune cells and rapidly dividing cancer cells as well as more quiescent cancer stem cells, and the resulting gene expression profile of a pooled population of tumor cells therefore provides only an ensemble average of all the cell types present. Analysis of pooled cell populations does not enable identification of the cell types that express certain genes but instead provides a virtual average of the multiple cellular components, which may well say very little about any specific cell type present. Similar problems have been encountered when pooled populations were used to assess gene expression associated with other disease conditions.

The past decade has witnessed powerful technological advances, enabling gene expression analyses to be carried out at much higher resolution than previously possible. Indeed, the expression level of every gene, even in a single cell, can now be defined. This technology, known as single-cell RNA sequencing (scRNA-seq), enables rapid determination of the precise gene expression patterns of tens of thousands of individual cells. Such analysis of the constituent parts—the single cells—provides much more meaningful insights into cell behavior than the analysis of aggregated blocks. For example, scRNA-seq of a tumor allows for separation of tumor fibroblasts, endothelial cells and cancer cells on the basis of their gene expression signatures. Moreover, each cell type can be further divided into subtypes; for example, tumor fibroblasts might be separated into fibroblast subtypes [1]. Such single-cell studies have also enabled identification of previously unknown cell types

[2–4] and have provided insights into the heterogeneity of non-cancer cell populations within tumors [5], highlighting the power of this research tool.

This technological revolution is providing stunning new insights into the underlying mechanisms of organ development and disease. Application of these new tools to the analysis of normal, developing and diseased tissue will enable a much deeper understanding of the human body by providing insights into developmental biology and how perturbed molecular pathways and processes can lead to disease. In this chapter, we discuss the fundamental concepts of single-cell RNA sequencing analysis, the most common scRNA-seq protocols in use today, the basics of data analysis and briefly discuss future directions in transcriptome analysis.

Single-Cell RNA Sequencing Techniques

Experimental Workflow

A variety of single-cell methods have been developed in the past 10 years with divergent approaches for cell capture and amplification as well as differences in mRNA transcript length, the number of target cells captured and read depth per cell [6]. Each method has unique advantages and disadvantages but, generally, all scRNA-seq techniques developed to date share a common workflow (listed in order): sample preparation, single-cell capture, reverse transcription and amplification, library preparation, sequencing, and analysis [7] (Fig. 14.1).

Adequate sample preparation is a prerequisite for generating robust single-cell transcriptome data. Given that every biological sample has its own set of characteristics, the protocol should be optimized depending on a broad range of factors, including tissue or cell type, culture conditions (for example, suspension versus adherent), extracellular matrix content and cell viability. A critical step during sample preparation, particularly for dense tissue and 3D organoid models, is single-cell dissociation, which is typically achieved enzymatically under gentle mechanical agitation (and in some cases with tissue perfusion) to limit excessive cell lysis and background noise. The choice of proteolytic enzyme (such as trypsin, collagenase or Liberase) and the duration of digestion should be carefully optimized to maximize yield while minimizing cell death. When cells are fully dissociated, they are separated into single cells using a variety of cell-capture techniques ranging from plate-based separation to microdroplet-based approaches. The maintenance of a high number of isolated viable cells, typically on the order of $\geq$10,000 cells, is key to improving data quality, and the method of cell capture is often determined by the properties of the sample of interest (such as cell size).

Of note, the dissociation process can induce the expression of stress genes, which lead to artificial changes in cell transcription patterns. This has been confirmed by a number of experiments. For example, Brink et al. found that the process of protease digestion at 37 °C induced the expression of stress genes, introducing technical error and causing inaccurate cell type identification [8].

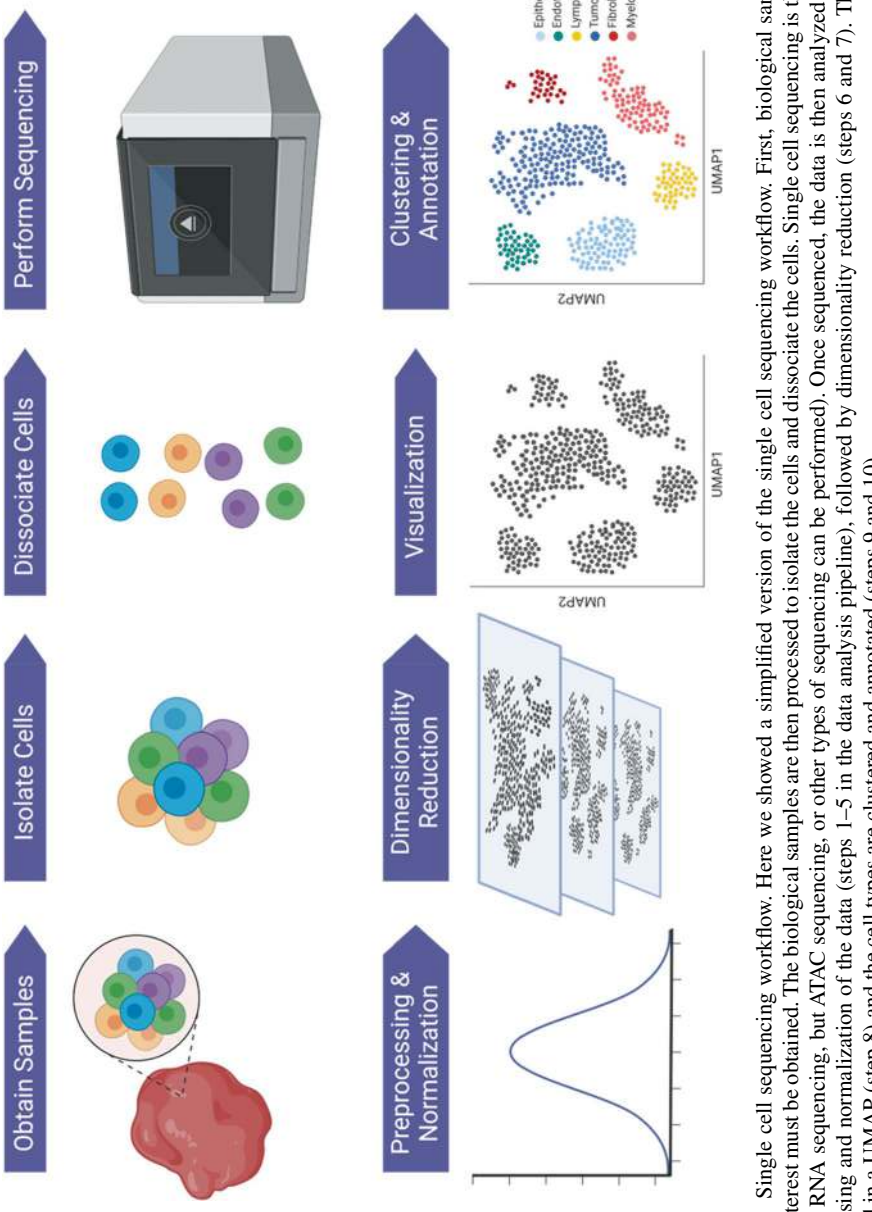

Fig. 14.1 Single cell sequencing workflow. Here we showed a simplified version of the single cell sequencing workflow. First, biological samples with the cells of interest must be obtained. The biological samples are then processed to isolate the cells and dissociate the cells. Single cell sequencing is then performed (typically RNA sequencing, but ATAC sequencing, or other types of sequencing can be performed). Once sequenced, the data is then analyzed. We start with preprocessing and normalization of the data (steps 1–5 in the data analysis pipeline), followed by dimensionality reduction (steps 6 and 7). The data is then visualized in a UMAP (step 8) and the cell types are clustered and annotated (steps 9 and 10)

For samples that are difficult to process, single-nucleus RNA sequencing (snRNA-seq) is a useful alternative method. Rather than sequencing the mRNA in the cytoplasm of cells, snRNA-seq only captures the mRNA in the nuclei of cells. The snRNA-seq method solves problems related to tissue preservation, allows for cell isolation from tissues that are not easily separated into single-cell suspensions, can be applied to frozen samples, and minimizes artificial transcriptional stress responses as compared to scRNA-seq [9]. SnRNA-seq is useful in many tissue types, such as muscle [10], heart [11], lung [12], pancreas [13] and various tumor tissues [14]. It is particularly applicable in brain tissue, which is difficult to dissociate into intact cells. However, it should be noted that snRNA-seq only captures transcripts located in cell nuclei, which may fail to capture important biological processes related to mRNA processing, RNA stability and metabolism. Despite these potential technical limitations, both technologies have allowed for a more nuanced understanding of cellular and biological processes and provided novel biomedical and cellular insights into disease pathogenesis.

Cell Capture Techniques

Plate-Based or Microwell-Based Methods

Current scRNA-seq techniques can be categorized according to the method of single-cell isolation and capture, which determines the throughput, depth and scale of the analysis. Plate-based or microwell-based protocols use either automated micropipettes or fluorescence-activated cell sorting (FACS) to segregate individual cells into 96-well or 384-well plates containing a lysis buffer and other processing reagents. A major advantage of this method is the possibility for long-term storage of the cell sample before analysis, which provides flexibility in experimental planning and coordination. Furthermore, this approach allows for the profiling of all cells independent of type or size and provides full-length transcript sequencing which facilitates the identification of isoform splicing in single cells. Plate-based platforms generally have high sensitivity and can reliably quantify up to 10,000 genes per cell. However, one downside of this approach is that reverse transcription must be performed in individual wells, which can slow down the workflow, limit throughput and increase noise in downstream analyses.

Microfluidic-Based Methods

Automated microfluidic-based platforms, such as the Fluidigm C1 system using Smart-seq [15], were among the earliest scRNA-seq techniques to be introduced and widely commercialized. The C1 system separates and isolates single cells using narrow parallel microfluidic channels, where cell capture, lysis, reverse transcription and multiplexing take place within an integrated fluidic circuit chip. A key feature of this approach is the option of viewing captured cells under the microscope before reverse transcription and amplification. In addition, the small volumes (<150 nanoliters per well) of cell suspension required for the technique help to reduce the risk of external contamination [16]. These methods are high in sensitivity

and use full-length transcript sequencing, allowing the characterization of isoform slicing, single-nucleotide variants and transcription start sites, and the detection of monoallelic and imprinted genes [17]. However, in many cases, microfluidic-based platforms require the input of >10,000 cells and are limited in scale and throughput (~100 to ~1000 cells per analysis) owing to the restricted number of single-cell capture sites per microfluidic array. Furthermore, this approach also requires homogeneity in cell size and is costlier than other techniques, limiting its use for large-scale experiments.

Droplet-Based Methods

The most popular current scRNA-seq methods employ microdroplets in place of microchambers [18, 19] (Fig. 14.2). Use of microfluidics technology enables hundreds of thousands of microdrops to be inexpensively generated. These aqueous microdrops, surrounded by oil, have a volume of ~2 nanoliters and contain a bead with a uniquely barcoded set of oligonucleotides and a single cell. Droplet-based methods involve the analysis of single cells encapsulated in oil droplets using DNA barcoding technology, substantially reducing the time and cost needed per analysis. Massive parallelization increases the number of cells profiled for a given run to up to ~10,000 cells per sample [19, 20]. Of note, the commercial Chromium system (10× Genomics) enables 3′-end or 5′-end sequencing of single cells with increased scale and throughput compared with plate-based or microfluidic based methods, albeit with a trade-off in sensitivity and read depth. Droplet-based methods quantify transcripts by 3′-end or 5′-end sequencing and have reduced transcript recovery rates (3–10%) compared with other existing methods (10–20%) [21], with the target read depth per cell ranging from 10,000 to 100,000 reads. Nevertheless, the

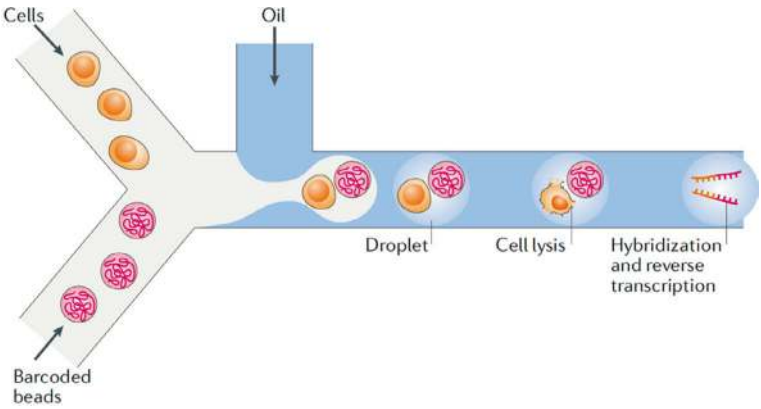

Fig. 14.2 Microdroplet-based scRNA- seq. A microfluidics system is used to make microdroplets, which contain cells mixed with beads that are encapsulated in oil. Barcoded gel beads are passed into a microfluidic device along with dissociated cells where they are captured in aqueous droplets in an oil solution. Cells are lysed within the droplets and mRNA is reverse transcribed to produced barcoded cDNA thereby allowing the sequences of those cDNAs to be assigned to a specific cell. (Modified from Potter et al. [7])

sensitivity of these protocols remains sufficient for the largescale profiling of complex heterogeneous samples and is expected to improve with continued protocol optimization and reductions in cost.

PCR Amplification and Sequencing

Once single cells are successfully captured, they are lysed and processed to create first-strand cDNA by reverse transcription and subsequently undergo second-strand synthesis and PCR amplification. The resulting cDNA are amplified by either polymerase chain reaction (PCR) or in vitro transcription (IVT) [22]. PCR as a nonlinear amplification process is applied in Smart-seq [23], Smart-seq2 [24], Fluidigm C1 [25], Drop-seq [19], 10x Genomics [26], MATQ-seq [27], Seq-Well [28], and DNBelab C4.

Currently, there exist two PCR amplification strategies. One uses SMART technology, which takes advantage of transferase and strand-switch activity of Moloney Murine Leukemia Virus reverse transcriptase to incorporate template-switching oligonucleotides as adaptors for downstream PCR amplification [29]. This method is the most used cDNA amplification method. The other strategy either connects the 5′ end of cDNA with poly(A) or poly(C) to build common adaptors in PCR reaction [30]. IVT is another amplifying approach and a linear amplification process, which is used in CEL-seq [31], MARS-Seq [20], and inDrop-seq protocols. It requires an additional round of reverse transcription of the amplified RNA, which results in additional 3′ coverage biases [22]. Both approaches can lead to amplification biases.

To overcome amplification-associated biases, unique molecular identifiers (UMIs) were introduced to barcode each individual mRNA molecule within a cell in the reverse transcription step, thus improving the quantitative nature of scRNA-seq and enhancing the reading accuracy by effectively eliminating PCR amplification bias. UMIs are adapted by the CEL-seq [31], MARS-seq [20], Drop-seq [19], inDrop-seq [32], 10x Genomics [26], MATQ-seq [27], Seq-Well [33], and DNBelab C4 protocols. Once the single cell-barcoded cDNAs are generated from single cells or single nuclei, the cDNA can be sequenced using a number of deep sequencing platforms. Different scRNA-seq technologies and experimental protocols are summarized in Table 14.1. Data generated from sequencing are then analyzed beyond simple quantification of gene expression to include in-depth examination of cell heterogeneity, lineage transitions and intercellular communication which will be discussed later in this chapter.

Table 14.1 Comparison of different single-cell RNA sequencing (scRNA-seq) technology and experimental protocols

Platforms	Isolation strategies	Tissue	Cell numbers	Targets	UMI	Amplification methods	Regions
Smart-seq	FACS	Dissociated cell	Hundreds of cells	/	No	PCR	Full-length
Smart-seq2	FACS	Dissociated cell	Hundreds of cells	/	No	PCR	Full-length
Fluidigm C1	Micro-fluidic	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	No	PCR	Full-length
Drop-seq	Microdroplets	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	Yes	PCR	3′ end
10× Genomics	Microdroplets	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	Yes	PCR	3′ end
MATQ-seq	FACS	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	Yes	PCR	Full-length
Seq-Well	Micro-fluidic	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	Yes	PCR	3′ end
CEL-seq	FACS	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	Yes	PCR	3′ end

MARS-seq	FACS	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	Yes	IVT	3′ end
inDrop-seq	Microdroplets	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	Yes	IVT	3′ end
DNBelab C4	Microdroplets	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	Yes	PCR	3′ end

FACS fluorescence-activated cell sorting; *IVT* in vitro transcription; *PCR* polymerase chain reaction; *UMI* unique molecular identifier; *MATQ-seq* multiple annealing and dC-tailing-based quantitative single-cell RNA-seq; *MARS-seq* massively parallel single-cell RNA-sequencing; *DNB-seq* DNA Nanoball Sequencing

Modified from Jovic et al. [70]

Multiomic Single-Cell Analysis

Single-cell molecular profiling technologies initially focused on the development of methods capable of accurately detecting a single aspect of the cell state. For example, scRNA-seq focuses on RNA expression within a cell, single nuclear sequencing (SNS) focuses on whole genome sequencing, and single-cell assay for transposase-accessible chromatin (scATAC-seq) focuses on chromatin accessibility. Although these technologies have yielded transformative insights into cellular diversity and development, this segregation is driven by methodological convenience and limits the ability to derive a deep understanding of the relationships between biomolecules in single cells. More recently, there has been considerable interest in the simultaneous profiling of multiple types of biomolecules within a single cell (paired multiomics) to build a much more comprehensive molecular view. Most commonly, paired multiomics is performed using single cell RNA sequencing and scATAC-seq, but paired multiomics can include genomic sequencing, DNA methylation, proteomics, metabolomics, and many other combinations of sequencing modalities (Table 14.2). Although a detailed discussion of these methods is outside the scope of this chapter, summaries of these technologies and their optimal methodology are available in a review by Stuart et al. [34]

Table 14.2 Current experimental methods for unimodal and multiomic single- cell measurements

Data types	Method name	Feature throughput	Cell throughput
Unimodal			
mRNA	Drop-Seq	Whole transcriptome	1000–10,000
	InDrop	Whole transcriptome	1000–10,000
	10XGenomics	Whole transcriptome	1000–10,000
	Smart-seq2	Whole transcriptome	100–300
	MARS-seq	Whole transcriptome	100–300
	Cel-seq	Whole transcriptome	100–300
	SPLiT-seq	Whole transcriptome	>50,000
	Sci-RNA-seq	Whole transcriptome	>50,000
Genome sequence	SNS	Whole genome	10–100
	SCI-Seq	Whole genome	10,000–20,000
Chromatin accessibility	scATAC-seq	Whole genome	1000–2000
	sciATAC-seq	Whole genome	10,000–20,000
	scTHS-seq	Whole genome	10,000–20,000
DNA methylation	scBS-seq	Whole genome	5–20
	snmC-seq	Whole genome	1000–5000
	sci-MET	Whole genome	1000–5000
	scRRBS	Reduced representation genome	1–10
Histone modifications	ssChIP-seq	Whole genome + single modification	1000–10,000
Chromosome conformation	scHi-C-seq	Whole genome	1–10

(continued)

Table 14.2 (continued)

Data types	Method name	Feature throughput	Cell throughput
Multiomics			
mRNA+spatial	osmFISH	10–50 RNAs	1000–5000
	STARmap	20–1000 RNAs	100–30,000
	MERFISH	100–1000 RNAs	100–40,000
	seqFISH	125–250 RNAs	100–20,0000
mRNA+cell surface protein	CITE-seq	Whole transcriptome+proteins	1000–10,000
	REAP-seq	Whole transcriptome+proteins	1000–10,000
mRNA+chromatin accessibility	Sci-CAR	Whole transcriptome+whole genome	1000–20,000
mRNA+DNA methylation	scM&T-seq	Whole genome	50–100
mRNA+genomic DNA	G&T-seq	Whole genome	50–100
DNA methylation+chromatin accessiblity	scNOMe-seq	Whole genome	10–20

CEL-seq cell expression by linear amplification and sequencing; *CITE-seq* cellular indexing of transcriptomes and epitopes by sequencing; *G&T-seq* genome and transcriptome sequencing; *LINNAEUS* lineage tracing by nuclease- activated editing of ubiquitous sequences; *MARS-seq* massively parallel RNA single-cells equencing; *MEMOIR* memory by engineered mutagenesis with optical in situ readout; *MERFISH* multiplexed error-robust fluorescence in situ hybridization; *osmFISH* cyclic single-molecule fluorescence in situ hybridization; *REAP-seq* RNA expression and protein sequencing assay; *scATAC-seq* single-cell assay for transposase- accessible chromatin using sequencing; *scBS-seq* single-cell bisulfite sequencing; *scChIP-seq* single- cell chromatin immunoprecipitation followed by sequencing; *scHi-C-seq* a single-cell Hi-C method for chromosome conformation; *sciATAC-seq* single-cell combinatorial indexing assay for transposase-accessible chromatin using sequencing; *sci-CAR* single-cell combinatorial indexing chromatin accessibility and mRNA sequencing; *sci-MET* single-cell combinatorial indexing for methylation analysis; *sci-RNA-seq* single-cell combinatorial indexing RNA sequencing; *SCI-seq* single-cell combinatorial indexed sequencing; *scM&T-seq* single-cell methylome and transcriptome sequencing; *scRRBS* single-cell reduced representation bisulfite sequencing; *scTHS-seq* single-cell transposome hypersensitivity site sequencing; *seqFISH* sequential fluorescence in situ hybridization; *snmC-seq* single-nucleus methylcytosine sequencing; *SNS* single-nucleus sequencing; *SPLiT-seq* split-pool ligation-based transcriptome sequencing; *STARmap* spatially resolved transcript amplicon readout mapping
Modified from Staurt et al. [34]

For the sake of brevity, we will focus on paired single cell RNA sequencing and single cell ATAC sequencing. While single cell RNA sequencing provides information about the transcriptional state of each individual cell in a sample, scATAC-seq provides information about genomic regulation. By assessing chromatin accessibility, ATAC sequencing can predict which genes are being actively transcribed, which genes are not accessible for transcription, and which transcription factors are active in the cells of interest. When performed in tandem, scRNA-seq and scATAC-seq can help researchers develop a more comprehensive understanding of how transcription factors regulate cellular states, and potentially find druggable targets for the disease

being studied. Many further analyses are possible and there are many potential prospects, both for paired RNA and ATAC sequencing, but more broadly for paired multiomics in general.

Overview of the Single Cell Data Analysis Pipeline

Once the single cell sequencing has been performed, the next step is to perform data analysis. Here we provide a high-level overview of a standard workflow for data analysis. Many more detailed resources are available for learning the nuances of single cell transcriptomic data analysis including the official web pages for Seurat [35]), Scanpy [36], and the Single Cell Best Practices Textbook [37]. At its core, data analysis consists of clustering cells with similar gene expression profiles and identifying clusters of cells based on differentially expressed genes. In practice, this requires many additional steps depending on the specific dataset.

Below, we will define some commonly used terms used in single cell transcriptomic data analyses:

- **Raw data:** Fastq files that are the output product from the sequencing facility and have not yet undergone any processing.
- **Feature:** Gene or transcript. Most commonly expressed as an Ensembl gene id or HUGO Gene Nomenclature Committee (HGNC) symbol but can be expressed in other terms as well.
- **Barcode**: Also known as unique molecular identifier (UMI), this refers to the molecular tag that is used to label the RNA in each cell. Each cell is given a unique tag, which allows RNA expression to be linked with individual cells in downstream analysis.
- **Feature-barcode matrix:** The single cell data matrix consisting of genes x cells (i.e., features x barcodes). These matrices are typically extremely large consisting of tens of thousands of genes by hundreds of thousands of cells. Additionally, these matrices are sparse (i.e., consist mostly of 0 s indicating that the vast majority of genes in the matrix are either not expressed or not detected).
- **Library or package:** Software that has developed for a specific step of data analysis typically within existing workflows (i.e., in R or in Python)

Next, we will provide an overview of a standard workflow for data analysis. Table 14.3 summarizes the steps involved in a standard workflow.

Step 1: Creation of Feature-Barcode Matrices from Raw Data

After single cell sequencing has been completed, researchers will often receive the raw data, the feature-barcode matrices, and a sequencing report from the sequencing facility. If this is the case, the processing of the raw data has already been performed by the sequencing facility. If this step has not been performed, it is prudent

Table 14.3 Single cell sequencing data analysis pipeline

Step	Description	Seurat function	Scanpy function
1	Creation of feature-barcode matrix	–	–
2	Raw data quality assessment	–	–
3	Choosing a data analysis strategy	–	–
4	Data preprocessing	PercentageFeatureSet(), subset()	sc.pp.calculate_qc_metrics(), sc.pp.filter_cells(), sc.pp.filter_genes()
5	Data normalization	NormalizeData()	sc.pp.normalize_total(), sc.pp.log1p()
6	Selection of highly variable genes	FindVariableFeatures()	sc.pp.highly_variable_genes()
7	Dimensionality reduction	RunPCA()	sc.tl.pca()
7.5	Batch correction	*external packages*	*external packages*
8	Plotting a UMAP	FindNeighbors(), RunUMAP()	sc.pp.neighbors(), sc.tl.umap()
9	Clustering cell types	FindClusters()	sc.tl.leiden()
10	Annotation of cell clusters	FindMarkers()	sc.tl.rank_genes_groups()

to coordinate with the sequencing facility to determine which tool will be optimal for generating feature-barcode matrices. Various tools are available for this step (i.e., Cell-Ranger [26]), which function to demultiplex the raw sequencing reads, perform sequencing alignment, and generate feature-barcode matrices. Typically, these tools are run from the command line.

Step 2: Quality Assessment of the Raw Data

Once the feature-barcode matrices have been generated, the first step is to assess the quality of the data. It is important to inspect the sequencing report to identify any issues with the samples or sequencing. Sequencing reports will typically display metrics such as the total number of reads, the total number of cells, the number of reads per cell, the number of valid UMIs (unique molecular identifiers), and many other metrics. If a researcher has not interpreted a sequencing report in the past, it is important to confer with the sequencing facility for help with interpretation. The expected quality metrics in the sequencing report can vary significantly from sample to sample and can depend heavily on the type and quality of tissue being inspected. For example, inactivated immune cells typically only express approximately 1200 genes, whereas activated immune cells can express upwards of 4000 genes. Following inspection of the sequencing report, it is also important to inspect the raw data files (i.e., fastq files). Many tools are available for this purpose (e.g., FastQC [38], MultiQC [39]) and will output metrics including a quality score, base content, and certain other relevant statistics.

Step 3: Choosing a Data Analysis Strategy

Following the assessment of quality control metrics, the next step is to choose a strategy for data analysis. Two major workflows have emerged as the tools of choice: Seurat (R) and Scanpy (Python). There are pros and cons to each tool, and ultimately the choice will depend on which downstream analyses are planned and the comfort level of the researcher with each programming language. Some of the benefits of using Seurat are that many libraries were developed for Seurat and often only Seurat will have access to the latest software. Some of the benefits of using Scanpy are that Python implementations of single cell software are often faster and more memory efficient than those developed in R. While these statements are true in a broad sense, they are a drastic oversimplification; some libraries in R can be fast and memory efficient and some of the newest packages are developed only in Python. Even if the analysis has been performed in one ecosystem but the library of interest is in the other, interconversion between the two workflows is possible with some extra effort. Finally, depending on the size of the dataset, a standard computer may not possess adequate processing capacity to analyze the dataset. If this is the case, researchers may opt to use a supercomputer processing center at their institution or may utilize proprietary computing solutions (e.g., Amazon Web Server).

Step 4: Data Pre-Processing

Depending on which workflow is chosen, there are minor differences in how preprocessing of the data is performed. More detailed tutorials are available on the Seurat webpage (https://satijalab.org/seurat/) and on the Scanpy webpage (https://scanpy.readthedocs.io/en/stable/). Generally, data, which is commonly stored either as a tab separated matrix (tsv file) or as an hdf5 compressed file (h5 file), is loaded into a storage object in either Seurat (Seurat object) or Scanpy (Anndata object).

Once the data is loaded, several pre-processing steps are available from each program by default. These include methods to filter out low-quality cells (cells expressing too many genes, too few genes, or too many mitochondrial genes) and low-quality genes (genes that have too few reads or are expressed in too few cells). The goal of these filtering steps is to remove data that would contribute little to, or even hinder, downstream analyses. Conceptually, these steps help to remove doublets (cells with double the number of expected genes may have been two cells accidentally processed together), low quality reads, and stressed/dying cells (which typically have a high percentage of mitochondrial reads). However, these methods are rough approximations and more advanced methods can be employed to more accurately preprocess the data.

Additional steps that can be performed at this stage include removal of ambient RNA and more advanced doublet removal. Removal of ambient RNA (e.g., using SoupX [40]) aims to identify cell-free RNA (or RNA present in the solution used to store the cells) and remove this ambient RNA from the feature-barcode matrix. Doublet detection has garnered much enthusiasm and many advanced methods have

been developed for doublet identification and removal (examples include scDblFinder [41], Scrublet [42], DoubletFinder [43]). These tools employ additional computational methods to detect doublets more accurately as compared to simply removing cells with high read counts.

Step 5: Data Normalization

Once pre-processing of the data has been completed, the data now can be prepared for cell clustering. But prior to the clustering, normalization of the feature-barcode matrix is performed. Several methods for data normalization exist, but the most common methods perform some version of counts per million (CPM) normalization (i.e., NormalizeData in Seurat, or normalize_total in Scanpy), followed by log transformation. Other methods for normalization employ more complex algorithms (e.g., ScTransform [44]) and may result in more accurate normalization. At this point, the data can also be scaled to ensure each cell has a similar range of gene expression values.

Step 6: Selection of Highly Variable Genes

Following normalization, or in some cases, prior to normalization depending upon the workflow, a set of the most highly variable genes in the dataset is selected. This is the set of genes that demonstrate the highest variability within the dataset and are most likely to be differentially expressed between different cell clusters. Both Seurat and Scanpy employ default methods for selection of highly variable genes. In each package, it is important to tune the parameters for highly variable gene selection as this can affect downstream results. More advanced methods for highly variable gene selection exist and utilize alternative methods for identification of highly variable genes (e.g., Pearson's residuals). Following selection of highly variable genes, the feature-barcode matrix can be subset into a smaller matrix of only the highly variable genes.

Step 7: Dimensionality Reduction

The next step in data processing is dimensionality reduction. In its current state, the feature-barcode matrix is still extremely large, consisting of hundreds of thousands of cells by tens of thousands of genes. Even with the inclusion of only highly variable genes, this matrix is still too large to efficiently perform cell clustering. Many dimensionality reduction techniques exist to further reduce the size of this matrix. But the most common of these is principal component analysis (PCA), which identifies the vector components in the high dimensional gene space that contribute the greatest variability in each cell's gene expression. The top components (the principal components) will explain most of the variability between cells. Once PCA dimensionality

reduction has been applied, researchers will be left with a matrix of hundreds of thousands of cells by a small number of principal components (typically 30–50). Optionally, at this point the data can be visualized in two dimensions using the top two principal components, and some preliminary cell clusters may be observed.

Step 7.5: Batch Correction

Depending on the dataset, batch correction can be performed at this point. Batch correction is utilized for datasets that have been run in multiple batches or for datasets consisting of multiple discrete datasets (e.g., collections of different cell atlases or experiments). Due to minute differences in run conditions, each instrument run of single cell RNA sequencing will provide a slightly different set of gene expression values. On downstream analysis, these differences will result in the clustering of cells by batch rather than by cell type. In other words, rather than identifying biological differences in the analysis, the researcher will erroneously identify technical differences. Countless batch correction methods exist (e.g., scVI [45], Harmony [46], Scanorama [47]) and which batch correction method is optimal is highly dependent on the dataset. If the dataset has all been run as one batch, this step is unnecessary.

Step 8: Calculating Cell-Cell Neighborhoods and Plotting

The final steps after dimensionality reduction are to generate a neighborhood graph of the cells based on cell-cell distance and to create a low dimensional embedding to visualize the cells. Both Seurat and Scanpy have default functions for creating a neighborhood graph (FindNeighbors for Seurat and neighbors for Scanpy). Again, these functions have parameters that should be tuned depending on sample characteristics. Most notably, the number of neighbors calculated for each cell can have a large impact on the final visualization; typically, the number of neighbors per cell is set to a number between 10 and 100. Following the calculation of a neighborhood graph, the uniform manifold approximation and project (UMAP) algorithm can be applied to reduce the data into x and y coordinates which can then be used to visualize the dataset. Again, both Seurat and Scanpy have default implementations of this algorithm, for which the parameters should be tuned. An alternative to UMAP that has mostly fallen out of favor is the t-distributed Stochastic Neighbor Embedding (tSNE) method, which performs a similar function to UMAP, but can require significantly longer computation times depending on the size of the dataset.

Step 9: Clustering Groups of Cells

You now have successfully created a UMAP of your single cell data. If everything went smoothly, the UMAP should demonstrate discrete cell clusters representing the different cell types in the sample(s). Once the UMAP has been generated, the next step is to

algorithmically cluster the cells. The most used clustering algorithms for this task are Leiden clustering and Louvain clustering. Both Leiden and Louvain clustering algorithms have specific parameters that determine the approximate size and number of clusters, and researchers will need to decide which parameters best represent the data.

Step 10: Annotation of Cell Clusters

Now that the cells have been clustered, the final step is to annotate the cell clusters according to which cell type they most likely represent. There are a multitude of different cell types that express different cellular phenotypes, and it is up the researcher to determine the appropriate granularity for annotation depending on the research question (for example, individually labeling specific types of immune cells *vs*. labeling all as immune cells). Furthermore, it is important to recall that not all cells will exist as discrete clusters and many cells can exist on a continuum between different phenotypes. Cell type annotation can be accomplished either using automated methods or manually.

Many algorithms exist for automated cell type annotation (e.g., Garnett [48], CellTypist [49], SingleR [50]). The quality of annotation with each of these algorithms can vary and many factors also can affect how well the algorithm performs. Some of these factors include what type of algorithm was utilized, the quality of the data used to train the model, and the quality of the researcher's data. It is often prudent to trial several automated annotation methods to determine which produces the best and most reliable results. Once the automated annotations have been assigned to the dataset, it is vital to confirm the legitimacy of the labels through manual inspection.

Manual annotation focuses on utilizing the differentially expressed genes within each cluster to define the cell type of the cluster. The differentially expressed genes between clusters can be identified with the FindMarkers function in Seurat and rank_genes_groups function in Scanpy. There are multiple statistical approaches (for example, t-test and Wilcoxon) to defining which markers are the most significant for each cluster, but for initial analysis, the default methods are often sufficient. Once these functions have been run, the researcher will be left with a set of genes that defines each cluster. These genes can be cross referenced with common databases (e.g., PanglaoDB, CellMarker) to identify which cell type is most likely represented in each cluster. Once the cell clusters have been annotated, researchers can validate the cluster annotations by checking the expression levels of canonical genes that are known to be expressed in each cell type.

Downstream Analyses

Now that the main pipeline for single cell data analysis has been completed, various other analyses can be performed. scRNA-tools.org is a website that maintains a comprehensive database of all packages that exist for single cell analysis. At the time of writing this chapter, over 1700 packages have been developed for single cell

data analysis, with 54% available in R and 46% available in Python. Many packages have been developed for downstream analysis to provide more nuanced biological information about the dataset of interest, and it is up to the researcher to determine which downstream analyses is most appropriate for their research question.

Some of the major categories of downstream analyses include compositional analysis, differential gene expression (DGE) analysis, gene set enrichment analysis (GSEA), gene regulatory network inference, trajectory inference (including pseudotime and RNA velocity analysis), cell-cell communication, and bulk deconvolution. These analyses are summarized in Table 14.4. This list is by no means comprehensive, and many other types of downstream analysis can be performed.

Table 14.4 Downstream analysis tools

Downstream application	Description	Possible use case	Example package
Compositional analysis	Identify the proportion of each cell type in different conditions	Calculate the percentage change in T cells before and after checkpoint inhibition	scCODA
Differential gene expression	Identify the upregulated and downregulated genes in different conditions or cells	Discover upregulated genes in T cells after checkpoint inhibition	DESeq2
Gene set enrichment analysis	Identify upregulated pathways or biological processes in different conditions or cells	Discover pathways upregulated in T cells after checkpoint inhibition	GSEA
Gene regulatory network inference	Identify putative transcription factors involved in the regulation of gene expression	Understand the transcription factors driving T cell activation after checkpoint inhibition	SCENIC
Trajectory inference (pseudotime)	Identify changes in gene expression during cellular differentiation	Understand changes in gene expression during hematopoietic stem cell differentiation into T cells	Monocle3
Trajectory inference (RNA velocity)	Identify cellular dynamics in the developmental process	Order hematopoetic stems cells according to the stage of differentiation	scVelo
Cell-cell communication	Identify putative receptor-ligand interactions between cells	Discover possible interactions between tumor cells and T cells involved in chemotaxis	CellChat
Bulk deconvolution	Estimate cell type fractions in bulk RNA sequencing data	Identify changes in T cell populations of bulk RNA sequencing data from tumor samples	CIBERSORTx

Compositional Analysis

The core premise of compositional analysis is that the proportion of cell types represented in each sample varies according to condition. This idea can be used to test various hypotheses. For example, a researcher may be interested in identifying whether the proportion of T cells in patient tumor samples are altered after treatment with immune checkpoint blockade. In this example, compositional analysis could be utilized to determine the proportion of T cells within treated samples and untreated samples and subsequently test whether there is a statistically significant difference. As might be expected, sample integrity and consistency are crucial to the success of this type of experiment. For example, sampling a section of tumor that has little immune infiltration could drastically affect the results. Several libraries exist for the purpose of compositional analysis (e.g., scCODA [51]).

Differential Gene Expression

Differential gene expression simply identifies the differentially expressed genes between two conditions. The conditions are defined by the researcher according to their hypothesis. For example, a researcher may be interested in identifying which genes are overexpressed in T cells derived from patient tumors treated with immune checkpoint blockade. In this example, the researcher could perform differential gene expression analysis between T cells from treated and untreated tumors. Differential gene expression analysis can be performed utilizing single cell-based methods and bulk RNA sequencing based methods. Single cell-based methods are more convenient to access but may yield inconsistent results due to issues with data sparsity. Single cell based differential gene expression methods are available by default in both Seurat and Scanpy workflows.

Bulk RNA sequencing based methods are methods originally developed for bulk RNA sequencing data (e.g., DESeq2 [52], limma [53], EdgeR [54]), but can be adapted for use with single cell data. Since these methods are not optimized to deal with data sparsity, cells are typically aggregated into pseudobulk samples prior to analysis. Pseudobulk is a technique that aggregates many different single cells from the single cell data into a single sample. For example, a pseudobulk of endothelial cells can be generated by identifying all cells annotated as endothelial in the dataset and performing a simple summation of each gene in these cells. Once the pseudobulk samples have been generated, differential gene expression analysis can be performed. Several factors are important to consider when performing pseudobulk. For example, a researcher should consider which cells to aggregate, how many replicates to create, and how to aggregate cells (e.g., sum *vs*. mean *vs*. median). Various libraries have been developed to generate pseudobulk data based on user defined parameters (e.g., decoupleR [55]). Once the pseudobulk samples have been generated, bulk RNA sequencing based methods can be utilized to perform the more robust bulk RNA sequencing based methods of differential gene expression analysis.

Gene Set Enrichment Analysis

Gene set enrichment analysis focuses on the core premise that certain groups (or sets) of genes are associated with certain biological processes. If a group of genes is overexpressed in a sample, it is highly likely that the associated biological process is also occurring in the sample. Gene sets can be related to many different biological processes (e.g., pathway activity, gene regulation, transcription factor activity, miRNA activity, oncogenic activity, immunologic activation, cell type, and many others). For a more comprehensive list, MsigDB [56] (https://www.gsea-msigdb.org/gsea/msigdb) curates a plethora of high quality gene sets. When performing gene set enrichment analysis, it is critical to inspect the quality of the gene set of interest. A low-quality gene set may result in spurious results and conclusions.

A multitude of algorithms have been developed for gene set enrichment analysis. The most well described is GSEA [57], which utilizes the Kolmogorov-Smirnov test to accurately estimate the likelihood that a gene set is enriched in a sample. Other notable methods include over representational analysis (ORA) and database for annotation, visualization, and integrated discovery (DAVID) [58], among others. Gene set enrichment analysis can be helpful for identifying the drivers of cell behavior. For example, if a researcher is interested in understanding what pathway is driving T cell activation in patient tumors treated with immune checkpoint blockade, he or she could perform gene set enrichment analysis with several gene sets that are known to be involved in T cell activation. Gene set enrichment analysis can be performed on single cell data directly (for example, using AUCell [59] or DecoupleR), or can be performed on pseudobulk samples of the data using GSEA. It is important to note that due to issues with data sparsity, single cell methods may be less robust than methods designed for bulk RNA sequencing.

Gene Regulatory Network Inference

At the genomic level, genes can be regulated by various transcription factors or regulatory elements (e.g., promoters, enhancers, repressors) that bind to the genome near the gene of interest and either activate or repress expression of the gene. Gene regulatory network inference focuses on identifying which transcription factors are most likely contributing to the transcriptional state observed in the samples. Gene regulatory network inference utilizes prior knowledge of the genomic binding sites of various transcription factors to infer the regulons (or groups of genes controlled by the activity of said transcription factor). Using this strategy can be effective for understanding the underlying biology driving a condition of interest. For example, if a researcher was interested in understanding the transcription factors driving T cell activation in patient tumors treated with immune checkpoint blockade, he or she could perform gene regulatory network inference on these samples. Many different libraries have been developed for this purpose including SCENIC [59].

Trajectory Inference, Pseudotime, and RNA Velocity Analysis

Single cell RNA sequencing is an excellent model system for studying tissue over various stages of development. While this technology cannot capture individual cells at multiple points in time, it can provide a spectrum of cells across various stages in the developmental process. Once this information is captured, cells can be ordered according to the stage in the developmental process (pseudotime), with lower values representing more primordial cells and higher values representing more differentiated cells. Trajectory inference leverages information from single cell experiments to understand the gene expression profiles driving cellular differentiation. For example, if a researcher was interested in studying what changes in gene expression drive a hematopoietic stem cell to differentiate into a T cell, he or she could perform trajectory inference on a dataset of bone marrow cells. This type of analysis could additionally be applied to various other developmental processes (e.g., differentiation of the fetal mesoderm into the different parts of the gastrointestinal tract). Many packages are available for trajectory inference, and these include Monocle3 [60], Palantir [61], and PAGA [62].

RNA velocity analysis is another form of trajectory inference, which attempts to order cells according to their stage in the developmental process. RNA velocity analysis is unique to pseudotime in that RNA velocity analysis attempts to utilize transcriptomic information to automatically label the developmental state of each cell rather than have the researcher assign cells as more or less differentiated as is performed in pseudotime analysis. RNA velocity analysis leverages information about RNA splicing kinetics (e.g., DNA is transcribed into unspliced pre-mRNA which is then spliced into mature mRNA) to make inferences about how transcriptionally active each cell is. Cells expressing high levels of unspliced pre-mRNA are considered more transcriptionally active. It is critical to confirm that the underlying assumptions of RNA velocity analysis hold true in the sample of interest and that the analysis is performed on tissue for which the developmental process is comparable to the half-life of RNA molecules. If these assumptions do not hold true, spurious results can occur. Several different libraries are available for RNA velocity analysis (e.g., scVelo [63], cellrank [64]). A simplified version of RNA velocity analysis, only utilizing the raw number of mRNA expressed in the tissue of interest can also be performed and appears to provide similar results in some developmental tissues (e.g., using Cytotrace [65]).

Cell-Cell Communication

Cell-cell communication seeks to infer intercellular signaling through the application of databases containing known or predicted receptor-ligand interactions. When compared between groups of cells, some clusters will have high expression of the ligands and some clusters will have high expression of the receptor, and these clusters will be predicted to be interacting. For example, if a researcher is interested in T cell chemotaxis to tumor cells, he or she may perform cell-cell communication

analysis between these two cell clusters to examine whether patterns known to be associated with chemotaxis are present. Cell-cell communication is highly dependent on the database utilized for receptor-ligand interactions and on the method used to infer interactions. A multitude of libraries has been developed for this purpose (e.g., cellchat [66], cellphoneDB [67]) and many of the libraries demonstrate markedly different results when performed on the same dataset. LIANA [68] is a package that was developed to attempt to address this issue by aggregating interaction scores across multiple different libraries. While cell-cell communication may have limited utility in single cell data, combining this analysis with spatial transcriptomics (discussed later) can provide additional power to the analysis.

Bulk Deconvolution

While single cell transcriptomics can provide a wealth of information about the biology of a sample, this technology is not yet widely used clinically. Bulk transcriptomics is much more common, cheaper, and is more readily accessible. Bulk deconvolution analysis attempts to leverage information gained through single cell RNA sequencing to better understand bulk RNA sequencing data. More specifically, by utilizing the gene expression profiles of each cell type in single cell RNA sequencing samples, bulk deconvolution can estimate the proportion of each cell type in bulk RNA sequencing data. For example, if a researcher wants to assess whether enrichment of T cells within tumor samples is associated with longer survival, he or she may perform bulk deconvolution on bulk RNA sequencing data from tumor samples obtained from a central database to determine if the estimated proportion of T cells is associated with survival. Several libraries exist for bulk deconvolution (e.g., CIBERSORTx [69]).

Future Directions

Single cell transcriptomics is a constantly advancing field. Since its inception over a decade ago, the field has evolved at a rapid pace. Furthermore, the cost of performing single cell transcriptomics has decreased dramatically, while the accuracy and depth of sequencing has increased significantly, leading this technology to be more accessible to researchers than ever. New technologies are constantly being developed to provide additional information to researchers allowing for more nuanced analysis of disease pathology. One of the most promising upcoming technologies is spatial transcriptomics.

Spatial Transcriptomics is similar to single cell RNA sequencing but differs in how the cells are barcoded prior to sequencing. By labeling cells with spatially resolved barcodes, cell location within samples can be determined post processing. In other words, data analysis for spatial transcriptomics is performed in a nearly identical fashion to single cell transcriptomics (including quality control, normalization, dimensionality reduction, clustering, and annotation), but once this is

complete, an additional step can be performed that maps cells to the spatial coordinates of the barcodes they were tagged with. This technology can provide extremely useful information for testing biological hypotheses. For example, let's say a researcher hypothesizes that T cell recruitment to tumors prevents tumor progression and that immune privileged areas of the tumor are responsible for tumor growth. He or she could test this hypothesis utilizing spatial transcriptomics. Specifically, using spatial transcriptomics, he or she could visualize which parts of the tumor are associated with T cells and which appear to be immune privileged, and use this information to assess whether there are differences between the gene expression profiles between these two areas of the tumor. Many technologies are in the process of development for spatial transcriptomics, and it remains to be seen what other ways this technology could be utilized.

Summary/Conclusion

Over the past several decades, transcriptomics has radically changed our understanding of human disease. The transcriptome, or the entire set of RNAs expressed by the cell or tissue of interest, is a core part of the central dogma of biology: that DNA is transcribed into RNA which is translated into protein. Leveraging this information has allowed researchers to understand cell biology better than ever before, including inferring the core programs that drive cell behavior. The field of transcriptomics has evolved hand in hand with the development of novel sequencing technologies. Starting with microarrays (which allow for the evaluation of large sets of predefined RNAs), technology progressed to bulk transcriptomics (which allows for the assessment of all RNAs in the tissue of interest), and finally single cell transcriptomics (which allows for the assessment of all RNAs in individual cells). The advent of single cell transcriptomics is nothing short of remarkable and has led to many discoveries about how certain cell types can contribute to or mitigate disease. To use a common analogy, while bulk transcriptomics analyzes a smoothie of all the different cell types in the tissue of interest, single cell transcriptomics parses out each individual fruit used to make the smoothie.

But using and interpreting single cell data can be challenging. Routinely, single cell datasets comprise of hundreds of thousands of cells, each with tens of thousands of genes. Together, these data require a huge amount of computational power to analyze. Many different approaches have been developed, but to date, the two most popular approaches are Seurat (R) and Scanpy (Python).

In this chapter, we discussed many of the basics of single cell RNA sequencing including experimental workflow, differences between single cell RNA sequencing and single nucleus RNA sequencing, and the potential for multiomic analysis. We also discussed a high-level overview of the approach to data analysis, including quality control, dimensionality reduction, clustering, and annotation, and showed some of the commands used by Seurat and Scanpy in this workflow. We described a plethora of potential downstream applications, including gene set enrichment analysis, trajectory inference, and cell-cell interactions, and described relevant

examples of each. Finally, we discussed some future directions for single cell transcriptomics, namely spatial transcriptomics, which both have the potential to provide a wealth of new information to researchers.

In sum, single cell transcriptomics represents one of the most data rich methods for studying cell biology and disease pathology that has ever been developed. While the learning curve for understanding single cell transcriptomics is steep, it is a worthwhile endeavor for those interested in understanding the nuanced biology of disease.

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Stem Cells and Tissue Engineering

15

Allison L. Speer and Troy A. Markel

Abstract

Regenerative medicine has become the most recent and innovative branch of the medical sciences. It deals with the restoration of damaged or injured tissues in patients suffering from catastrophic injuries or chronic diseases in which their own body's regenerative capacities are not functioning properly. Regenerative medicine typically involves the study of stem cells and the engineering of biological tissues or organs.

Stem cells are unique cells that have the capability of dividing continuously and differentiating into other end organ cells. Pluripotent stem cells and adult/somatic stem cells are the two main classifications of stem cells. They provide protection to injured tissues through cell differentiation, heterotopic cell fusion, and by the release of paracrine factors and exosomes. These methods work to limit inflammation, promote tissue restoration, and limit damage by reactive oxygen species.

Principals of tissue engineering include the study of stem cells as well as the study and engineering of biological tissue scaffolds. 3D printers and bioreactors have been developed to facilitate the production of biologic tissues and organs for therapy. As technological capabilities increase, whole organs may be eventually engineered for transplantation into humans to combat chronic diseases and organ failure.

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Introduction

Regenerative medicine has become the most recent and innovative branch of the medical sciences. It deals with the restoration of damaged or injured tissues in patients suffering from catastrophic injuries or chronic diseases in which their own body's regenerative capacities are not functioning properly. The study of regenerative medicine continues to advance knowledge about how human beings develop and how healthy cells function to replace damaged cells following injury. Regenerative medicine typically involves the study of stem cells and the engineering of biological tissues or organs.

The use of stem cells in research has become very common over the last decade, as scientists continue to search for novel ways to repair and replace damaged organs. Stem cells have the unique ability to replicate indefinitely and to differentiate into multiple cells in the body, thereby making them ideal substrates for tissue repair. In this chapter we will examine several facets of regenerative medicine including: (1) the types of stem cells available for use, (2) the mechanism of action that these cells use during therapy, and (3) principles of tissue engineering such as scaffolds, bioreactors, and 3D printing.

Types of Stem Cells

Stem cells are a unique group of unspecialized cells that are distinguished from other cell types by two specific principals. First, stem cells are capable of indefinite self-renewal through cell division. Second, under specific physiological conditions, stem cells can be induced to become a specific organ or tissue specific cells. In this regard, stem cells have the unique potential to repair or replace damaged or senescent cells all over the body.

Stem cells are typically characterized by their "potency", or their ability to divide into more specialized cells. Totipotency is defined as the ability of a single cell to develop into any and all cells of the body, including extraembryonic tissues, such as the placenta. Totipotent cells are limited to the mammalian zygote and the early embryonic blastomeres up to the four cell stage [1]. Cells up to the four cell blastomere were able to independently support term pregnancies and produce live animals [2]. Pluripotent stem cells (PSCs), on the other hand, are cells that are able to differentiate into any of the intraembryonic tissues including the three germ layers: ectoderm, mesoderm, or endoderm, as well as germ cells. They can give rise to any fetal or adult cell type [1], but lack the potential to develop into an organism. PSCs include embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst, as well as induced PSCs (iPSCs), which are derived by exposing somatic

cells to reprogramming factors such as Oct3/4, Sox2, Klf4, and cMyc to induce pluripotency [3]. Multipotent stem cells are further restricted, having undergone lineage establishment, and thus, are able to develop into a limited set of cells typically from one germ layer. Whereas pluripotent cells do not age and can proliferate indefinitely, multipotent stem cells have aging problems and eventually stop proliferating. Oligopotent cells are limited even more, for example, a myeloblast is already committed to the myeloid line and differentiates to become a neutrophil, eosinophil, or basophil, and can not become a lymphoblast or lymphocyte. Unipotent cells are only able to differentiate into a terminal cell type, such as a neural progenitor differentiating into a neuron or a skeletal muscle stem cell differentiating into a striated muscle cell. The two main types of stem cells include PSCs (ESCs and iPSCs) and multipotent adult/somatic stem cells (ASCs) (Fig. 15.1).

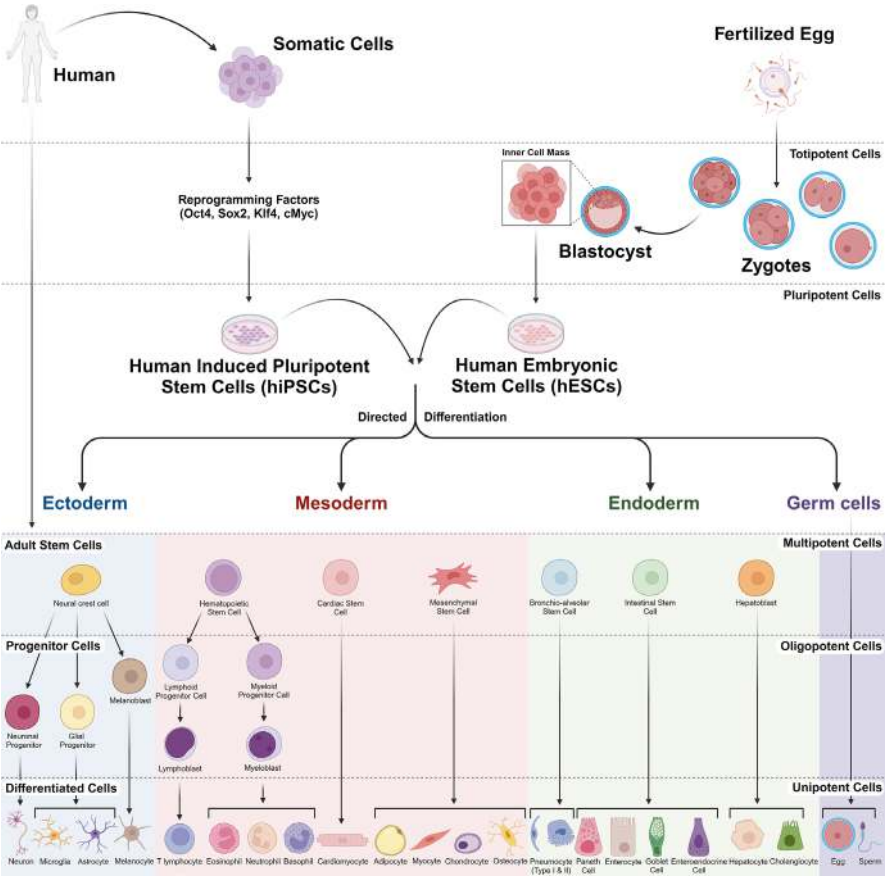

Fig. 15.1 Stem cell hierarchy: The two main types of stem cells are pluripotent stem cells (embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)) and adult/somatic stem cells (ASCs). All have the potential to differentiate into various cell lineages

Embryonic Stem Cells

The ability to derive ESCs from mouse embryos was derived nearly 40 years ago by two independent laboratory groups. Martin Evans and Matthew Kaufman from the University of Cambridge published first in July of 1981 where they revealed a new technique to culture mouse embryos in the uterus, allowing for an increase in cell number and the derivation of ESCs from these embryos [4]. In December of the same year, Gail Martin from the University of California San Francisco published her work and coined the term "Embryonic Stem Cell" [5]. These studies led to the development of specific ESC lines from discarded human embryos in 1998 by James Thompson and Jeffrey Jones from the University of Wisconsin [6].

Embryonic stem cells are easily grown in culture and they divide readily without differentiating in appropriate cell culture conditions (Fig. 15.2). The appeal to these cells is that they are pluripotent, meaning that they can divide into any cell from all three germ layers with the proper stimulation and environmental factors. Several stage specific embryonic antigens (SSEAs) such as SSEA-1, SSEA-3, and SSEA-4 help identify embryonic stem cells. Other markers in the cluster of differentiation (CD) family are also utilized. These include CD324, CD90, CD117, CD326, CD9, CD29, CD24, CD59, CD133, and CD31. Other markers include TRA-1-60, TRA-1-81, Frizzled5, Stem cell factor, and Cripto [7].

Despite the appealing scientific benefits, many in the community felt that it was unethical to use stem cells from aborted fetuses or unused embryos. Therefore, on August 9, 2001, President George W. Bush limited federal funding to only the 60 already established ESC lines, and forbid federal funding to establish any further lines [8]. Eight years later though, President Barack Obama signed Executive Order 13505 which removed the restrictions that were previously put in place for human stem cells. This effectively allowed the National

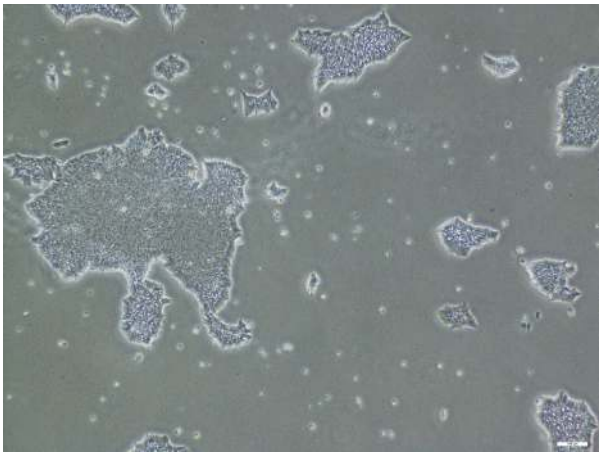

Fig. 15.2 Human embryonic stem cells: Human embryonic stem cells (H9) growing in culture on a Matrigel-coated surface under phase contrast microscopy. Scale bar = 100 μm

Institutes of Health to provide funding for human ESC research [9]. That same year, the Food and Drug Administration provided approval for Geron Corporation to perform a limited phase 1 trial of their human ESC (hESC) derived treatment for spinal cord injuries [10].

In October of 2010, the first patient was enrolled in the Geron Spinal Cord Injury Trial to receive hESC-derived oligodendrocyte progenitor cells. Only five patients of the intended ten were enrolled before the study was halted. No official results have been published but preliminary results presented at the 2011 American Congress of Rehabilitation Medicine would suggest that no changes to the spinal cord or the patients' neurological conditions were found [11]. Multiple clinical trials using ESCs have been undertaken as of 2020 and are found in the UMIN Clinical Trials Registry or ClinicalTrials.gov [3]. These clinical trials address a variety of disorders including: macular degeneration, retinitis pigmentosa, Parkinson's Disease, amyotrophic lateral sclerosis, spinal cord injury, Type I diabetes, and citrullinemia type I [3]. Due to the challenges of enrolling patients, interpreting the data, and long term follow up, many of these trials do not yet have long-term results.

Induced Pluripotent Stem Cells

In 2006, the Yamanaka group made a remarkable breakthrough by identifying specific conditions that would allow certain adult somatic cells to be genetically reprogrammed to assume a pluripotent state. These iPSCs functioned like ESCs, and were able to replicate and differentiate accordingly in a mouse model [53]. Unlike ESCs, where the progression from mouse to human ESCs took 17 years, the advancement from the generation of mouse to human iPSCs was rapid and first described in 2007 [12, 13].

The original reprogramming method as described by Yamanaka utilized retroviral transduction of Oct4, Sox2, Klf4, and c-myc into mouse fibroblasts derived from mice expressing B-galactosidase-neomycin fusion protein, which is typically expressed in pluripotent cells [14]. The downside to the original reprogramming strategies though was that the efficiency of reprogramming was fairly low, and it used viral vectors to transmit the reprogramming genetic code into the cells [15]. In some instances, the viral DNA became incorporated into the genome, while in other cases tumors were formed.

Newer methods of reprogramming have been undertaken, including methods that do not involve viruses. These methods include single cassette reprogramming vectors with Cre-Lox mediated transgene excision, use of non-integrating viruses, use of mRNA and miRNA, as well as minicircle and episomal plasmids [15]. All of these methods have varying degrees of efficiency and the optimal method of transformation has not yet been identified.

iPSCs can be generated from many end organ cells, but typically these are fibroblasts. Furthermore, these cells have ushered in a stage of precision medicine because differentiated cells can be reprogrammed into iPSCs and then differentiated

into other cell lines to ameliorate disease. Dr. Yamanaka discusses remaining challenges such as tumorigenicity, immunogenicity, and heterogeneity of iPSCs as well as promising therapeutic applications in a recent review [3]. Similar to ESCs, a number of clinical trials are currently being conducted utilizing iPSCs for diseases such a macular degeneration, retinitis pigmentosa, heart failure, Parkinson's Disease, spinal cord injury, graft versus host disease, and cancer immunotherapy [3].

Adult Stem Cells

ASCs are multipotent, and thus by definition have less potency than ESCs and iPSCs. While they are more restricted in what they can divide into, they nonetheless still have enormous potential in terms of applications. ASCs are typically derived from specific tissues and their harvesting processes are labor intensive. In addition, the amount of stem cells acquired is typically small, thereby making them more difficult to use immediately for clinical applications. ASCs are divided into further categories, and include cells such as mesenchymal, hematopoietic, neuronal, and other tissue specific stem cells. These tissues include, but are not limited to, heart, intestines, and liver. Many studies focusing on stem cell therapy have used these cells as there is less controversy over their origins.

Hematopoietic Stem Cells

Hematopoietic stem cells can develop into all types of blood cells, including myeloid and lymphoid cells. They are typically found in the peripheral blood or the bone marrow. Human hematopoietic stem cells are denoted by being $CD34^{+}$. $CD59^{+}$, $CD117^{+}$, and lin^{-} [16]. The discovery of hematopoietic stem cells occurred through the appreciation of a transplantable multipotent bone marrow cell line that maintained hematopoietic activity. These experiments first occurred in irradiated mice, in which the transplanted cells were observed to protect the mice from a lethal dose of irradiation and replace the destroyed blood forming cells [17]. Further experiments demonstrated that rare, mainly quiescent hematopoietic cells could give rise to not only members of the myeloid lineage, but also those of the lymphoid lineage [18].

Hematopoietic stem cell transplant is clinically applicable in childhood leukemias, in which the chemotherapy destroys the host native blood forming cells [19]. In these instances, allogeneic stem cells from a matched donor or from a saved umbilical cord can be used [20]. Other hematologic diseases can also be treated with hematopoietic stem cells. These include sickle cell anemia and thalassemia [21, 22].

Mesenchymal Stem Cells

Definitive evidence that bone marrow includes not only hematopoietic stem cells but also cells that generate mesenchymal tissue-forming cells was originally provided by the pioneering work of Alexander Friedenstein in the 1960s. He performed several experiments that defined the existence within the bone marrow of different precursors for osteoblasts and fibrous tissue. One to two weeks after

seeding bone marrow cells in culture, he observed discrete colonies that adhered to plastic, were not phagocytic, and were elongated with a fibroblastic appearance. The clonogenic cell at the origin of each colony was shown to be quiescent at culture inception. Each colony seeded under the renal capsule of semi-syngeneic animals gave rise to fibrous tissue, bone, and bone marrow. Using chimeric animals, he further demonstrated that hematopoietic cells within the bony spaces were derived from the recipient, contrary to bone cells or fibrous tissue that were derived from the donor [23].

These cells were later named mesenchymal stem cells. They are multipotent cells that can differentiate into mesodermal lineages, including osteocytes, chondrocytes, myocytes, and adipocytes. MSCs are $CD44^{+}$,$CD73^{+}$, $CD90^{+}$, $CD105^{+}$, $CD34^{-}$, $CD45^{-}$, $CD11b^{-}$, $CD14^{-}$, $CD19^{-}$, and $CD79a^{-}$ [24]. These cells are typically harvested from the bone marrow, adipose tissue, umbilical cord, placenta, or amniotic fluid [25, 26].

Mesenchymal stem cells have been used for the treatment of a number of human diseases. A search of ClinicalTrials.gov demonstrates over 1200 current clinical trials associated with use of mesenchymal stem cells [27]. These include studies for bronchopulmonary dysplasia and refractory pulmonary diseases, chronic autoimmune diseases, inflammatory bowel disease, peripheral vascular disease, and chronic renal failure to name just a few. Due to their ability to differentiate into multiple tissue lineages, mesenchymal stem cells are attractive for clinical use.

Neural Stem Cells

Neural cell death is a characteristic of aging and acute central nervous system injury. Therefore, several trials are ongoing to transplant or stimulate native neural stem cells in order to mitigate disease progression [28]. Neural stem cells are tissue specific stem cells that differentiate to give rise to neurons, astrocytes, and oligodendrocytes [29]. Adult neural stem cells were first isolated from mouse striatum in the 1990's [30]. Neural stem cells, like many other cell types, can be classified by cell surface markers. A 2009 study in *Stem Cells* was able to determine cell surface markers in ESCs that progressed down a neural lineage. They found that $CD15^{+}$/$CD29^{hi}$/$CD24^{low}$ defined neural stem cells. $CD15^{-}$/$CD29^{hi}$/$CD24^{low}$ revealed neural crest like cells and mesenchymal phenotypes. $CD15^{-}$/$CD29^{low}$/$CD24^{hi}$ selected for neuroblasts and neurons [31].

The enteric nervous system, much like the central nervous system, is a highly specialized collection of neurons and synaptic connections. It contains more than 500 million neurons, which is more than the entire spinal cord [32]. Most of the enteric nervous system is derived from vagal neural crest cells, but some stem from sacral neural crest cells and Schwann cell precursors. Research groups have recently described protocols to efficiently derive vagal neural crest cells from PSCs (ESCs or iPSCs) [33–35]. The use of neural stem cells has also been studied to treat various gut motility disorders, such as diabetic gastroparesis, achalasia, Chagas disease, or congenital absence of the enteric nervous system such as seen in Hirschsprung's disease [36].

Intestinal Stem Cells

Gastrointestinal stem cells possess the ability to differentiate into all cells that occupy the villus, including enterocytes, endocrine cells, and goblet cells [37]. Under normal conditions, these stem cells may divide asymmetrically, giving rise to one stem cell and one cell that differentiates into the different types of epithelial cells. Under conditions of stress though, these stem cells may divide symmetrically, giving rise to two progenitors that replace injured intestinal cells [38].

Many years ago, two distinct and non-overlapping intestinal stem cell populations were described. These were the crypt base columnar cells (CBC) described by Leblond, and the +4 label retaining cell described by Potten [39]. Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as Gpr49) has become the predominant marker for the CBC population. Lgr5+ is controlled by Wnt signals and acts as the receptor for a series of Wnt agonists known as R-spondins [40]. Other markers including Ascl2, OlfM4, and Smoc2 have been found to be on these cells as well [39]. Markers such as *Musashi*, which had traditionally been thought of as a specific intestinal stem cell marker, may actually extend into the lower crypts to mark a broader cell line rather than specifically the intestinal stem cells [41, 42].

Lgr5+ cells are long lived and multipotent, and can be used to grow organoids in culture. Gene expression profiling of these cells has revealed an Lgr5+ signature which has been identified as contributing to the cell's stemness [39]. Generation of GFP labelled reporter mice allowed for the visualization of these cells. The cells were uniform in morphology, touched the Paneth cells, and divided uniformly on a daily basis [39]. These studies found that each crypt housed approximately 15 of these Lgr5+ cells.

The identification and characterization of the +4 cells has been a bit more scrupulous. Many researchers in the field still question the presence of this population. Original staining markers for these cells were located at the +4 position, hence how the name of these cells was derived. Several markers have been identified including Bmi1, HopX, and Lrig1. These cells appear to be relatively quiescent and resistant to radiation [39].

What is most interesting though is the likelihood of a reserve stem cell line, one that gives rise to the Lgr5+ population. This concept would suggest a stem cell hierarchy within the crypts. A recent study used diphtheria toxin to selectively kill off the Lgr5+ population [43]. Despite the death of the Lgr5+ cells, the crypts remained intact, implying that self-renewal could be achieved in the absence of the Lgr5+ cell. Once the toxin was removed the Lgr5+ cells reappeared. Lineage tracing of Bmi1 was performed and it showed that the new CBCs were derived from Bmi1 cells and acquired Lgr5 positivity. These studies would suggest the presence of two distinct stem cell lines in the intestine. One which is more rapidly dividing to repopulate the crypt villus structure, and one that is more reserved.

Liver Stem Cells

The stem cell for the liver has been defined as the progenitor cell that gives rise to both the hepatocytes and the cholangiocytes, the two types of liver epithelial cells [44]. Hepatoblasts seem to serve as the liver progenitor cell during fetal

development. Suzuki and colleagues found that the liver fraction of E13.5 mouse cells that were CD45$^-$, TER119$^-$, c-Kit$^-$, CD29$^+$, and CD49f$^+$ contained colony-forming cells with the potential to differentiate into hepatocytic and cholangiocytic lineages [45]. In many cases these cells were examined and found to express liver specific genes, be clonogenic, bipotent, and were able to repopulate the liver [44].

Other studies were able to select hepatoblasts based on a single specific marker. Deltalike 1 homolog (Dlk1), also known as Pref-1, is expressed in liver buds at E9.0 in the mouse embryo. Dlk1 expression is gradually decreased in the liver by the neonatal time period and becomes undetectable as the animal ages. Dlk1+ cells isolated from livers form highly proliferative colonies composed of the hepatocyte and cholangiocyte lineages *in vitro* [46]. E-cadherin, an epithelial-specific marker, was also utilized to isolate hepatoblasts [47, 48]. E12.5 liver epithelial cells were shown to specifically express E-cadherin, Dlk1, and Liv2 [49].

Hepatoblasts, however, likely change their characteristics during the course of development. Furthermore, *in vitro* expanded hepatoblasts are required in very high numbers to engraft a mouse, in comparison to a single hematopoietic stem cell. Additionally, engraftment of transplanted hepatoblasts to bile ducts has not been convincingly demonstrated, likely due to inadequate animal models. So even though hepatoblasts are likely the liver progenitor cell in the fetus, they lack the stringent criteria of "stemness" that other stem cells possess [44].

Because liver hepatocytes turnover slowly, there is question as to whether stem cells exist at all in the adult liver. It is felt that routine maintenance and repopulation of liver cells comes from mature hepatocyte proliferation. Despite this, many groups have succeeded in isolating "liver stem cell" populations from the adult liver based on marker gene expression and flow cytometric cell purification. These liver progenitors in adult livers can be defined as "stem cells"because they are [1] clonogenic with high growth potential, [2] able to differentiate into both hepatocytes and cholangiocytes, and, in some cases, [3] are capable of repopulating the liver upon transplantation [50]. Although these cells can be considered as liver stem cells because they can be functionally defined in culture, it remains unclear whether and where they exist *in situ* and how they behave under physiological conditions.

Cardiac Stem Cells

The first reported primitive cardiac stem cells (CSCs) were identified and isolated based on the expression of stem cell factor receptor CD117 or c-kit. c-kit$^+$ cardiac stem cells are also positive for Sca-1, MDR-1 (ABCG2), and other markers identified on adult cardiac stem and progenitor cell populations, such as CD105, CD166, PDGFrα, and CD90. c-kit$^+$ CSCs do not express CD34, CD31, CD45, or tryptase, which allows for separation from c-kit$^+$ endothelial progenitor cells.

CSCs are multipotent, self-renewing, and exhibit the ability to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells [51, 52]. They can be propagated over a long period of time in cell cultures and can remain in an undifferentiated and stable state without exhibiting senescence [53]. In the adult heart, most of the CSCs reside in the atrium and the ventricular apex, albeit at a very low density (1 cell per every 10,000 myocytes) [51]. When injected into an ischemic

heart, these cells were able to regenerate functional myocardium [51]. A subsequent study in the *Proceedings of the National Academy of Science* showed that when given after injury, these cells targeted injured myocardium and differentiated and fused with host cardiomyocytes [54].

Multiple clinical trials are now enrolling or have completed enrolling patients to study the use of cardiac stem cells to treat cardiomyopathy and ischemic heart disease. Intracoronary injection of CSCs demonstrated increased left ventricular ejection fraction and decreased infarct size in patients with ischemic cardiomyopathy [55, 56]. Animal studies showing the safety and efficacy of these cells showed no microembolism and very little retention of these cells in the heart after implantation, suggesting a possible paracrine mechanism of protection [57].

Cardiospheres have also been shown to play a positive role in myocardial repair. These cellular spheres, which range in size up to 150 μm, have been generated from heart biopsy specimens, and contain cardiac stem cells in the central core and other cardiac derived progenitors and differentiated cells at the periphery. The three dimensional construct of these cardiospheres reportedly protected the cardiac stem cells from oxidative stress and maintained their stem-like properties for longer periods of time [58]. The success of various *in vivo* studies using cardiospheres led to a phase 1 randomized controlled trial known as CADUCEUS. It showed a significant decrease in scar mass but no change in left ventricular ejection fraction [59].

Mechanism of Action

Stem cell therapy has shown remarkable potential in the treatment of end organ injury. Once stem cells are activated, they can either differentiate into end organ cell types, fuse with pre-existing cells, or secrete various bioactive factors that act in an autocrine or paracrine manner to attenuate damage following injury.

Cellular Differentiation

Stem cells can be isolated from almost every type of tissue in the body and possess a multipotent ability to differentiate into various cell lineages. This characteristic resulted in the initial hypothesis that upon entry into damaged tissue, transplanted stem cells engraft and differentiate into the phenotype of the injured tissue, thereby restoring the diseased organ with healthy, functioning cells.

While differentiation has been demonstrated for long term repair in animal models of acute myocardial infarction [60], acute renal failure [61], dermal wound repair [62], gastric perforation [63], and chronic lung injury [64, 65], the notion that stem cells consistently provide therapeutic efficacy via cellular differentiation is not completely convincing for the acute phase of injury. This is predominantly due to multiple studies that have shown therapeutic benefit in a very short period of time (minutes to hours), in which cellular differentiation could not have taken place [66, 67].

Heterotopic Cell Fusion

Heterotopic cell fusion involves the merging of two cells from different lineages. Inflammation triggers the migration of stem cells to areas of damage and stimulates an increase in the frequency of fusion events between stem cells and injured resident cells within the tissue [68]. The nucleus, functional genes, or ATP from the stromal cell is transferred into a degenerating cell, so as to protect and restore the dying cell's activity. Cell fusion reactions following administration of stem cells have been observed in multiple tissue types. It has been hypothesized that these newly formed multinucleated cells are preferentially selected for survival, able to withstand a greater degree of stress, and promote tissue homeostasis and regeneration [26].

Paracrine Mediators

Growing evidence favors the paracrine mechanism of stem cell-mediated organ protection. While cellular differentiation and heterotopic cell fusion events have been observed, the secretion of anti-inflammatory and proangiogenic factors by stem cells has been shown to be the most likely mechanism of protection. Numerous studies have suggested that applying cell free media that has been conditioned by stem cells provides equivalent protection as the cells themselves [69–73], suggesting that the paracrine factors produced by the cells in the media are what drive their therapeutic benefit. Additionally, the rate of transplanted stem cell engraftment and survival is so low that cellular differentiation and fusion events are likely too few to directly influence recovery of tissue function [74]. In addition, multiple studies have appreciated post-injury improvements in tissues located elsewhere from where stem cells engrafted, suggesting that stem cell homing to the actual site of damage is not mandatory for overall therapeutic benefit [75–77].

In addition to growth factor production, stem cells have been shown to secrete growth factor-containing exosomes into the extracellular milieu. The exosomes bind to target cells in the extracellular space and either alter target cell signaling or unload their vesicular contents into the target cell cytoplasm [78]. Studies analyzing the alterations generated by stem cell exosomal contents have observed enhanced angiogenesis and epithelial and endothelial wound healing in tissues [79, 80]. Therefore, the primary mechanism by which stem cells appear to combat the progression of tissue damage is via a multifactorial paracrine-mediated process, in which immunomodulation, tissue restoration, and reactive oxygen species scavenging occurs.

Immunomodulation

Stem cell paracrine mediators appear to play a role in the attenuation of the inflammatory response. The induction of transcription factor NF-kB following insult regulates the production of pro-inflammatory cytokines by activated macrophages and other cells of the innate immune system. Various studies have observed the capacity

of stem cells to decrease the activation of NF-kB in animal models of injury [81, 82]. Subsequently, stem cell therapy has been associated with decreased levels of proinflammatory cytokines, particularly TNF-α [82, 83], IL-1β [83], and IFN-γ [84]. These changes favor T cell differentiation into Th2 and T regulatory cell types, minimize inflammatory mediated intestinal destruction, and decrease the risk of systemic sepsis.

Studies have also observed increased production of anti-inflammatory cytokines in injured tissues treated with stem cells. One of the anti-inflammatory cytokines, IL-10, was observed to block NF-kB signaling, downregulate the expression of Th-1 inflammatory cytokines, and moderate COX-2 activation. While elevated IL-10 levels were initially believed to be made from the stem cells [85–87], studies now suggest that IL-10 may be produced by monocytes induced by stem cells [88]. Previous studies demonstrated that an elevation in stem cell derived IL-6 triggered the differentiation of monocytes to IL-10 secreting macrophages [89]. Stem cell immunomodulation in injury through increased production of anti-inflammatory cytokines and decreased synthesis of inflammatory mediators minimizes tissue damage and reduces the risk for multi-organ failure and death [26].

Tissue Restoration

Through the release of various bioactive factors, stem cells facilitate angiogenesis, inhibit apoptosis, alter the coagulation cascade [90], and stimulate resident stromal cells to proliferate and restore injured tissue. Upon entering hypoxic environments, stem cells upregulate mRNA expression of numerous growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2), and transforming growth factor-β (TGF-β), resulting in enhanced tissue restoration [91]. The upregulation in growth factor expression is believed to be mediated by a p38 mitogen-activated protein kinase (MAPK)-dependent mechanism [92].

Each growth factor modulates various parts of the healing process and their beneficial effects are multifactorial. VEGF and FGF2 are key promoters of angiogenesis following acute ischemia and inflammation [93–95], and appear to enhance stem cell survival upon transplantation [96, 97]. TGF-β appears to be a key mediator of tissue remodeling and enhances the expression of tight junction proteins which may limit cellular leak [98].

Anti-Oxidant Properties

While the reestablishment of blood flow to injured tissue is vital to salvaging the tissue, the generation of reactive oxygen species adds insult to injury. Stem cells have been observed to produce various antioxidants that work to fight the accumulation of these reactive oxygen species. Superoxide dismutase, catalase, and glutathione peroxidase are highly expressed by stem cells and work collectively to convert oxygen free radicals to water and oxygen [99, 100]. By scavenging oxygen radicals and converting them to innocuous particles, stem cells prevent further disruption of tissue barriers, decrease endothelial dysfunction and minimize inflammation.

Tissues Engineering Principles

Perhaps the most important potential application of human stem cells is the fabrication of tissues and organs that could be used for transplantation. Today, donated organs and tissues are often used to replace ailing or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Stem cells, which can be directed to differentiate into specific cell types, can be combined with unique tissue scaffolds in order to engineer tissues and organs for therapy.

Two main approaches are utilized to produce engineered tissue. First, scaffolding can be used as a cellular support mechanism for cells that are seeded onto the scaffold *in vitro*. Cells are then prompted to lay down a matrix that promotes tissue generation. This process usually occurs in a bioreactor, an enclosed *in vitro* system that mimics the native *in vivo* environment. The second approach to tissue engineering involves using the scaffold as a growth factor or drug delivery device. Once the scaffold and growth factors are implanted, cells from the host are recruited *in vivo* to seed the scaffold and form tissue [101].

Scaffolds

The principals of tissue engineering include the use of two main biological components: a cellular component capable of regeneration and a scaffold on which to seed the cellular component. The engineered graft must allow for ingrowth of native tissue and neovascularization, and ideally, would be biodegradable after neovascularization has occurred. In addition, the ideal scaffold material utilizes surface properties that promote cell adhesion, proliferation, and differentiation without inducing proinflammatory reactions after implantation [102, 103].

Scaffolds can be generically divided into those made of natural or synthetic materials. Natural materials typically include proteins and polysaccharides such as fibrin, hyaluronic acid, or collagen. Natural materials are usually biocompatible and biodegradable, mimic the native matrix, and can easily promote tissue ingrowth. However, there is the possible transfer of pathogens as the biomaterial usually comes from living donors. They also require modification or decellularization, and their degradation can be quick or even unpredictable. Synthetic materials, such as polyglycolic or poly-L-lactic acids, are typically composed of metals, ceramics, or polymers. These materials are easy to handle and have a controlled degradation pattern. Because they are not harvested from living tissues, they do not transmit pathogens. However, they often require special processing to promote cell attachment, and their degradation may yield unwanted, or even toxic byproducts [103].

Guiding Cells to Integrate

Cells can be guided to specific locations on the scaffolds using growth factors or polymerized/charged particles. For example, impregnating VEGF in the scaffold

has been shown to promote angiogenesis and vascularization of various scaffold materials [104]. Other groups have attached factors such as basic fibroblastic growth factors to the surfaces of alginate beads to provide a microenvironment that permitted the growth and development of neuronal stem cells [105].

Even more appealing is the opportunity to place concentration gradients onto polymers to guide cell incorporation or to control matrix ingrowth. This concept is known as "zoning" and allows for the regionalized release of specific factors to initiate specific physiological processes at specific sites on the scaffold. This concept was demonstrated in a study in which PLA/PEG microparticles were loaded with trypsin, BMP-2, or horseradish peroxidase. The particles were put in distinct layers on the scaffold and the BMP-2 particles were able to localize osteogenic differentiation of specific cells in vitro at that location [106].

In addition to the use of growth factors to create gradient zones of interest, the ability to change the adherence properties of the scaffold can allow for tight control of where on the graft cells incorporate. This can prevent aggregation of cells at the periphery and can promote better integration into the center of the scaffold. For example, deposition of plasma polymerized allyl amine allows stronger cell adherence, while deposition of plasma polymerized hexane strongly discourages cell adhesion and integration [107]. Generating gradients of surface chemistries in layers can promote cells to move from the non-adherent surface layer, to a more adherent core, thus encouraging 3D cellular incorporation to form the desired organ shape [101].

Bioreactors

Bioreactors are indispensable in cell and tissue based therapies. These devices are used to maintain well controlled environments and to regulate cell growth and tissue development. They provide strict and reproducible growth products to cells and facilitate removal of waste from cell cultures. They assist in controlling the spatiotemporal delivery of the various biochemical and biophysical stimuli that are essential to tissue engineering [108].

Cell expansion bioreactors are becoming more prevalent as treatments for a variety of clinical diseases turn to cell-based therapies. Given that large numbers of allogeneic cells will be needed for these applications and the cost will likely be too high for traditional hospitals, many are turning to centralized commercial facilities to develop Good Medical Practice products. Therefore, a demand has risen for industrial scale, high density suspension culture reactors that are able to generate homogenous populations of specific lineage specific cells.

The ability of cells to adhere to surfaces or grow in suspension affects the type of bioreactor needed. For adherent cells, suspension cultures can be achieved by the use of hollow fibers in perfusion systems, encapsulation, or microspheres, which are able to increase the surface area of the bioreactor and increase production [109]. Challenges in these sectors though include assuring homogenous distribution of growth factors and nutrients to all cells and adequate removal of waste to prevent

cell death and differentiation. Cells without needs of adherence can be grown in rotating flasks, rotating wall, or stirred-tank bioreactors [110].

Organ-on-a-chip bioreactors use minute quantities of cells grown in micrometer scaled wells to test various drug and tissue interactions. In addition, these technologically advanced bioreactors have incorporated physiologic factors such as airflow, with mechanical factors associated with breathing [111, 112], or vasculature integration and blood flow with cardiac contractile cells [113]. These more accurately capture native physiological effects while retaining the ability to study these cells *in vitro*.

3D Printers

Bioprinting provides an economical and reproducible method to create spatially organized and mechanically stable biological constructs that mimic the natural organization of the native organ. Multiple types of printers and inks are available. Common printers include inkjet, laser induced forward transfer (LIFT), and extrusion printing [114] (Fig. 15.3).

In inkjet printing (Fig. 15.3a), a cartridge is filled with biomaterial (cellular or polymer) and applied in droplet format to the stage. Pressure pulses created by electrically heating the print head or by applying sound waves to the print head eject the

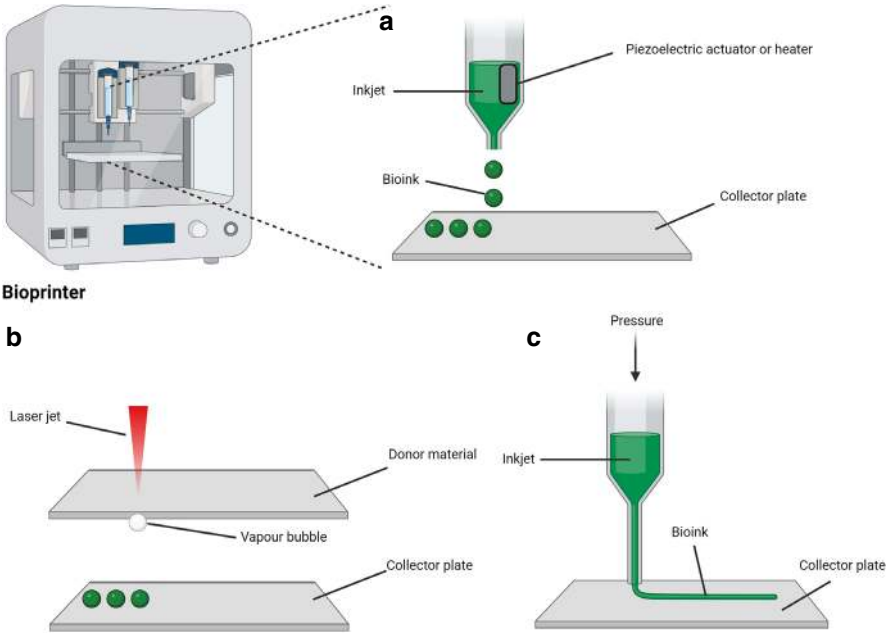

Fig. 15.3 **3D Printers**: (**a**) Inkjet printing, (**b**) Laser induced forward transfer, and (**c**) Extrusion printing

droplets. Printing resolution can be fairly high, but the changes in temperature and shear forces associated with bioink dispersal can limit the types of cells that are utilized. LIFT technology (Fig. 15.3b) uses a laser to excite a donor layer ("the ink") which vaporizes this layer and allows it to cascade onto the collector plate [115]. Finally, in extrusion printing (Fig. 15.3c), the bioprinter dispenses continuous cylindrical strands of hydrogels by using air or pneumatic force. This is equivalent to squeezing icing onto a cake. Mechanical extrusion printers use piston or screw mechanisms to project the hydrogel. These types of printers allow a vast array of ink viscosities and the cell densities can be fairly high [116]. Its resolution is a bit lower than other modalities and the impact of the shear stress on the cells/ink needs to be considered when designing the graft.

Modern 3D printing machines allow for the fabrication of complex multicellular tissues and organs secondary to their ability to incorporate multiple print heads that are loaded with different biomaterials. Several groups have used varying approaches to fabricate living organs such as ovaries, bone grafts, aortic valves, and liver tissue. Despite these successes, the ability to fabricate a life sized, fully functional human organ is still in its infancy [117].

Conclusion

Regenerative medicine has become a robust and promising area of study, particularly over the last decade. Use of pluripotent cells as therapy for disease brings new light to the treatment of many diseases that previously had no cure. In addition, the potential application of tissue engineered grafts is limitless considering organ shortage and immunosuppression associated with transplantation. Bioreactors and 3D printers are components of advanced technology that will allow tissue engineering to become more mainstream in the near future.

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Animal Models in Surgical Research

16

Michelle M. Holland, Allan M. Goldstein, and J. Bart Rose

Abstract

Scientists have long utilized model organisms to answer fundamental questions about biology. Zebrafish, rodents, swine, and primates represent just a few of the many models commonly leveraged for biomedical research. They are especially important in translational research and advancing our knowledge of human disease. For surgeon-scientists, modeling the conditions that affect our patients is essential to elucidating the underlying pathophysiology and to discovering novel therapies. Familiarity with the various model organisms available and their respective strengths is therefore essential so that the best system is selected to answer a scientific question. Here we present a selection of model organisms and the uses, drawbacks, and practical aspects thereof to provide a brief review for the surgeon-scientist.

Keywords

Animal models · Translational research · Chicken · Tree shrew · Zebrafish · Rodents · Swine · Sheep · Primates · Xenotransplant · Basic science

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Introduction

For centuries, scientists have used model organisms to answer fundamental questions about human biology. Model organisms including yeast, worms, flies, teleosts, avians, rodents, and primates represent basic tools for biomedical research that are critically important in advancing our knowledge of human health. For surgeon-scientists, modeling the conditions that affect our patients is essential to elucidating the underlying pathophysiology and to discovering novel therapies. Familiarity with the various model organisms available and their respective strengths is therefore essential so that the best system is selected to answer a scientific question. Factors to consider in selecting one model organism over another might include the ease and cost of housing and maintenance; number of animals required; ability to perform genetic studies, surgical procedures, drug screens, or other applications; and relevance of the biology and pathology to human physiology and disease.

Surgeons routinely leverage zebrafish, chick, rodent, swine, and other species to advance surgical knowledge. But, while these models have advanced our understanding of human disease and enabled powerful discoveries, they also have limitations, which partly explains why many promising results have failed to translate when applied to humans. Here we present a selection of model organisms and the uses, drawbacks, and practical aspects thereof to provide a brief review for the surgeon-scientist. We hope that this will inspire investigators to utilize animal models in their research, as the complexity of these organisms provides a rich context in which to study the pathophysiology of disease and to discover new therapies.

Chicken

Expense: $.
PROS: Easy embryologic manipulations, xenografting.
CONS: Poor human translation, difficult transgenics, scarce species-specific antibodies, non-mammalian.

Many experimental animal models have contributed significantly to our understanding of embryonic development, including chick, zebrafish, drosophila, xenopus, mouse, and *C. elegans*. While each of these has advanced the field of developmental biology, the chick (*Gallus gallus*) embryo has been particularly important for deciphering many fundamental aspects of vertebrate development. The avian embryo is an attractive model for multiple reasons: (1) it is a vertebrate, (2) its husbandry is simple and inexpensive, requiring only an egg incubator that regulates temperature and humidity, and (3) embryos are readily accessible for experimental manipulation by windowing the egg and then sealing it closed with clear tape. The chick embryo has a long history as a model organism and served as the basis of some of the most fundamental discoveries in development, including formation of the germ layers, migration and fate of neural crest cells, cellular movements associated with

gastrulation, discovery of B-lymphocytes, limb bud patterning, and the initial steps leading to left-right asymmetry [1].

One of the major benefits of the avian model is the ability to perform many different experimental molecular and surgical manipulations on the developing embryo *in ovo*. The chick can be used for classic embryologic manipulations, such as cell lineage experiments, targeted tissue ablation, generation of chick-quail chimeras, chorioallantoic membrane grafting, and tissue transplantation into the coelomic cavity. Furthermore, germ line transgenics and transient genetic inhibition or misexpression are an important part of the avian embryo armamentarium. Efficient gene overexpression can be accomplished by injection of a virus expressing the desired transgene or by delivery of a plasmid followed by electroporation, in which an electrical field is applied across a region or tissue, causing the cell membranes to become permeable and to take up the DNA [2]. These methods allow overexpression or ectopic expression of a gene, or its inhibition using, for example, dominant-negative constructs or small interfering RNA (siRNA).

From the surgeon's perspective, the avian embryo has allowed important advances in our understanding of congenital diseases. For example, studies in chick have revealed many of the cellular and molecular processes governing development of the enteric nervous system and helped elucidate the causes of Hirschsprung disease [3]. The chick model has also been of great value to cardiovascular research, both in elucidating the mechanisms of angiogenesis and the identification of antiangiogenic compounds as well as in characterizing the development of the coronary vessels, heart valves, and outflow tract [4, 5]. Given the close relationship between mechanisms underlying development and cancer, it is not surprising that the chick embryo has been useful in cancer biology research. The chick's highly vascularized and easily accessible chorioallantoic membrane, which readily accepts xenografted cells, offers an ideal environment to study tumor growth and metastasis, to define gene regulation during tumor progression, and to perform drug screens for novel anticancer agents [6, 7]. Surgical investigators should consider how they can exploit the many strengths of the avian model in their research programs, bearing in mind the potential drawbacks of any model system. In the case of the avian system, these include the difficulty in producing transgenic chickens, the challenge of housing them post-hatching, and the relative scarcity of species-specific antibodies and other reagents as compared to rodents.

Tree Shrew

Expense: $.
PROS: Mammalian, human translation.
CONS: Lack of inbred lines, novel model.

The tree shrew (*Tupaia belangeri*), a squirrel-like mammal from Southeast Asia, is emerging as a relatively new model for a variety of benign diseases and cancer. The tree shrew combines advantages from the non-human primate (NHP), swine, and

rodent models, while simultaneously mitigating some of the limitations of these models. Similar to rodents, tree shrews have short reproductive and life cycles, high reproductivity (4–6 months from birth to adulthood, with 2–6 offspring per litter), and moderate size (adult weight 120–150 g), which make them an ideal cost-effective mammal for biomedical research [8]. However, the significant genetic differences between rodents and humans, which makes clinical translation often difficult, is not seen with the tree shrew. Studies have demonstrated that the tree shew is more closely related to primates and exhibits pathological, physiological, and immune responses similar to those of humans [9]. Moreover, genome sequencing reiterated this connection by showing that the nervous, immune, and metabolic systems of the tree shrew are very close to those of humans and non-human primates [10].

Tree shrews have historically been used to study viral infection, particularly HBV and HCV, since the only other existing natural-infection animal model for these is the chimpanzee. Recently, cancer models are starting to emerge, including hepatocellular carcinoma, glioblastoma, breast cancer, lung cancer, basal cell carcinoma, and pancreatic ductal adenocarcinoma (PDAC) [11]. With regards to PDAC, the tree shrew is able to reproduce human biological features that have been previously unachievable in rodent models [12].

In short, compared to rodents, the tree shrew is more similar to humans and NHPs with respect to pathologic, immunologic, and physiologic responses. At the same time, they avoid the major obstacles of breeding, domestication, handling and ethics seen with NHPs. However, standardization of experiments is currently difficult due to the lack of inbred tree shrew strains. This lack of defined genetic background can introduce unwanted variability, particularly problematic with cancer modelling. Additionally, gene knockout techniques have yet to be applied to this novel animal model. Though still early in its utilization and adoption as an animal model, the tree shrew has shown promise as a tool for the study of human diseases.

Zebrafish

Expense: $$.
PROS: Short life cycle, high reproduction, transparent embryos, established transgenics, mutant strains.
CONS: Poor human translation, non-mammalian.

Zebrafish (*Danio rerio*) is a commonly used model organism which, in the 1990s, became the first vertebrate species in which large-scale mutagenesis screens were performed, leading to the discovery of many genes associated with organ formation and function [13]. The zebrafish is an appealing model organism for many reasons: (1) short generation time (sexually mature at 3 months), spawn frequently, and produce hundreds of offspring; (2) development occurs outside the mother and embryos are optically transparent, allowing visualization of internal structures in the living animal; (3) well-established techniques for modulating gene activity *in vivo*,

including generating transgenic animals; and (4) many mutant strains are available [14]. The combination of a short life cycle and large clutch sizes makes zebrafish powerful models for genetic research. Genes can be overexpressed by injecting mRNA at the one-cell stage, or they can be knocked down using morpholino oligonucleotides, which bind to complementary RNA sequences to inhibit their expression. Knockout zebrafish can be produced using genome editing techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR) or transcription activator-like effector nuclease system (TALEN).

For the surgeon-scientist studying developmental biology, the zebrafish has much to offer. Zebrafish have uncovered important knowledge about the development of the structure and function of the heart, ranging from early cardiac mesoderm specification to formation of the valves and conduction system [15]. Similarly, the zebrafish has taught us about the genetic, molecular, and cellular events that guide craniofacial development and the formation of orofacial clefts [16, 17]. Zebrafish have also contributed to the study of melanoma due to the availability of abnormally pigmented mutant lines that contain defects in melanocyte differentiation or function, including genetic models of zebrafish with melanoma [18]. Taking advantage of the transparency and immunoprivileged environment of the zebrafish embryo, and the fact that many cancers recapitulate embryologic processes, investigators have used fish for *in vivo* cancer studies and high-throughput chemical screens to identify new anti-oncogenic compounds [19]. Importantly, zebrafish research is not limited to the study of embryos. Transparent adult zebrafish have been produced, and this allows introduction of fluorescently labeled stem cells or tumor cells and the ability to follow *in vivo* their engraftment, proliferation, differentiation, and, in the case of cancer, metastasis. Zebrafish are also an excellent vertebrate model for high-throughput drug screening and are often used for this purpose [20].

Rodents

Expense: S$.
PROS: Mammalian, transgenics, knockout strains, high reproduction.
CONS: Poor human translation, difficulty generating transgenics in rats.

Mus musculus, the house mouse, is the most widely used model system in biological research. As a mammal, it shares anatomic and physiologic similarities to humans and is an attractive model for translational research. The mouse has been indispensable in the study of gene function *in vivo*, and the mouse genome has been extensively sequenced, studied, and manipulated. Genetically modified mouse strains are readily available from repositories at Jackson Laboratory and Charles River Laboratories, and new strains are routinely under development. The NIH knockout mouse project (KOMP) aims to create genetically targeted knockout strains for every gene in the mouse genome, enabling a multitude of discoveries [21]. The manipulability of the mouse genome has become even more facile with the

development of the CRISPR-Cas9 system, as mentioned above, wherein a guide RNA targets site-directed genomic modification, allowing researchers to manipulate one or more genes in an animal [22]. Tissue-specific driver lines and inducible cassettes also allow cell-type-specific labeling and timed targeting of molecular effectors [23].

The contributions of mouse research to the understanding of human biology are too numerous to count. Of interest to surgeons, for example, the mouse provides an accessible mammalian system for the study of trauma. The molecular underpinnings of neuronal cell death following traumatic brain injury and concussion have been investigated [24]. Surgeons have also studied the metabolic response to bariatric surgery in mice and even created a novel method of coating the bowel lumen to mimic the effects of weight-loss surgery [25, 26]. Unfortunately, many promising results in mice have not translated to human biology. One must be cognizant of the many evolutionary divergences between rodent and human biology and interpret data carefully before extrapolating results directly to human disease. For example, rodent models of sepsis have yielded confounding results, likely due to fundamental differences in the biologic response to infection, including for example significant differential cellular responses to LPS between mice and humans. Unfortunately, multiple clinical trials for sepsis utilizing targets identified in animal models have failed to yield promising results [27].

Though use of *Mus musculus* currently is predominant, the rat (*Rattus norvegicus*) was once the most commonly used model organism in laboratory research. An outbred albino strain, Sprague Dawley, is frequently used because of its calm demeanor and history of extensive study. Because of their larger size, rats are better suited to surgical procedures and repeat blood draws for serial analysis. Some aspects of rat biology more closely mimic human biology when compared to their murine counterparts. Important contributions to the field of hormone-responsive breast tumors have been made using rat models of breast cancer, as induced mammary tumors in mice are almost exclusively ER-negative [28]. Detailed protocols for many surgical procedures are available for the intrepid surgical researcher, including donor and partial auxiliary liver transplantation and bariatric procedures [29, 30]. The difficulty of genomic engineering in rats, however, has limited their use in transgenic studies.

Swine

Expense: $$$.
PROS: Mammalian, human translation, short generation times, large litters, editable genomes.
CONS: Increased need for space, feed, and specific management protocols.

Domesticated pigs (*Sus scrofa domesticus*), which are readily obtained given their widespread use in the food industry, are genetically and anatomically very similar to humans. Indeed, porcine valves have been used for over 30 years in human heart

valve replacement. Porcine models have gained momentum over the past several years since public acceptance of the use of dog and primate models has declined. Pig has largely replaced dog as a model for surgical training and for testing surgical devices or developing new surgical procedures prior to human use. Porcine models for deliberate practice have been developed for laparoscopic surgery, natural orifice transluminal endoscopic surgery (NOTES), trauma, pediatric surgery, cardiac surgery, and more. For the surgeon-scientist wishing to experiment in large animals with established surgical protocols, the domesticated pig provides an excellent model. A variety of breeds are available for use, including outbred and purebred strains and pigs of different ages and sizes.

Pig skin bears many similarities to human skin with respect to thickness and density of hair follicles and has therefore been widely used as a model of human wound healing [31]. Rodent models fall short in these studies due to their thin epidermis and dense fur. Many skin models relevant for surgeons have been developed in pigs, including models of diabetic wounds, hypertrophic scars, and burns [32]. As an example, the timing of excision and grafting in acute deep dermal burns was tested in a pig model in a study that established early excision at postburn day 3 (versus 14 or 21) as being superior for avoiding hypertrophic scarring [33].

As in primates, pigs are also important models in the study of transplantation, which is further expounded upon in the xenotransplantation section below. They are also used as models of pediatric brain trauma [34]. In short, the porcine model has allowed for great advances in surgical research, but must be balanced with the increased costs associated with housing and feeding these large animals. While established management protocols are typically beneficial for scientists, the strict and specific swine protocols limit flexibility in study design, which should be taken into consideration when in the preliminary stages of experimental planning.

Sheep

Expense: $$$.
PROS: Mammalian, similar to human anatomy, easier to maintain compared to swine and primates.
CONS: Transgenics, poor human translation.

Sheep are a particularly useful model with several distinct advantages, including the fact that they are easier and cheaper to maintain than primates and easier to handle than swine. For pediatric surgeons, the fetal lamb is similar in size to human fetuses and amenable to *in utero* manipulation, which has made it a valuable model for fetal surgery. Surgeon-scientists have studied *in utero* repair of myelomeningocele using either open or endoscopic techniques in lambs, and these studies have led to human clinical trials showing significant improvement in outcomes [35]. Fetal lambs are also commonly used as a model of congenital diaphragmatic hernia (CDH) as they are large enough to create a diaphragmatic defect, undergo fetal repair, and receive mechanical ventilator support [36]. These ovine studies led to the clinical use of

fetal tracheal occlusion for high-risk CDH patients. Recent work used the lamb to model extreme prematurity and demonstrated the ability to support these animals outside of the womb using an innovative *ex utero* system that includes a pumpless arteriovenous circuit and a closed amniotic fluid environment [37]. Aside from pediatric surgery, sheep are also useful in other fields of surgery, including vascular surgical research, because of their similar vascular anatomy, biology, and pathophysiology [38].

Non-human Primates

Expense: $$$$.
PROS: Mammalian, human translation.
CONS: Ethically fraught, long life cycle, low reproduction.

The close evolutionary relationship of non-human primates (NHP) to humans, and their highly similar physiology, allows unparalleled insight into topics of critical importance in many fields of medicine, including infectious disease, reproduction, neuroscience, and immunology. These attributes have facilitated important discoveries, such as the identification of CD4+ T cells as a primary target of HIV infection [39]. Cynomolgus and rhesus macaques are most frequently utilized for animal studies, but several other species are also commonly used across North America [40]. For preclinical testing, few models offer better insight into human biology. Many of the advantages of using NHP, however, are the very reasons why experimental research on NHP is ethically fraught. The well-developed cognition and sophisticated socialization of NHP naturally evokes concerns about misuse. Debates regarding the need for the use of NHP in biomedical research have led to attempts to reduce and refine the use of NHP, with primates comprising less than 0.3% of experimental animals in the US [41]. A few high-profile events, including the closure of the New England Primate Research Center—one of the premier primate research centers in the country—reignited this ongoing discussion [41]. Working with NHP requires a high level of individualized care, careful consideration of the impact of experimental manipulations on the well-being of the study animals, a sophisticated facility, and an attentive and well-trained staff.

NHP models have played an indispensable role in the field of organ transplantation. Research that targets minimizing the use of immunosuppression is of great interest clinically, as these medications cause toxicity and potentially fatal complications. Recently, durable graft survival without prolonged immunosuppression has been observed in NHP following renal allografts with delayed bone marrow transplantation [42]. Discussion regarding the NHP model for human xenotransplantation, however, has been replaced with the swine model due to limited feasibility of a large scale xenotransplant program using primates.

Another clinical area where primate models are unmatched is in the study of the brain. Given the similarity between the human and primate brains, models of surgical and medical treatments are more likely to be aligned. Neurosurgeons have used

NHP to study complex neurocognitive development and to pioneer possible new avenues of treatment, including in the area of deep brain stimulation. For example, primates are used to study the effects of deep brain stimulation on a multitude of cognitive tasks, including associative learning [43]. The treatment of hyperkinetic conditions and other movement disorders such as Parkinson's disease has also benefitted from the use of NHP models. Our understanding of the basal ganglia circuitry in the brain comes from data collected in NHP [44]. In sum, while researchers must carefully consider the circumstances for which the use of non-human primate models is appropriate, some questions are best answered in this model.

Xenotransplantation

Currently, there are over 100,000 people in the United States on an organ transplant waitlist, with 17 people dying each day waiting for an organ according to the Health Resources & Service Administration. Moreover only 46,000 transplants were performed in 2023 in the United States; 28,000 of which were organs from deceased donors. According to the World Health Organization, only 10% of the global demand for organ transplants is being filled. Though not a new concept, xenotransplantation was less of a focus in the past two decades due to failure of previous xenotransplant attempts from the 1960s to 1980s as well as zoonotic concerns.

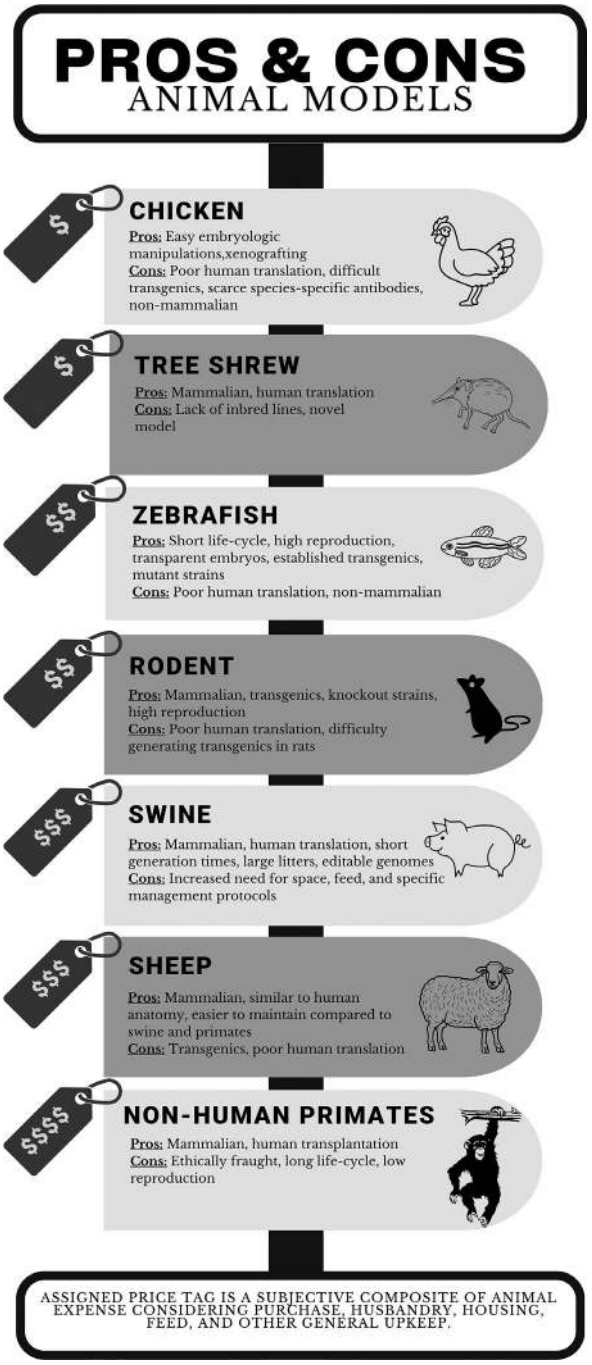
With the arrival of CRISPR technology, successful use of porcine heart valves, and advances in transplant science, discussions regarding solid organ xenotransplantation have been renewed in the biomedical field with the swine and NHP models predominating. Furthermore, the critical shortage of organs available for transplantation underscores the importance of developing alternative methods of providing organs for patients in need. Pig-to-primate transplantation studies aim to induce immune tolerance of xenografts through simultaneous transplantation of additional solid organs, such as the thymus, or via mixed hematopoietic chimerism with bone marrow transplants [45]. Primates, as our closest phylogenetic relatives, provide the best model for study, but are overarchingly not feasible due to the inability to source enough organs from NHPs, making swine models more accepted [46].

With regards to xenotransplant in the swine model, cardiac allograft tolerance has been studied extensively, and studies would suggest that co-transplantation with other organs may enhance tolerance of allografts [47, 48]. In tissue engineering, porcine organs have been decellularized and used as scaffolds that are recellularized with autologously derived cells to generate organs for transplantation [49]. Xenotransplantation of pig organs to human recipients holds a great deal of promise, but faces hurdles that include potential immunological barriers and concern for infection of human cells by porcine endogenous retroviruses (PERVs) which are scattered in the swine genome. Recently, CRISPR was successfully used to inactivate PERVs in a genome-wide fashion from porcine cells *in vitro*, and then, via somatic cell nuclear transfer, PERV-inactivated animals were generated. This advancement opened the door for using PERV-inactivated swine organs for transplantation in the future [50]. Currently, the scientific community has witnessed

several xenotransplants of pig kidneys to humans who were declared brain dead, and transplantation of a pig heart to a patient who was not a candidate for allotransplantation, although results have been variable [51].

Conclusion

Animal models are a critical component of translational research. By modeling human biology and pathology, these model systems allow us to explore molecular and cellular processes and to discover and test novel diagnostic and therapeutic strategies. While many investigators use a single model system, taking advantage of multiple animal models can be a very useful approach. For example, a surgeon studying a congenital anomaly may exploit the strengths of the zebrafish for a mutagenesis screen to identify causative mutations, the mouse embryo to study the role of that gene during mammalian development, and ultimately a large animal like the lamb to test innovative approaches for treating that congenital defect. By understanding the uses, advantages, and limitations of each animal model, the surgeon-scientist can leverage their respective strengths to perform clinically relevant and impactful translational research.


PROS & CONS
ANIMAL MODELS
$
CHICKEN
Pros: Easy embryologic manipulations,xenografting
Cons: Poor human translation, difficult transgenics, scarce species-specific antibodies, non-mammalian
$
TREE SHREW
Pros: Mammalian, human translation
Cons: Lack of inbred lines, novel model
$$
ZEBRAFISH
Pros: Short life-cycle, high reproduction, transparent embryos, established transgenics, mutant strains
Cons: Poor human translation, non-mammalian
$$
RODENT
Pros: Mammalian, transgenics, knockout strains, high reproduction
Cons: Poor human translation, difficulty generating transgenics in rats
$$$
SWINE
Pros: Mammalian, human translation, short generation times, large litters, editable genomes
Cons: Increased need for space, feed, and specific management protocols
$$$
SHEEP
Pros: Mammalian, similar to human anatomy, easier to maintain compared to swine and primates
Cons: Transgenics, poor human translation
$$$$
NON-HUMAN PRIMATES
Pros: Mammalian, human transplantation
Cons: Ethically fraught, long life-cycle, low reproduction
ASSIGNED PRICE TAG IS A SUBJECTIVE COMPOSITE OF ANIMAL EXPENSE CONSIDERING PURCHASE, HUSBANDRY, HOUSING, FEED, AND OTHER GENERAL UPKEEP.

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Overview of Advances in Live Animal Imaging

17

Kirsten Remmert, Jonathan M. Hernandez, and Thinzar M. Lwin

Abstract

Modern multi-modal imaging has transformed our ability to observe and understand biological processes in living systems. This chapter explores key imaging modalities that have enabled unprecedented visualization of in-vivo processes, including ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET), as well as optical imaging approaches such as fluorescence and bioluminescence. We examine how these tools enable unprecedented visualization of in-vivo processes. We explore the integration of imaging technologies and experimental model systems - from invertebrates to mammals - highlighting their role in bridging laboratory research with clinical applications. Attention is also given to emerging platforms such as organoids and organ-on-chip platforms that offer alternative approaches to tradi-

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tional animal studies. Versatile understanding of the technical considerations, limitations, and cost-effectiveness of these tools will continue to provide valuable insights into anatomical, functional, and molecular aspects of diverse biological processes.

Keywords

Live animal imaging · Imaging modalities · Animal models · Alternatives to animal imaging

Introduction

The fundamental objective of scientific research is to expand our understanding of disease states through systematic investigation. Animal models with live imaging provides an important avenue for the study of disease in complex biological systems. Importantly, live imaging allows for visualization of processes that more closely approximate the human, and bridges the gap between basic laboratory research and clinical applications to bring us closer to translational relevance. As surgeon-scientists it is important to have a versatile understanding of the applications, strengths, and limitations of available techniques for models and read-outs to effectively utilize them.

Imaging Technologies

Animal imaging encompasses a diverse array of techniques, but careful consideration must go into the selection of the appropriate imaging modality. The scale of the process to be studied, resolution and penetration required, as well as the time required to visualize the process vary significantly among different imaging modalities (Fig. 17.1: Imaging modalities and scale of imaging [1]). Each technique comes with limitations that one should be aware off when designing an experimental workflow.

Among the most widely used imaging modalities are ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and optical imaging including fluorescence and bioluminescence. These modalities provide powerful tools to visualize molecular and cellular events to enhance our understanding of biologic processes for translational applications. One or more of these approaches can be combined to overcome the limitations of a given technique to maximize the knowledge gained from each model system.

Ultrasound (US)

This approach uses high frequency sound waves that echo off-of the subject of interest [2]. The captured signal creates dynamic images, and the information has been used to monitor development, assess disease progression and studying

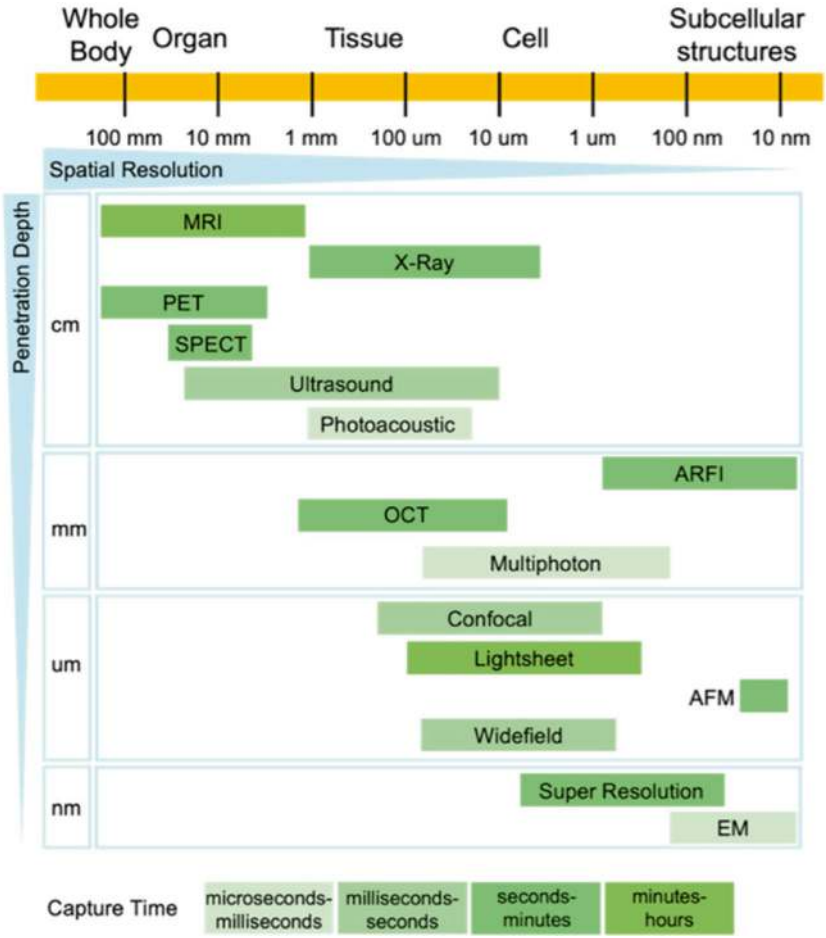

Fig. 17.1 Imaging modalities and scale of imaging. (Adapted from Huang et al. [1])

cardiovascular function. Ultrasound is non-invasive, cost effective, and rapidly provides real-time data of soft tissue, organs, and circulation. There is no need for ionizing radiation and the method can be repeated over time which provides longitudinal data across subjects. However, it is operator dependent, introduces user variability, and has limited resolution at very shallow and deep tissue depths. At shallow depths, the sound waves are very close to the transducer due to the need for appropriate acoustic coupling, but higher frequency ultrasound waves can be used to improve resolution [3]. At increased tissue depths, ultrasound waves start to lose energy due to absorption, scattering, and reflection from surrounding tissue. This reduces the strength of returning echoes at the detector, resulting in decreased image quality at increased depth [4]. Advanced in ultrasound technology are being developed to facilitate live small animal imaging. Photoacoustic imaging, a technique which combines ultrasound with light (usually in the form of lasers), is being used in

research settings to provide higher-resolution images as well as provide therapy, offering new insights into tissue composition and function [5]. Micro-bubble based contrast agents are enhancing the resolution of the images acquired using ultrasound [6]. 3-D whole animal ultrasound is being developed to decrease user variability. These advancements provide a valuable opportunity to use ultrasound in small animals and enhance the specificity and physiology information obtained from each experiment.

Computed Tomography (CT)

This approach uses X-rays at multiple different angles that pass through the subject of interest, collected by the detector to reconstruct 2-D or 3D images. CT provides non-invasive, rapid, precise, high-resolution images, and can image through bony structures and or implants [7]. CT has been used to image dental, skeletal, and neurologic structures. Micro scale CT scanners, providing higher spatial resolution (voxel size <= 100 μm) have been developed for imaging small animal models of disease [8]. However, the cost of imaging equipment is high, exposes the subject to ionizing radiation, and has limited soft tissue contrast and may require administration of exogenously administered contrast agents which can add to the difficulty in conducting studies [9].

Magnetic Resonance Imaging (MRI)

This approach uses radiofrequency pulses within magnetic fields to excite protons from a subject of interest and captures the signal emitted when the protons realign. MRI provides detailed, high-resolution images of different types of soft tissue, joints, and inflammation without the use of ionizing radiation. MRI has further advantages in that it can provide functional and physiologic data with information on blood flow, oxygenation, and activity which been used to neurologic [10] and inflammatory disease processes [11]. However, MRI is costly, even more so than CT, requires specialized facilities (shielded to protect from magnetic interferences), takes a prolonged time period to conduct (40–50 min vs. less than 1 min for CT), and requires subjects to remain completely still to obtain a clear image which have limited its widespread use [12].

Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT)

This approach detects emitted positrons from administered radiopharmaceuticals labeled with a radioactive isotope. PET relies on detection of positions from radiotracer decay while SPECT detect gamma rays [13, 14]. These approaches are valuable for their ability to visualize metabolic and functional processes at a molecular

level with the ability to quantitatively measure biologic processes, offering data on pharmacokinetics and biodistribution of agents [15]. The techniques are highly sensitive, with the ability to detect minute amounts of tracers. However, the scans are costly, require specific radioactive probes that are tailored to the experiment at hand, require specialized interpretation, and spatial resolution of the images are lower as compared to CT or MRI [16]. Commonly used radionuclides available for PET (^{18}F, ^{64}Cu, ^{68}Ga, ^{124}I) or SPECT imaging (^{99m}Tc, ^{111}In, $^{123/125}$I, ^{68}Ga) in patient care are also available for live animal imaging. PET and SPECT imaging is often performed in combination with CT or MRI to obtain anatomic co-registration and optimize the information obtained.

Optical Imaging

This approach uses the detection of light energy (photons) from a subject of interest to construct an image. Fluorescent molecules require energy transfer from photons for excitation and emission. Bioluminescence on the other hand does not require external light energy for excitation since the light is produced biochemically. These agents either directly conjugated to a target molecule or genetically encoded within a gene of interest. Advantages of optical imaging include high sensitivity, high resolution, detection of biologic processes at a molecular level, the ability to quantitatively measure the signal, and cost-effective imaging strategy without the use of radioactivity. Limitations of optical imaging include tissue depth penetration (only up to few mm), making it challenging to image deeper internal structures, subject to photobleaching and autofluorescence, and the necessity of external agents or genetic modification to obtain a signal. Despite its drawbacks, it is highly effective and efficient, and thus often utilized for live animal imaging research.

Bioluminescence refers to the production and emission of light that takes place in living organisms. It is the result of a biochemical reaction where a light-emitting molecule (a luciferin) reacts with an enzyme (a luciferase) in the presence of oxygen and other co-factors to produce light energy [17]. There is a diversity array of luciferin and luciferase combinations based on the biologic system, leading to variations in color and intensity of the light produced (ie: firefly, jellyfish, dinoflagellate, fungi, and bacteria). The genes for expression of the luciferase enzymes are genetically engineered into living organisms such that their expression can be detected using sensitive cameras in the presence of luciferin and other cofactors at a cellular and macroscopic level [18]. This permits observation of gene expression, cellular localization, disease progression, and therapeutic efficacy within live animals. As compared to fluorescence, bioluminescence signal is produced only by the specific chemical reaction which comparatively decreases signal from off-target signal resulting in a high signal-to-noise ratio and increases the sensitivity of detection [19]. However, the reaction requires that the substrate (luciferin) and co-factors reach the cells or tissue-of-interest to produce the reaction [20]. This leads to a limited spatial and temporal resolution for bioluminescence.

Fluorescence is the emission of light by a substance (the fluorophore) that has absorbed light energy. Like bioluminescence imaging, fluorescence permits observation of dynamic processes in living organisms, but compared to bioluminescence which can only be obtained by genetically engineering, a fluorescence signal can arise through endogenous or exogenous approaches. Endogenous expression of fluorescence arises from genes encoding the expression of fluorescent proteins such as the green fluorescent protein (GFP) [21] which can be engineered into cells to track biologic processes like that of the gene encoding for luciferin. Fluorescent proteins are available in multiple colors (red [22], yellow [23], orange [24], cyan [25]) and endogenous expression of different fluorescent proteins can be used simultaneously to image multiple cell types or tissue structures within the same animal. Further, a fluorescence signal at the target of interest can be obtained through exogenous administration of a fluorescent agent. Fluorescent dyes can be linked to probes to permit detection of a wide array of targets. Fluorescent dyes can be linked to quenchers and enzymes to permit the study of dynamic molecular processes [26]. The probes can be multiplexed and can be detected at very low concentrations (pico- to femto-molar), allowing for highly sensitive imaging at both the macroscopic and microscopic level. While fluorescent signals are brighter than bioluminescent signals, there are very low levels of auto luminescence, which results in an improved contrast with bioluminescent imaging [27, 28]. Given that a fluorescence signal requires excitation by an external light source, there is increased background signal from tissue auto-fluorescence [29]. In fluorescence imaging, the tissue depth penetration of visible wavelength fluorophores is limited to several mm, but the use of near-infrared fluorophores has led to improved tissue penetration, allowing for deeper depth of tissue imaging in live animals. Fluorescence has advantages in that signal detection is rapid (signal acquisition is in seconds, rather than minutes for bioluminescence). Surgical implantation of tissue fragments in ectopic or orthotopic locations combined with fluorescent reporters or probes have permitted detailed studies of oncologic processes (Fig. 17.2). There are strong correlations demonstrated between photon emission and target cell number, permitting quantitative monitoring of biologic processes [30].

Knowing the relative strengths and drawbacks of each imaging modality is important in guiding selection. These imaging technologies provide a valuable, non-invasive means to study complex biological processes longitudinally, reducing the need for euthanasia and allowing for more humane and ethical research practices.

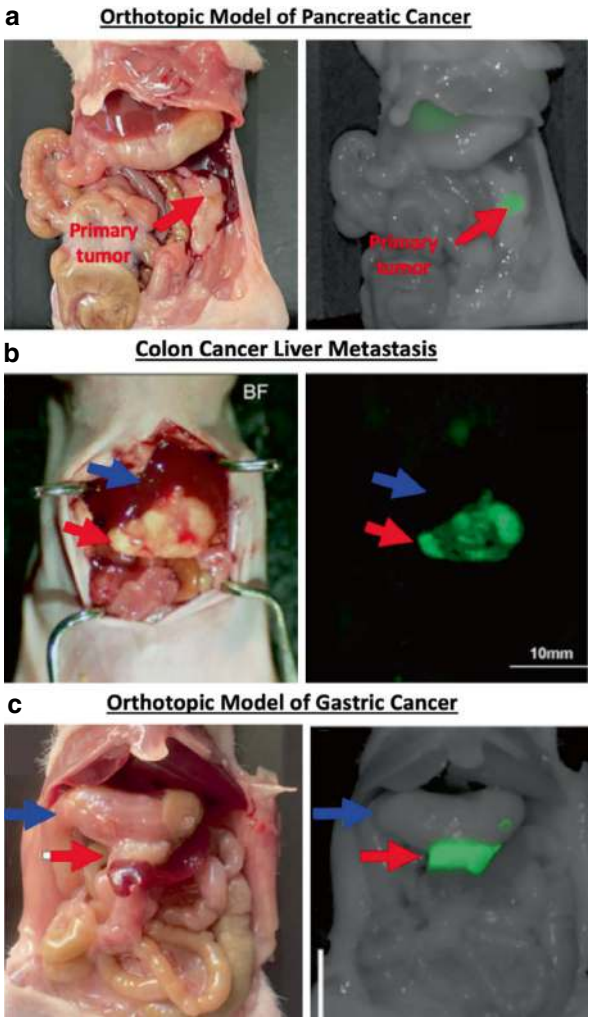

Fig. 17.2 Optical imaging of surgically implanted orthotopic mouse models using reporter fluorescent proteins or exogenously administered fluorescent probes. (**a**) An orthotopic mouse model bearing human pancreatic cancer is visualized with an anti-CEA nanobody tagged to a near-infrared fluorophore. (**b**) An orthotopic mouse model of colon cancer tumor liver metastatsis is visualized using the GFP reporter. (**c**) An orthotopic mouse model of gastric cancer visualized using an anti-CEA antibody tagged to a near-infrared fluorophore. (Red arrows indicate tumor, blue arrows indicate normal tissue)

Technical Considerations for Live Animal Imaging

The accuracy, efficiency, and reproducibility of the imaging process must be counterbalanced with safeguarding the safety, comfort, and ethical well-being of the organism being studied. To meet this goal, the choice of imaging modality should align with the specific requirements of the study, considering factors such as resolution needs, depth of tissue penetration, and the physiological or behavioral state of the animal. Proper preparation of the animal is essential, which may involve anesthesia to prevent movement during imaging and to minimize stress and pain. The physiological effects of anesthesia must be carefully monitored and managed to avoid influencing the study outcomes. Additionally, the timing of imaging

relative to experimental interventions, such as drug administration or surgical procedures, is critical to capture relevant data [31]. Calibration and maintenance of imaging equipment are also vital to prevent artifacts and ensure consistent, high-quality images. For imaging modalities that require administration of exogenous contrast agents or radioactive tracers, the timing and route of administration must be considered. Furthermore, ethical considerations should guide all aspects of animal imaging to minimize animal use and suffering. Attention to these technical considerations is essential for obtaining reliable and reproducible data from animal imaging studies.

Animal Models for Live Animal Imaging

Animal models have been indispensable in scientific progress, serving as critical proxies for studying physiology, pathology, pharmacology. Models commonly employed range from simple organisms to complex primates, each with their advantages and disadvantages. Selection of the appropriate model significantly influences the relevance and success of planned scientific studies. Key considerations include the biological relevance of the organism selected, availability and cost, ease of manipulation, scale-ability, reproducibility, ethical and regulatory considerations, and historical data and precedence in existing literature [32]. Other factors to consider include duration of life cycles, maintenance cost, space requirement, and ease of manipulation. Organisms such as drosophila, zebrafish, and chick embryos have been used to study embryonic development and genetic studies due to their relatively short life cycle, large number of offspring, and cost effectiveness. Rodents are often used due to their genetic similarity to humans and ability to mimic the human disease process, and a large body of established literature. Larger mammals such as rabbits, swine, canines, or non-human primates provide unparalleled insight of biologic processes in more complex systems, but at a higher cost, time, and ethical/regulatory concerns. The intent of this chapter is to educate the surgeon-scientist about the suitability of various model systems to be interrogated through common live animal imaging techniques.

Drosophila Model

The fruit fly *Drosophila* melanogaster is a widely used multicellular model to study general biological processes. Initially the adult fly represented a model for chromosomal inheritance whereas the fly embryo was used to interrogate developmental biology. Over the past decade research using Drosophila to study cancer biology has increased tremendously [33]. Several features make Drosophila a very attractive model system: Fruit flies are small, easy to handle, and their maintenance is very cost effective. They have a short life span and produce many offspring, which permits high throughput experiments and aids statistical analyses of the data. With large scale mutagenesis screens and the subsequent mapping /cloning of

genes as well as CRISPR/Cas9 knock-out/knock-in and overexpression strategies [34, 35]. Genes and alles related to human diseases can be studied and manipulated in the fruit fly; remarkably, 60% of all human genes and 75% of disease-related genes have orthologs in *Drosophila* [36]. The publicly available database "FlyBase" provides researchers with a valuable resource [37] and tools are plentiful and readily shared within the fly community. The Drosophila embryo lends itself to imaging approaches since it is small, optical transparent and immobile. Larvae and adults are more challenging imaging objects since they move and develop light scattering features, but techniques have been reporting applying lightsheet/confocal and two photon microscopy techniques to study the fly in the late developmental stages [38, 39].

Zebrafish Model

Zebrafish (Danio rerio) is a tropical freshwater fish that became a powerful vertebrate animal model of development starting in the 1980's and more recently has been integrated in disease modeling [40, 41]. 70% of human genes have at least one zebrafish orthologue and a similar degree of overlap in disease-related genes, which makes it an attractive model to study normal physiology and disease processes relevant to humans [42]. The zebrafish's genome can be manipulated with ease and the existence of a well-established zebrafish research community sharing resources and knowledge make it easy for a beginner to setup a zebrafish husbandry and laboratory. A multitude of mutants in protein-coding genes have been generated and a series of transgenic fish lines exist to study human diseases. Small size, high fertility, short generation time, rapid embryonic development, and external fertilization as well as fully developed organ systems, including heart, intestine and blood vessels within 48 h. after fertilization benefit study length and make for economical maintenance and lower space requirements than rodent models. From an imaging perspective zebrafish is a great model since it is optical translucent in the embryonic stage. Furthermore, the pigmentation process can be delayed with melanin synthesis inhibitors [43] or prevented altogether with pigment mutants [44]. Fluorescent reporter lines enable imaging of the embryonic stages and adult zebrafish at the cellular and subcellular level. Microscopic techniques such as light sheet microscopy and confocal microscopy allow for prolonged imaging with minimal interference of physiological processes that occur during either regeneration or development [45, 46]. Luciferase-based transgenic zebrafish lines have been designed for imaging deep in the tissue [47]. Recovery from lesion-induced damage has been observed using ultrasound or magnetic resonance imaging [48–51].

Avian Models

Avian models, and the chicken embryo in particular, have made significant contributions to our understanding of development. The process of development is

evolutionary conserved among chick and human and has led to extensive research in embryonal development as well as development of the heart, muscle and vasculature amongst other tissues. The chick embryo is an attractive model due to its widespread availability, ease of in-ovo and ex-ovo manipulations, and its simple and cost-effective husbandry. Newly developed techniques as grafting, electroporation, and microinjection provide the research community with excellent tools for genetic manipulation and has sparked interest in using this vertebrate model for disease modeling and drug screening efforts. For researchers interested in imaging of the living organism, the chicken embryo became even more attractive with the generation of transgenic avian models stably expressing a host of fluorescent reporter proteins [52]. The chorioallantoic membrane (CAM) is of particular interest for imaging studies focusing on in vivo angiogenesis experiments [53], as well as human tumor growth, metastatic outgrowth and drug screening [54–56]. While a valuable model in the repertoire of available research techniques due to its innate immunodeficiency (permitting engraftments), it is a limited system to study the involvement immune cells during tumor growth and dissemination.

Rodent Models

Rodent models, particularly mice and rats, have been widely used in research to model mammalian biological processes due to their anatomic and physiologic similarities to humans. There is over 95% homology between the human genome and that of the mouse and rat [57, 58]. Due to their genetic manipulability, ease of handling, and biologic parallels that closely mimic human benign and pathologic diseases, they have been indispensable research tools.

The laboratory mouse is the most widely used in-vivo model in biologic research. Genetic manipulation of the mouse genome was first described over 35 years ago [59]. Since then, the mouse genome has been well mapped and there is a significant body of literature in its manipulation [57]. Commercially available and academic repositories of genetically modified mouse strains are available for translational research [60]. Custom genes can be readily knocked out and knocked in, via homologous recombination using site-specific selection cassettes and the Cre/lox recombinase system, or more recently, via the CRISPR/Cas9 gene editing technology with single guide RNA's [61]. These advances have allowed for precise investigation of molecular signaling and disease mechanisms.

Mice are well suited for live-animal imaging of biologic processes given their small size, ease of handling, and compatibility with the imaging modalities described above, making them versatile subjects for in-vivo studies. Given their value in research, imaging modalities have been adapted to accommodate for the scale of murine anatomy, such that reliable and reproducible non-invasive whole body imaging with ultrasound, CT, MRI, PET/SPECT is possible [62–64]. While the scale of mouse anatomy is larger than that of the drosophila and zebrafish, the depth of tissue permits robust application of serial optical imaging approaches. Bioluminescent enzymes and fluorescent reporter proteins can be encoded downstream of genes or

interest or tagged in tissue of interest [65]. Exogenous fluorescent agents can easily be administered to highlight the target being studied such as tumor antigens [66], pH [67], enzymatic activity [68], calcium channel function [69]. Advancements in optical imaging technology such as that of enhanced biocompatible cyanine dyes, near-infrared /second-window infrared wavelengths, highly sensitive and quantitative detectors, highly sensitive charge-coupled device (CCD) detectors, narrower bandpass filters or electronically tunable filters, and improving software algorithms for multi-spectral or time domain analyses are permitting the application of optical imaging beyond the traditional limits of light penetration [70–73]. For example, optical imaging using near infrared windows is now possible through bony structure such as the calvarium which previously required surgically created skull windows [74].

Particularly interesting to surgeon-scientists is the ability to perform survival surgeries in mouse models to model surgical pathologic processes. It is a reliable mammalian system with similar anatomic structures able to tolerate surgical procedures and model neurovascular [75], cardiopulmonary [76], hepatobiliary [77], bariatric [78], endocrine [79] and/or GI interventions [80]. Especially valuable for surgeon-scientists studying oncologic processes is the ability to model neoplastic processes in an orthotopic location to maintain native tissue/stroma signaling [81–83]. Along with anatomic advantages, the mouse immune system can be manipulated to provide an immunodeficient system to study human xenografts, or immunocompetent systems to evaluate the immune cell interaction with the biologic processes of interest, a feature that is limited in other live-animal models. These approaches can be combined with imaging techniques to dynamically study the immune response for example, in immunotherapeutics and cellular therapies [84, 85].

Since the mouse model is smaller in size and generally more cost effective than the rat model to maintain, they are often a first choice in rodent studies. However, the rat model is a valuable rodent platform for larger mammalian studies; particular for surgical procedures. They may also be preferred in metabolic and cardiovascular studies as well as behavioral studies [86]. Given that many small animal imagers are designed and optimized for the mouse model, there are fewer devices available for the rat models for live-animal imaging, and more limited applicability in this field.

Larger Mammals

While not often utilized due to their increased cost, time, and complexity in handling, large animal models still provide crucial role in advancing biomedical research and translational medicine. These models, which often include pigs, dogs, sheep, and non-human primates, offer significant advantages over smaller animals due to their physiologic and anatomic similarities. Particularly in the field of live animal imaging, given their larger sizes, standard scale imaging devices can be used with greater resolution and detail. This enhanced imaging capability enables the study of more complex biological processes, disease progression, and therapeutic

interventions in real-time. These large animal models are often a vital bridge between preclinical studies in small animals and human clinical trials, offering valuable insights into drug efficacy, safety, and pharmacokinetics [87]. However, the use of large animal models comes with ethical considerations, higher costs, and logistic challenges that must be carefully balanced against the potential scientific and medical benefits. The FDA Modernization Act 2.0 (S.5002) was recently passed by the U.S. Senate in 2022 to authorize the use of alternatives to animal testing using cell-based assays or computer models and removes the mandatory requirement to use animal studies to obtain a license for new drug [88]. Despite these changes, the unique advantages offered by large animal models in live imaging play a critical role in driving important advancements in biomedical research.

Alternatives to Animal Models

Although animal models recapitulate many facets of human physiology, there is a constant debate as to whether the results are translationally relevant to humans, which is of course the overarching aim of most research undertaken by the surgeon scientist. In recent years, a wealth of research focusing on the development of alternative human ex vivo models [89] has led to the establishment of experimental approaches beyond tradition 2-dimenstional cell cultures. Models such as organoids, ex vivo culture of human tissue (organotypic tissue slice cultures), tissue co-culture models, and organ on a chip are sophisticated alternatives that are amenable to complex imaging modalities. A brief overview of these alternative approaches is discussed with a focus on their use in imaging bio-physiologic processes.

Organoids

Organoids are miniaturized organ-like 3D in vitro structures that can be generated from pluripotent stem cells (PSC) or adult stem cells (ASC) from both human and animal tissue. Generation of ASC-derived, epithelial organoids is restricted to tissues with regenerative compartments (growth recapitulates adult tissue repair) whereas PSC-derived organoids simulate development and are potentially more complex containing mesenchymal, epithelial and endothelial constituents [90, 91]. Both types of organoids require presence of extracellular matrix to mimic the basal lamina and can be genetically modified using CRISPR-Cas9–based techniques [92–94]. The ability to generate ASC-derived organoid cultures from healthy tissues and their diseased counterparts makes organoid culture an attractive model to study disease in general and cancer biology and precision medicine in particular [95]. One major limitation of organoid culture is the absence of an intact TME including immune and stromal cells, which is sought to be overcome by co-culturing tumor-derived organoids with exogenous immune cells to improve studying individualized drug responsiveness [96]. In addition, ASC-derived organoids exhibit low

establishment rate due to multiple technical and logistical factors. Generation of ASC-derived organoids requires skillful preparation of primary cells from patient tissue and limited access to fresh patient tissue often makes their generation impractical, although efforts are being made to generate organoid biobanks [97–99] and access to tissue is rarely a limiting factor for surgeons.

Organotypic Tissue Slice Cultures

Organotypic tissue slice cultures can be established from animals or humans, from a variety of normal and diseased solid tissues and cultured in vitro. Slices of 200–500 μm thickness are prepared using vibratomes. This model has been used extensively to study brain physiology in small rodents, the assessment of pharmacological efficacy and more recently in the development anticancer drugs/precision medicine, especially in the field of immune-oncology [100–102]. In vivo tissue complexity as well as physical, chemical, and biological elements within tissue are preserved in the context of an intact microenvironment and native extracellular matrix. Slice cultures can be easily generated, manipulated in vitro and maintained for prolonged periods of time (4–16 days) making them amenable for testing drugs and reporting results via imaging [103, 104]. It can permit the study of tissue types that have traditionally be difficult to cultures such as appendiceal cancer [105] and neuroendocrine cancers [106]. The drawback of this culture system is that the cutting process can introduce artifacts due to compression distortion and cell damage as well as limited light penetration. Additionally, sample sizes are usually limited and processing can present a logistical challenge.

Tissue Models

To mimic an even more physiological setting with intact extracellular matrix, endogenous immune and stromal cells without the need to cut the tissue in the z dimension, the tissue co-culture model utilizes a system in which resected normal and tumor-bearing tissue is mounted onto custom-designed platforms and cultured in patient derived plasma for up to 4 days (Fig. 17.3). We have found that this is most readily accomplished using small peritoneal metastasis as would be encountered during a diagnostic laparoscopy or during cytoreductive surgery. Platforms can be removed from the culturing system and repeatedly imaged using fluorophore-conjugated antibodies targeting cells of interest. Imaging depth (50–200 μm) can be easily achieved by confocal/two photon microscopy since the peritoneum is a transparent membrane with reduced light scattering properties. The limitations of this approach include time for experimental (drug) effect if applied outside the patient, and number of parameters that can be visualized (<5) with current technology. Never-the-less, this technique offers the opportunity to perform a large number of multi-omic evaluations following fixation after live imaging as certain fluorophores are retained after paraffin-embedding.

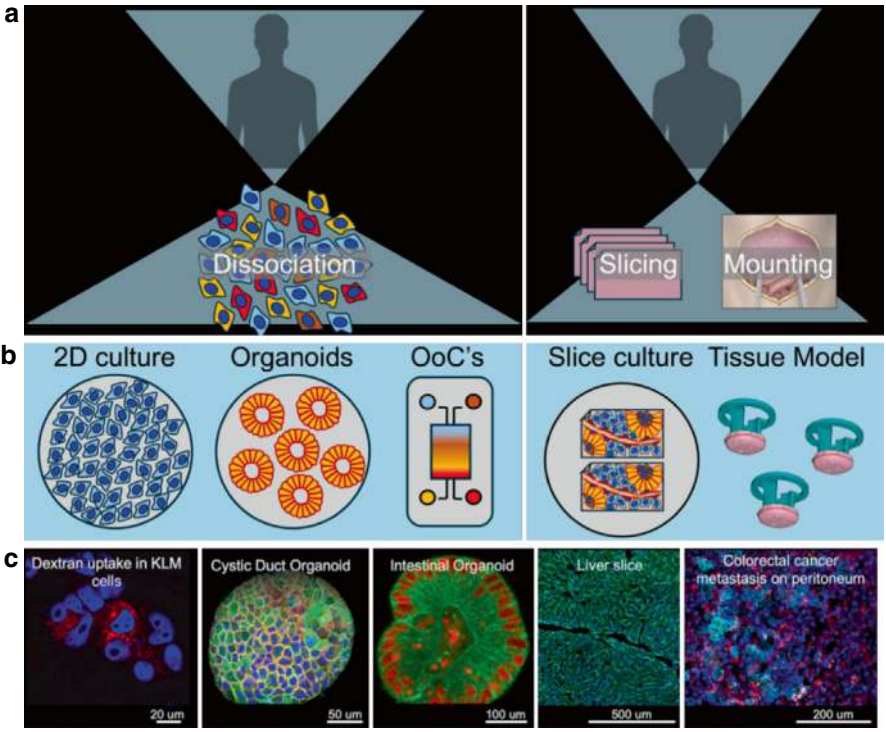

Fig. 17.3 Establishment of alternatives to animal models from human tissue biopsies. (**a**) Biopsy treatment ex vivo (**b**) Different models can be established post dissociation, slicing or directly mounting on customized platforms (**c**) Fluorescent imaging of representative examples of 2D culture (KLM cell line ingesting fluorescently labeled dextran (red)), Organoids (fixed Cystic duct organoid stained for EpCAM (red), panCytokeratin (green), F-actin (cyan) and nuclei (blue); live intestinal organoid expressing GFP-Myosin IIA (green) and RFP-Histone (red)), tissue slice incubated with anti CD49a antibody (cyan), SirActin (green) and NucBlue (blue) and peritoneal tissue with colorectal lesion incubated with antibodies against CEA (cyan), CD45 (red) as well as NucBlue (blue)

Organ-on-a-Chip

The Organ-on-a-Chip (OoC) technology combines human primary as well as stem cell-derived cells and microtechnology to generate systems that recapitulate important aspects of human physiology [107, 108]. Multiple microfluidics systems have been developed to test pharmaceutical and chemical compounds, biomaterials, model human diseases or test single/multi-organ function. Since imaging is an important readout for cell and tissue function, most of the OoC systems incorporate optically transparent media to allow for fluorescent imaging and even high-content imaging. They are useful tools to overcome disadvantages of conventional cell culture and animal models but come with their own disadvantages. The microenvironment and

cell compositions are artificially constructed, often from multiple patient sources and used in artificial quantities Their physiological relevance is less validated than other technologies (e.g. organoid-based technologies), system setup and customization of assays to relevant readouts are time consuming and the complexity of human physiology is exceeding bioengineering capabilities up until now [109].

Conclusion

The field of live animal imaging has undergone remarkable advancements in the recent years, offering researchers increasingly powerful tools to investigate biological processes, disease progression, and therapeutic interventions in living subjects over time. The array of imaging techniques discussed, provide unique insights into anatomical, functional, and molecular aspects of animal physiology. These imaging modalities, most of which are non-invasive and permit longitudinal studies in the same organism, have revolutionized preclinical research reducing the number of animals required while providing more statistically robust data. This has enhanced the scientific understanding of disease processes and treatment efficacy, while also addressing ethical concerns by seeking to minimize the numbers of animals used. Each model offers distinct advantages and limitations for live animal imaging. The choice of animal model and imaging modality must be carefully considered based on the specific research questions, accounting for factors such as resolution, sensitivity, cost, and translational relevance.

Looking forward, the field of live animal imaging will continue to evolve rapidly, and is already being applied to ex vivo human tissue models. Technological advancements will drive improvements in spatial and temporal resolution, sensitivity, and quantification accuracy across all modalities. The integration of artificial intelligence and machine learning algorithms will further improve the quality, volume, and interpretation of acquired live animal images, potentially accelerating scientific discoveries. The ultimate goal of imaging in live animal models is to bridge the gap between preclinical studies and clinical need. As surgeon scientist, this can be accomplished through the use of the imaging modalities described above to both animal models and tumor tissue, thereby leveraging our unique advantage of access to fresh tissue. We see this approach as an effective means to translate results obtained to therapeutic interventions in the human. The surgeon-scientist must play a pivotal role in this field by providing critical insights that translate these imaging findings from animal models and human tissue to the clinic.

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The Role of the Microbiome in Surgical Diseases and Their Management

18

Ryan M. Thomas

Abstract

Much interest has been generated in the lay and scientific press regarding the microbiome in human health and disease. This coverage is most certainly warranted and represents one of the most exciting and rapidly developing fields in research that has the great potential to help the medical community understand and treat human disease. Because the specialty of surgery is so integrally related to the treatment of many benign and malignant diseases, addressing the intersection of the microbiome with surgical diseases is critical. The chapter will provide the reader who is interested in this topic, a framework for discussion and further independent reading through the comprehensive reference list of notable studies. While there is limited discussion of microbial taxonomic changes in order to maintain an approachable narrative to all readers, the astute reader will notice themes and trends in bacterial taxa discussed in this review. In this chapter, a broad overview is presented on the microbiome, methodologies to investigate and conduct microbiome studies, the role of the microbiome in surgical health and disease, and areas of future investigation to advance this exciting and critical field.

Keywords

Microbiome · Microbiota · Surgery · Surgical disease

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Introduction

Few fields in scientific and medical research have taken as prominent a role in the lay and scientific press as that of the microbiome. While the term has been liberally used, knowledge of the nuances of this expanding area of research are sometimes limited amongst medical personnel and demands introduction to clinicians with limited knowledge, or perhaps a great interest, to understand the multiple facets that the human microbiome plays in human health and disease. The term "microbiome" and "microbiota" have both erroneously been credited to Lederberg and McCray as first used in 2001[1] when in fact, both terms have been used for many years beforehand, particularly in the ecology literature. In general, the *microbiota* is the assemblage of microorganisms present in a defined environment. As it relates to humans, this may refer to the intestinal microbiota, lung microbiota, urinary tract microbiota, among others—all assemblies of microorganisms specific for a particular environment. While the most abundant and well-studied microbiome in humans is the gut bacterial microbiome ("bacteriome"), one would be remiss not to recognize the viral microbiome ("virome"), fungal microbiome ("mycobiome"), and any other collection of microorganisms that form their own microbiome. As is the case for much of the literature, the discussion that follows will mainly refer to the bacterial microbiome (and in particular that of the gut) but the reader should be aware that the discussion can be adapted to other microbiomes as well. For its current usage today, and for purposes of the research which is the focus of this discussion, the *microbiome* was defined in 1988 by Whipps et al. as:

> *A convenient ecological framework in which to examine biocontrol systems is that of the microbiome. This may be defined as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity.*[2]

An important distinction in this definition is the "theatre of activity" which takes into account the effect that a microbiome has on its environment. The MicrobiomeSupport Association held a workshop that concluded that the definition proposed by Whipps and colleagues was the most comprehensive but offered additional clarification in terms of the members of the microbiome, interactions among its members, spatial and temporal microbiome characteristics in their environment, the "core" microbiota (stable members versus transient), functional predictions and phenoytpes, and host-microbiome coevolution and interactions. Given this rapidly expanding field, every surgeon should have a familiarity with the terminology used in microbiome research. Following is a discussion of the components of microbiome experimentation and analysis, role of the microbiome in the context of surgical diseases, implications for its potential therapeutic use, and future applications.

Microbiome Experimentation and Analysis

Definitions and Background

An introduction to the terminology used in microbiome research will provide a framework for subsequent examples and discussions. Microbiome analysis can broadly be divided into compositional analysis (what *comprises* the microbiome in question) and functional analysis (what the microbiome in question is *doing*). Depending on the hypothesis to be tested, the analysis and the necessary tools will differ. The most widely used method for compositional analysis is marker gene analysis. With the cost of next generation sequencing (NGS) decreasing significantly over the past 10 years, this straightforward and informative method has become the first stop for microbiome projects. Marker gene analysis relies on the use of primers targeting a specific gene of interest that is highly variable but flanked by conserved sequences to allow primer binding. In this way, amplification of the variable region allows characterization of taxonomy. In the case of bacteria and archaea, the 16S rRNA gene is amplified, and for fungi, the Internal Transcribed Spacer (ITS) gene is targeted [3, 4]. The advantage of this technique is that it is of low cost, high throughput, and informative for low biomass samples or those contaminated with host DNA (such as a patient's tumor sample). However, this technique can be biased based on the region sequenced and does not give species-level information as it is typically only limited, at best, to the genus level. Should species-level information be needed based on the hypothesis being tested, whole metagenome sequencing (sometimes referred to as metagenomic sequencing, shotgun sequencing, or shotgun metagenomics) sequences the entire genome present in a sample [5]. This method therefore sequences all genomic material in the specimen: prokaryotic, eukaryotic, and viral. This yields extremely robust data that can resolve sequences to the species level for the population present since the entire genome is sequenced as opposed to a single gene as in the case of 16S rRNA gene sequencing [6, 7]. Because of this, not only will species be identified but their relative abundance in the sample and the functional capacity of the metagenome as well [8]. The drawback to this approach is that it is more costly compared to 16S rRNA gene sequencing secondary to sample preparation costs and the deep bioinformatics analysis necessary. Additionally, like marker gene analysis, this method cannot discriminate between live/dead cells and gives no information on microbial activity [9, 10]. Metatranscriptomic analysis is the final method to analyze the microbiome and rectifies some of these shortcomings but is the most expensive and labor-intensive approach [11]. As the name implies, metatranscriptomics profiles the transcriptome of the entire community ("meta") present within the sample. In this way, the gene expression profile of the microbiome, and functional capacity, is characterized. Metatranscriptomics therefore analyzes only components of the microbial community which are actively undergoing the process of transcription and thus excludes dead cells. However, this method is skewed toward highly active microbes and the

presence of ribosomal RNA (rRNA) results in limited utility of the sequencing output given that 80–90% of the total bacterial RNA output is rRNA [12–15]. Depletion of rRNA is therefore necessary to enrich the fraction of desirable messenger RNA (mRNA) that will yield the most useful information [16, 17]. Selection of one of the aforementioned methods is dependent on the project and hypothesis. These three methods will undoubtedly yield a large amount of data that need to be converted into usable information and the quality of the bioinformatics analysis is therefore critical to produce the best characterization of the microbiome.

Microbiome Experimental Considerations

Appropriate detail to the experimental design is critical to produce the most informative and biologically meaningful microbiome data. The choice of microbiome sequencing method and pipeline analysis will be meaningless if attention is not paid to the laboratory and/or clinical phase(s) of the study. It is during the design phase that confounding variables must be taken into consideration and mitigated (Fig. 18.1). One way to mitigate these issues is with meticulous record keeping of all metadata associated with study participants, sample collection processes, experimental details, and bioinformatic software, commands, and pipelines utilized. Reporting guidelines for human microbiome research have been proposed [18] and the Genomic Standards Consortium (https://www.gensc.org//) is an organization

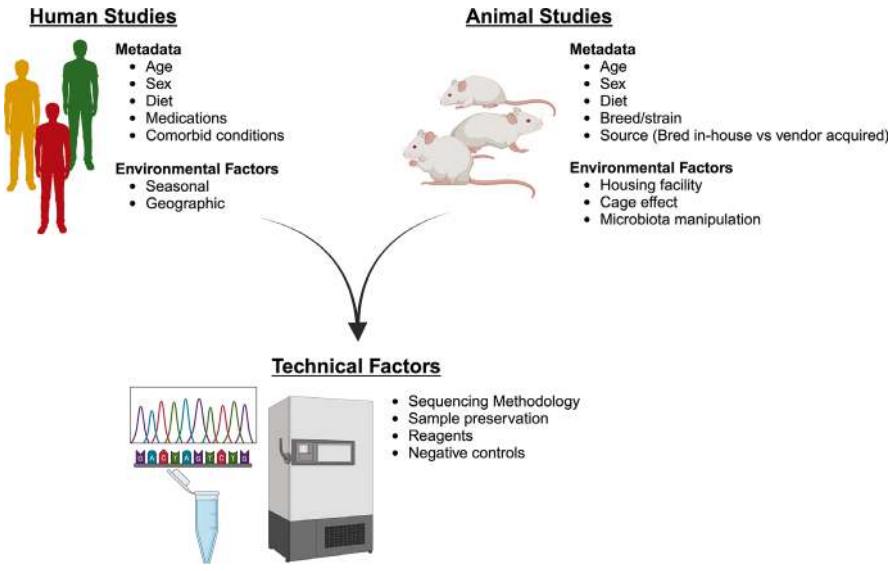

Fig. 18.1 Experimental factors to consider that may confound microbiome studies
Three main areas of experimental consideration are illustrated which may influence microbiome studies. These should be taken into account and if they cannot be controlled for, meticulous record keeping is necessary in order to take these factors into account during microbiome analysis

that has set minimum standards for information collection so that microbiome experiments can be reproduced. Examples of human metadata to be collected include but are not limited to age, sex, diet, medications, comorbid conditions, and if longitudinal studies are performed, annotating the time and location of specimen collection as seasonal and geographic variations exist. This information is important to derive meaningful conclusions from the sequencing data and cohort comparisons. From an animal microbiome experimental point of view, the source of animals needs to be noted (in-house breeding versus acquired from a vendor) [19], diet, sex, and cages in which the experimental animals are housed given the coprophagic nature of rodents that can skew microbiome data. Finally, technical aspects of sample collection and processing need to be accounted for [20, 21]. Depending on the hypothesis to be tested, the sample preservation method is very impactful [22]. If metatranscriptomics are planned, preservation in RNase-free media or with RNase inhibitors to maintain RNA integrity is critical. If the desire is to retrieve culturable bacteria from a sample, preservation media that prevents bacterial overgrowth and preserves anaerobic bacteria should be utilized [23, 24]. Finally, if metabolomic profiling (to be discussed later) is the goal of the study, preservation agents that will not interfere with downstream metabolite extraction or analysis is necessary. Controls also must be thoughtfully planned out specific for microbiome experiments. For example, given potential DNA contamination of reagents [25], negative controls that have gone through the same sample processing steps as all other samples are necessary in order to determine any potential bacterial carryover into treatment groups merely from reagents. Given all of the complexities in microbiome experimentation, consultation with a microbiome expert and someone with expertise in microbiome bioinformatics is highly recommended.

Animal Models

Studies focused on the role of the microbiome in human disease have moved well beyond simple associative studies that report changes in the microbiome in the presence of a disease or intervention. Now, mechanistic studies and alterations in the transcriptome and metabolome of the microbiome are increasingly more important. In order to accomplish this, experimental models that allow manipulation of the host microbiome is necessary. Because humans cannot be rendered germ-free and long-term broad-spectrum antibiotics to deplete the human intestinal microbiota is not feasible, animal models are critical to evaluate the effect of single changes on the microbiome with disease progression or other experimental conditions (Fig. 18.2) [26]**.** The ability to manipulate the microbiome is therefore a powerful tool to study host-microbiome interactions. Germ-free (GF) animals, also referred to as axenic, are completely devoid of microorganisms, having been born and raised under sterile conditions from a GF mother, and have become critical to this area of research. Gnotobiotic, from the Greek "gnosto" (known) and "biote" (life), refers to a known microbiota which could be either no microbiota at all (ie axenic) or composed of a specific known microbe or microbial consortium [27, 28]. While various GF animal

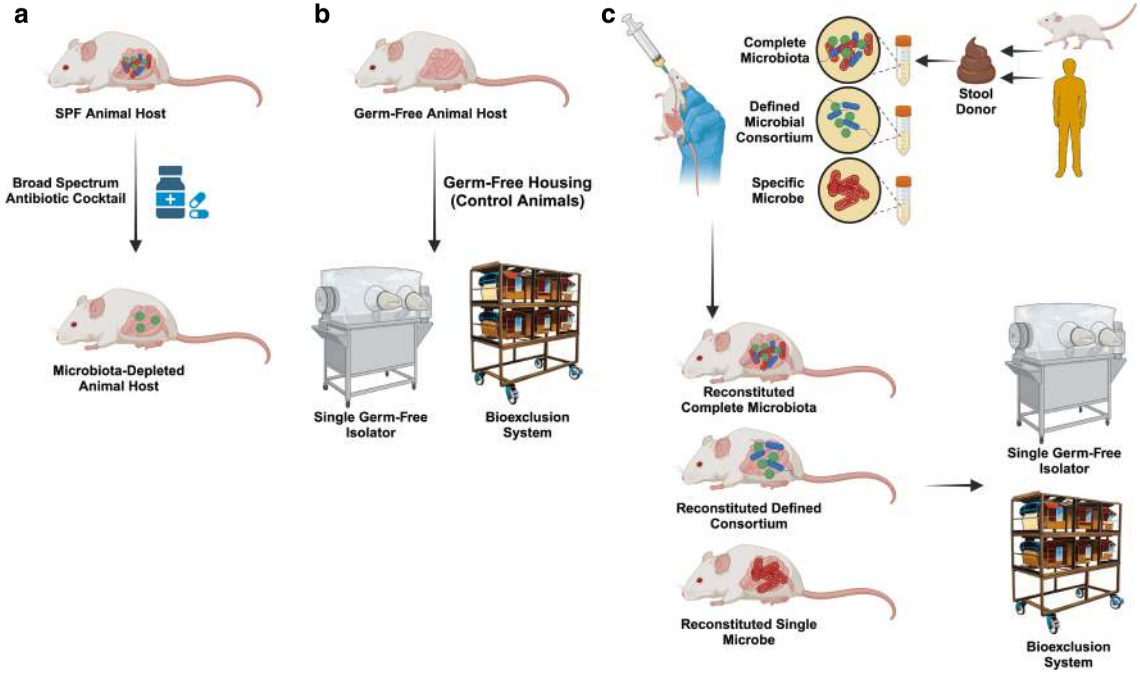

Fig. 18.2 Methods of gut microbiota manipulation in animals
The most economical method for microbiota manipulation involves the administration of a cocktail of broad-spectrum antibiotics to deplete the microbiota in specific pathogen-free animals (SPF; a). Depending on the antibiotics utilized, the diversity and abundance will both be reduced and reconstitution with other microbe(s) can be performed. However, one must be aware that this is not a complete elimination of the microbiota but can yield insight into phenotypes produced by microbiota depletion. Only germ-free animal hosts are completely devoid of microbes (b). These animals are housed in either single germ-free isolator which are ideal for breeding but not experimental manipulation because of cost and only one experimental condition can be carried out per isolator. Instead, bioexclusion systems have been developed to maintain germ-free in each case as they are individually ventilated and filtered. Finally, to test the effect of microbes in the intestinal tract on a specific intervention, various microbiota can be reintroduced into the germ-free host through oral gavage (c). This can vary from a complete microbiota derived from the stool of a human or animal donor, a defined microbial consortium consisting of several known microbial species, or even a single microbial species. Animals are then housed in either single germ-free isolators or a bioexclusion system

models exist [29–31], use of GF mice is the most common because of ease of manipulation, experimental familiarity, existence of many disease models to investigate the effect of the microbiota, and physiologic similarities to humans [26]. Special conditions must be considered to maintain the GF or gnotobiotic status during breeding, routine housing, or experimentation. The GF mice must be housed in sterile isolators to ensure their axenic status but are costly and labor intensive to maintain. Experimentation in these isolators is difficult because any experimental microbe introduction exposes the entire colony in the isolator. Each isolator would therefore have to be its own experimental condition or control. Because of setup costs and maintenance, this is simply not financially sound. To remedy this, systems such as the Techniplast IsoCage P Bioexclusion system have been designed that include HEPA-filtered, individually positive pressure ventilated cages. This system allows robust manipulation of microbial content in a cost-effective manner and allows bioexclusion of individual cages. An alternative approach is the antibiotic-mediated depletion of the intestinal microbiota with a cocktail of broad-spectrum antibiotics. This cost-effective approach allows one to gather insight on microbiome changes and allows for microbial manipulation without the cost associated with GF studies [32]. However, the utilization of antibiotics will be a confounding factor that must be taken into account. The manipulation of the microbiota in GF models typically involves monocolonization with a single microbe [33], a minimal microbial consortium (low diversity of microbes but of known identity) [34], or a complex consortium that may be defined or undefined (such as the transfer of the intestinal microbiota from one host to a new GF host) [26]. Several considerations need to be made regarding the colonization of a GF host. These include the method of colonization (oral gavage, co-housing) [35–37], duration and dosage of colonization, age of the colonized host, and confirmation of colonization. All of these factors influence the ability to successfully colonize and may have an impact on the experiment. Thus, surgeons involved in microbiome studies will need to become familiar with these issues.

Analysis Methodologies for Microbiome Studies

While an in-depth discussion of the bioinformatic tools and pipelines available to analyze various data generated from microbiome is out of the scope of this chapter, a brief overview will be presented to familiarize the reader with these concepts as in-depth reviews have already been published on this topic [38–41]. As discussed previously, there are three primary types of microbiome sequencing data to analyze: marker gene, metagenome, and metatranscriptome (Fig. 18.3). Analysis of marker gene datasets (16S, 18S, ITS) has traditionally began with the removal of sequencing errors by computational construction of operational taxonomic units (OTUs) which are similar sequencing reads that are clustered and differ by a specified error threshold, typically 3% [42]. This sequencing error threshold is based on the idea that similar organisms will have similar sequences and that any error (up to 3% in this case) will be trivial and not affect the identification of taxa. Once OTUs are

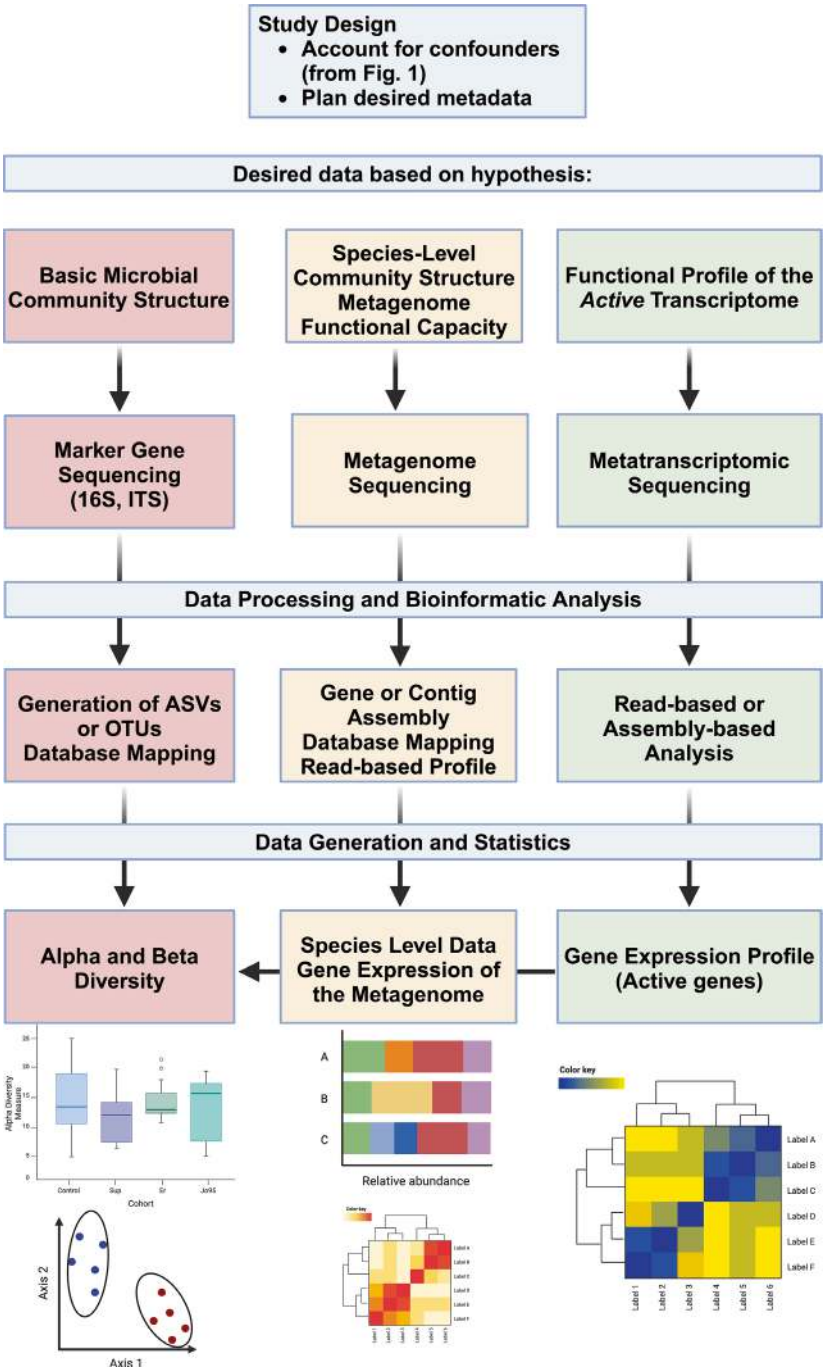

Fig. 18.3 Workflow for microbiome sequencing options based on hypothesis to be tested
Flow diagram presenting the workflow of microbiome studies starting from study design to identifying the best sequencing option that will test the hypothesis to data generation and reporting

generated ("clustered"), the taxonomic classifications of the OTUs are determined by comparison to a database. Typically, a *closed reference* database with known OTU sequencing information is utilized to identify sample taxonomy. Closed reference databases, such as SILVA or Greengenes, cannot capture potential biologic variability or novel taxa since they are defined datasets [43, 44]. However, one benefit is that OTUs generated from different studies can potentially be compared if they were classified utilizing the same dataset. More recently, amplicon sequence variant (ASV) aims to provide a more precise identification of microbes as it is not a cluster of similar sequences as is the case for OTUs [45, 46]. Utilizing ASVs involves determination of the exact sequence that was read and how many times that sequence was present in the sample. Rather than clustering similar reads together and assuming an error threshold rate, ASVs are combined together and an error model is individually determined for each sequencing run that essentially creates a p-value for each sequence. Studies utilizing ASVs can therefore be compared since these are exact sequences and comparison to a reference database allows my precise characterization potentially down to the species level (which OTUs do not allow).

As described earlier, metagenomic sequencing sequences the entire genome present in a sample, including eukaryotic and DNA from other cells, all of which need computationally removed or "filtered" out after sequencing. Individual genes or contigs (sets of overlapping DNA segments to produce one continuous region of DNA) are assembled and subsequently mapped to known genes, contigs, or genomes utilizing various software platforms [8, 47, 48]. In this way, information to the species level can be acquired and even metabolic profiling can be inferred based on the number of sequencing reads for a particular gene or pathway. An excellent in-depth review has been published on this topic that would serve the interested reader well [5]. Finally, metatranscriptomic analysis yields detailed information of gene expression profiles within a sample set. Its analysis is performed in much the same way as metagenomics with one difference being the need to deplete samples of abundant rRNA and host mRNA with subsequent read-based or assembly-based analysis [8, 47, 48].

Once all data has been analyzed, results are compared between cohorts typically beginning with broad patterns of variation through reporting alpha and beta diversity. Alpha diversity refers to diversity within a sample and can be compared between different sample groups. This is often reported as the number of species within the sample, otherwise termed species "richness". Beta diversity, in contrast, refers to the similarity (or dissimilarity) between pairs of samples and allows the comparison of diversity between treatment groups. Several computational pipelines are available for these statistical tests with QIIME2, mothur, and R statistical software packages being the most common [49–52]. Alpha diversity can be measured with a variety of statistical modeling with Chao1 bEing the most common whereas the Shannon index takes into account the number of species ("richness") and their relative abundance ("evenness"). Beta diversity is determined utilizing either quantitative metrics with Bray-Curtis or weighted UniFrac which take into account abundance data. Contrast this to unweighted UniFrac or Jaccard index which simply take into account the presence or absence of features (in microbiome studies this

would refer to the presence/absence of particular microbes). These analyses produce large amounts of data and typically in order to visualize them in a meaningful way, principal coordinate analysis is used to plot Bray-Curtis beta diversity data into colorful dot plots to convey the (dis) similarity of cohorts that many readers have likely encountered. The field of microbiome bioinformatics is large and the curious reader should familiarize themselves with the more complicated nuances of these analyses should they enter the field of microbiome research [38, 53, 54]. It is vital that those pursuing studies in the microbiome collaborate with individuals who have training and expertise specifically in microbiome bioinformatics.

Microbiome in Surgical Health and Disease

Human Microbiome and Health

The human microbiome is the conglomerate of microbes that make up a symbiotic and pathobiont relationship with its human host [55]. The various microbiomes are established early in life [56, 57] with initial transmission of microbial flora from mother to infant being dependent on factors such as route of delivery [58, 59] and breast-feeding status [60]. A stable microbiome is established typically by the second to third year of life at which time it begins to resemble that of an adult [61]. After birth, there is a steady increase in the diversity of the microbiome which contributes to this stability, and inter-individual variability decreases [62]. What defines a "healthy" microbiome is a matter of debate as a specific definition of "healthy" has not been established as microbiome community structure differs between individuals to varying extents. The term "dysbiosis" has been introduced to describe a change, or shift, in either the diversity (how many different types of species are present) or abundance (how much of a species is present) of the microbiome. However, this is a misnomer as this implies there is something wrong or bad ("dys-") with the state of the microbiome when, in fact, any change may simply be an appropriate response to environmental or interventional pressures. Therefore, it is more useful to think of microbiome stability and functionality as influencing health and disease. It is now known that each microbiome community possesses core functions that are responsible for homeostatic mechanisms. As there are metatranscriptomic similarities between species within taxonomic classifications, it makes sense that there is redundancy of function and that shifts in the microbiome, whilst maintaining an otherwise stable taxonomic community structure, will have limited effect on the functional profile of that microbiome community [63]. However, with chronic selective pressures such as diet [64], smoking [65], and medications [66], a change in the host microbiome homeostatic functions involved in nutrition metabolism, inflammatory regulation, and immune modulation, as just several examples, may develop over time and lead to various disease processes. Having a diverse microbiome therefore allows greater redundancy of function and resistance to various pressures. In fact, most data support that diversity in microbial communities is associated with health [67] and as will be discussed later, improved response to anti-cancer

treatment and survival. A secondary concept is that of resilience, or the ability of the microbiome to return to its baseline state after some pressure or intervention [68]. For example, antibiotic use disturbs the intestinal microbiome and is a factor in *Clostridioides difficile*, formerly *Clostridium difficile*, colitis (CDC) [69–71]. Under normal conditions, however, the intestinal microbiome reverts to its pre-antibiotic state after a few weeks [72] but repeated antibiotic exposure may also result in permanent changes to the community structure [73]. Microbiome resilience may therefore be as important, if not more so, than the impact of specific microbes on human health and disease [74]. Investigations involving microbiome stability and alterations in the functional output will be of paramount importance

The Role of the Microbiome in Surgery

Up to this point, a broad overview of the microbiome has been presented with a focus on experimentation, analysis, and health. The microbiome has increasingly been demonstrated to have an interplay with the *preoperative*, *operative*, and *postoperative* care of patients (Fig. 18.4). As technical improvements have reached their limit, understanding the role of the microbiome within these various phases of surgical care offer a novel opportunity to improve patient outcomes. While the discussion that follows focuses primarily on gastrointestinal (GI) surgery, this does not imply that the microbiome does not play a role in non-GI surgery, merely that the vast majority of research is in this area secondary to the ease of studying the fecal microbiota and abundance of bacteria in these specimens. It does, however, underscore the opportunity to study the role of the microbiome in non-GI surgical care.

As a first example of the microbiome role in the *preoperative* phase, interventions such as diet alterations (limitation to clear liquids, NPO status) [75, 76], exercise [77, 78], preoperative antibiotics [69, 70], and surgical bowel preparation [79] have a profound impact on the intestinal microbiome—the consequences which may not be fully realized at the time. Specifically, mechanical bowel preparation has been shown to decrease the abundance of *Bifidobacterium* and *Lactobacillus* [80], both genera generally considered to be beneficial to the gut microbiota, such as the case of antibiotic-associated diarrhea prevention [81]. As mentioned previously, *C. difficile* colitis represents a state of a disrupted intestinal microbiota, often secondary to broad-spectrum antibiotics, that surgeons often must attend to. Evidence exists that bile acids play a role in the pathogenesis of CDC whereby primary bile acids promote *C. difficile* growth and secondary bile acids inhibit its growth [82]. Specifically, the secondary bile acid deoxycholic acid (DCA) has been shown to inhibit *C. difficile* growth while the primary acid taurocholic acid (TCA) can trigger its germination [83, 84]. Patients may therefore be at increased risk for CDC preoperatively and represent a potential area for preemptive intervention. Finally, physiologic insults may occur to a patient prior to any surgical intervention. Such is obviously the case with penetrating or blunt trauma, and burns. It is now clear that in these scenarios substantial shifts in the intestinal microbiota occur. For example, in the case of burn injury in humans it has been shown that a major reduction in the

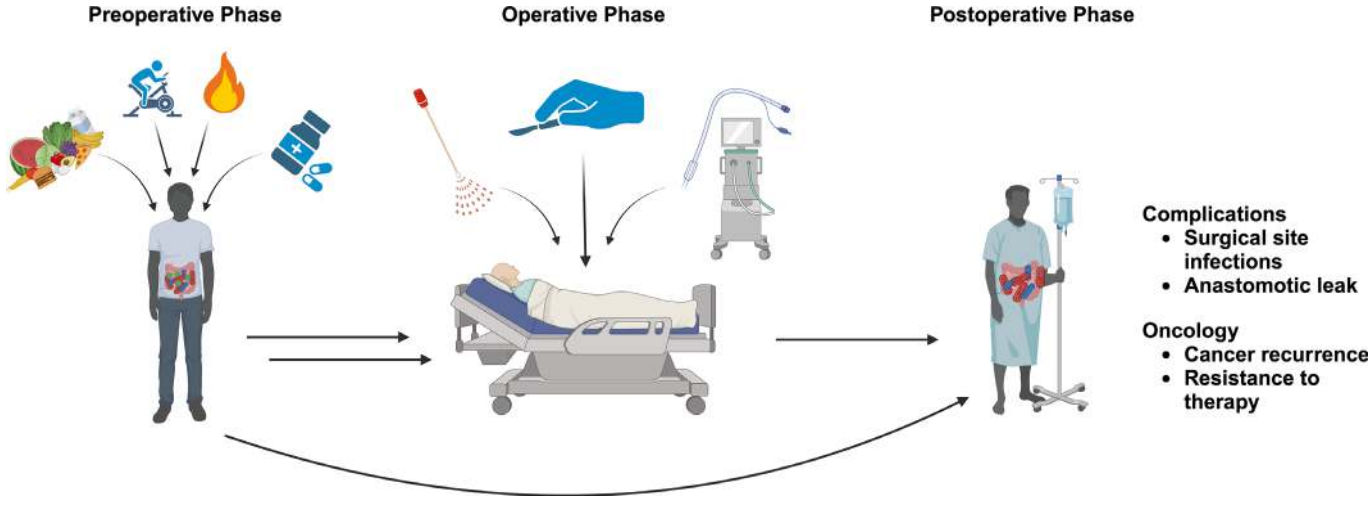

Fig. 18.4 Factors that influence the microbiome of the surgical patient and potential areas for future research
In the preoperative phase, the microbiome has been shown to be influenced by diet, exercise, trauma/burns, and medications (left panel). The implications of these changes come to bear both on the operative phase (center panel) and postoperative phase (right panel). In the operative phase, skin preparation, the surgical procedure itself, and anesthesia have all been demonstrated to impact the microbiome which impacts postoperative recovery. In the postoperative phase, the microbiome changes play a role in postoperative complications as well as oncologic outcomes

phyla Bacteroidetes and Firmicutes occurs with an increase in the pathogenic *Enterobacteriaceae* family of bacteria [85]. Burn injury has been shown to disrupt the integrity of the intestinal mucosal barrier [86, 87] and such a disruption in combination with blooms of pathogenic bacteria may be related to septic complications in burn patients [88–90]. Similarly, a model of acute trauma involving pulmonary contusion and hemorrhagic shock has demonstrated shifts in the microbiome that continued with persistent stress, but which began to correct with cessation of the stress [91]. Interestingly, microbiota changes, typically characterized by decreased diversity with concomitant increased abundance of pathogenic bacteria, appear related to the type of injury, whether that be traumatic brain injury, hemorrhagic shock, or multi-organ injury [92]. How these many microbiome changes affect patients in the immediate perioperative period or long-term are areas of active research but failure of the microbiome to revert to its pre-trauma state has been associated with worse outcomes and cognitive issues, for example [74, 93–95]. These studies highlight the various ways that the microbiome can not only be impacted preoperatively but how these changes can have repercussions long-term. Research must focus on ways to mitigate immediate microbiome changes in order to reduce morbidity and mortality in the operative and postoperative phases of surgical care.

The *operative* phase of surgical care is the defining area of this specialty. While the goal of the technical aspects is to provide diagnostic information, cure of disease, or symptom alleviation, these actions also have notable implications on the microbiome. Any operative intervention creates physiologic changes, and it is now known microbiome changes as well. Given surgical antiseptic preparation to reduce surgical site infections (SSI) [96], it is natural to consider the implications of this on the local (skin) microbiome outside of SSI risk reduction. Studies have demonstrated the rapid, but short-lived alteration in the skin microbiota after chlorhexidine treatment [97, 98]. One pilot study characterized the change of the forearm skin microbiota with 16S rRNA gene sequencing at 24-h intervals (x72 h) after initial preparation with chlorhexidine and used the untreated contralateral forearm as control [97]. This small study (n = 10) demonstrated *Cutibacterium* (formerly *Propionibacterium*) *acnes* and *Staphylococcus epidermidis* as the most common skin flora but while there were species-specific differences between treated and control arms, the overall difference in community structure was not significant at the various timepoints indicating either a quick repopulation of the skin flora, small sample size, or limitations in methodology (treated arms were only "swiped" 3 times with a 2% chlorhexidine-impregnated cloth, certainly less than normal preoperative skin prep for surgery). Another commonality between patients during the *operative* phase of surgical care is the use of general anesthesia. Even inhaled anesthetics have been shown to alter the diversity and abundance of the lung [99] and intestinal microbiome [100], independent of any surgical intervention. The universal utilization of inhaled anesthetics in major operations therefore underlies changes in the microbiome which may have profound systemic effects. Finally, human and animal studies have demonstrated the effect of gastrointestinal surgery on the intestinal microbiota. This may be secondary to transient

ischemia incurred during resection of a portion of the gastrointestinal tract or exposure of anaerobic bacteria in the colon to atmospheric oxygen during resection and anastomosis [101]. For example, the microbiota of transplanted small bowel undergoes significant shifts predominated by strictly anaerobic *Bacteroides* and Clostridia to facultative anaerobes *Lactobacillus* and Enterobacteria. Interestingly, this shift was found in patients with ileostomies who had not undergone transplantation, suggesting that the exposure to oxygen may play a role [102]. Additionally, in a rat colectomy model, a significant increase in the abundance of *Enterococcus*, *Escherichia*, and *Shigella* were noted [103]. While these studies highlight taxonomic changes incurred in the microbiota of various sites, they do not go further to investigate the impact that these changes have on the physiologic or metabolic processes in the host which may have significant implications during the *postoperative* phase.

All *preoperative* and *operative* care coalesce into the *postoperative* phase of the surgical patient. Potential issues during the first two portions of care can have significant issues for patient recovery during the last. One important concept related to microbiome alterations is the resolution, or recovery, of any change back to baseline. Failure of this recovery, as described earlier, has been referred to as dysbiosis but it is the functional impact, not simply the microbial community structure change, that is more important and of interest [104, 105]. It is the physiologic changes associated with surgery that result in adaptation of the microbiome to this stress. Thus, microbiota sensing of surgical environmental stressors is likely nothing more than a survival mechanism for microbes that consequentially express a more virulent phenotype to the detriment of the patient [103, 106–108]. Clinically this process may be interrupted with the use of perioperative antibiotics, limitation of opioids, as well as early enteral nutrition [109]. However, on occasion this is insufficient to prevent virulent microbial overgrowth such as in CDC (described earlier) [110] or other septic complications [106, 111, 112]. How quickly the microbiome can recover likely plays a critical role in postoperative recovery. It is suggested that this process can occur as quickly as 6 h [101] but in animal models, the failure of recovery is associated with increased morbidity [91]. Advances in this area of research to identify the temporal relationship of site-specific microbiome changes and its recovery will be critical. Finally, changes in the local intestinal microbiota at the time of colectomy has been shown to result in increased risk of anastomotic leak. Specifically, *Enterococcus faecalis* has been shown to have the ability to degrade collagen in healing intestinal anastomoses and activate host matrix metalloprotease through gelE and sprE gene activity, leading to increased rates of anastomotic leaks [113–115]. Interestingly, commonly used preoperative antibiotics did not eliminate *E. faecalis* nor prevent anastomotic leaks in a rat model. This suggests that identification of patients harboring this strain of *E. faecalis* or careful selection of preoperative antibiotics can mitigate anastomotic leak rates in colorectal surgery. Such personalized information centered on the microbiome is the next step to potential groundbreaking surgical care and will move the field beyond associative studies.

The Role of the Microbiome in Malignant Surgical Diseases

Perhaps the most notable area that has implicated the microbiome in the care of surgical patients is that involved with oncology. The microbiome has been investigated in many cancer types and once restricted to associative studies, exciting developments in this area of research have outlined the role of the microbiome in cancer development, tumor immune suppression, and treatment response [116]. While the World Health Organization recognizes specific microbes as cancer-causing agents, many more have been implicated. The theorized mechanisms whereby bacteria impact colorectal cancer (CRC) pathogenesis is becoming extensive and a detailed explanation is outside of the scope of this chapter. However, several mechanisms bear mentioning [117]. First, bacteria have been shown to accelerate CRC through the production of genotoxins. The genotoxin colibactin is produced by bacteria that possess the *pks* gene island, such as certain strains of *E. coli*. Colibactin has been shown to induce DNA crosslinks that produce a specific mutational signature associated with CRC. Additionally, several bacteria produce genotoxins with the ability to degrade DNA in a non-specific manner. Most studied is the cytolethal distending toxin (CDT) produced by *C. jejuni* but recently UshA (from *Citrobacter rodentium*) and indolimines (from *Morganella morganii* isolated from patients with inflammatory bowel disease) have demonstrated DNA-degrading ability as well [117]. Bacteria have also been implicated in the upregulation of oncogenic gene transcription. For example, *Fusobacterium nucleatum* produces the Fusobacterium adhesin A (FadA) protein that can bind E-cadherin to upregulate β-catenin expression and cellular proliferation. This bacterium has been implicated in several surgically-treated cancers [118–121]. Another oncogeneic pathway upregulated in a variety of cancers, PI3K-Akt, has been shown to be activated by *Peptostreptococcus anaerobius* through binding of the bacteria to integrins overexpressed in colon cancer cells. Finally, reactive oxygen species (ROS) and persistent inflammation are a hallmark of carcinogenesis and bacteria have been demonstrated to take part in both of these processes. Notably, enterotoxigenic B*acteroides fragilis* (ETBF) secretes the *B. fragilis* toxin (BFT) that has been shown to have various functions. Besides being able to upregulate β-catenin expression (oncogenic gene transcription previously described), the BFT has been demonstrated to increase spermine oxidase production that leads to ROS generation and DNA damage. This pathway, in particular, has been shown to facilitate mismatch repair gene mutation and host-dependent alterations in the *APC* gene [122]. Given that inflammation is immune-mediated, several aforementioned bacteria (*F. nucleatum* via short-chain fatty acid production), ETBF (via BFT), as well as *Clostridioides difficile* (via *Clostridioides difficile* toxin B; TcdB) facilitate the recruitment of Th17 cells into the colonic microenvironment with resultant IL-17 production and inflammation that leads to cancer formation. As mentioned earlier, the majority of research, and this discussion, has focused on colorectal carcinogenesis but that should not give the impression to the reader that these bacteria and mechanisms are limited only to colorectal carcinogenesis. On the contrary, the impact of bacteria through many of these same mechanisms in

pancreatic, hepatic, and breast cancer have been made clear [34, 123–125]. While the bacteria may be different, the mechanisms whereby the microbiome imparts its carcinogenic influence are similar.

Future Directions and Conclusions

The surgical community likely will not have a "silver bullet" of one bug equals one disease. Rather, each individual microbiome community structure and the resultant metatranscriptome will help to determine the path to disease progression and potential for treatment. The microbiome holds great promise for the diagnosis, prevention, and treatment of surgical diseases. Already investigations have suggested that the microbiome can be utilized to diagnose pancreatic cancer [126] and hepatocellular carcinoma [127] or predict treatment response in lung cancer [128], as just a few examples with additional trials underway [129, 130]. Furthermore, distinct microbial communities have been demonstrated in patients with benign pancreaticobiliary and breast diseases [131, 132]. While preliminary and associative in nature, studies such as these demonstrate the possibility to utilize the microbiome in surgical disease diagnosis or risk. Of course, with identification of increased disease risk, the medical community attempts to mitigate such risks. Numerous trials have been designed to utilize the microbiome for risk reduction associated with surgical diseases (Table 18.1). One common theme in these preventative trials is the role of nutritional interventions on the microbiome and subsequent risk reduction. We now know that dietary manipulation has a significant impact on the gut microbiota and production of beneficial short-chain fatty acids [133]. Recent research demonstrates the feasibility to metabolically profile the short-chain fatty acid profile of the gut microbiota for a potential individual-tailored approach to gut health and surgical disease prevention [134]. As discussed previously, microbiome manipulation as a means to treat surgical diseases would be aimed primarily at the treatment of morbidity associated with surgical treatment. This may be to mitigate or treat SSI risk, anastomotic leak, sepsis-related injury as a result of burn injury or other traumatic insult. Significant effort has recently been placed into investigating the role of the microbiome in augmenting chemotherapy and immunotherapy efficacy [135–140]. Should global changes in the microbiome community structure be required, nutritional and probiotic intake, as well as intake of specific bacterial consortia, will be of major interest in order to alter the metatransciptome toward the production of beneficial metabolites [141, 142], whether that be to enhance the host anti-tumor immune function or modulate chemotherapy metabolism [143, 144]. Finally, a directed approach utilizing a bacteriophage to target specific culprit bacteria may be of interest where a specific microbe is at fault for disease development or progression [145, 146].

The study of the microbiome is a rapidly expanding field in surgical disease prevention, diagnosis, and management. Surgeons should make themselves aware of this developing field, especially as it relates to clinical trials. That said, surgeons are best positioned to investigate the role of the microbiome in surgically related

Table 18.1 Recent and current clinical trials investigating the role of risk reduction in surgical diseases

Title	ClinicalTrials.gov ID	Disease	Intervention	Long-term objective
Mediterranean diet and weight loss: Targeting the bile acid/ gut microbiome Axis to reduce colorectal cancer (bridge CRC)	NCT04753359	CRC	Diet Weight loss	CRC risk reduction
Lactobacillus lozenges as preventative care in low-grade dysplasia	NCT05707702	Low-grade oral dysplasia	Probiotic oral lozenge	Regression of oral dysplasia
The beans to enrich the gut microbiome vs. Obesity's negative effects (BE GONE) trial	NCT02843425	CRC	Diet	CRC risk reduction
PRevention using EPA against coloREctal cancer (PREPARE)	NCT04216251	CRC	Diet supplement	CRC risk reduction
Modifying diet to improve gut microbiome	NCT03924778	Obesity	Diet	Obesity and obesity-related disease prevention
Effect of preoperative diet on perioperative gut microbiome	NCT05027763	Any surgical colectomy	Diet	Surgical complications
Microbiome, exercise tracking study: Among individuals at high risk for colorectal cancer (METS)	NCT02780284	CRC	Exercise	CRC risk reduction
Evaluating the impact of a virtually supervised exercise intervention and group counseling on inflammation and the microbiome of smokers at high risk for lung cancer, BE FIT trial	NCT06445192	Lung cancer	Exercise	Lung cancer risk reduction
Manipulation of the gut microbiome by a standardized preoperative diet to prevent colorectal cancer recurrence and metastasis following surgery (DIET)	NCT06349590	CRC	Diet	CRC recurrence

diseases because of their ability to easily procure patient specimens important to microbiome studies, such as saliva, feces, or tumor and should work with experts in the microbiome community to facilitate such trials. Finally, much work is yet to be done on the role of the virome, mycobiome, and other microbial communities in surgical disease pathogenesis and treatment. Such studies are beginning to appear

and likely will demonstrate the importance of these microbial communities in surgical health and disease much as the bacterial microbiome has [147–153].

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Systems Biology: Generating and Understanding Big Data

19

Serena Zheng and Timothy R. Donahue

Abstract

Systems biology is a multidisciplinary field that incorporates a multitude of experimental and computational approaches to investigate complex biological systems using a holistic approach that underscores the integrated and dynamic networks of interacting components (genes, proteins, cells, organisms, and their interactions). Systems biology relies largely on multi-omic approaches to generate differential data, which has been facilitated by advancements in biological research techniques. Large biological datasets are generated from high-throughput experiments, such as microarrays, mass spectrometry, and high-throughput drug screening. Many comprehensive shared datasets are even available in numerous online portals and can provide unparalleled access to valuable information. Analysis of data from genomic, transcriptomic, proteomic, and metabolomic experiments can elucidate changes caused by perturbations like disease process and therapeutic interventions. Although each type of "omics" dataset on its own can provide important insights, integrating data from multiple omics experiments and dimensions (e.g., genome and proteome) can provide a more comprehensive understanding of how different dimensions of biology

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interact with and inform one another. Systems biology is instrumental in studying biological complexity and can pave the way for advances in addressing biomedical challenges.

Keywords

Systems biology · Bioinformatics · Databases · High-throughput experiments · Omics experiments · Data analysis

Introduction

Systems biology is, at its core, the study of complex biological systems through understanding the bigger picture—in other words, by piecing the parts together. Whether this is done at the level of the cell, tissue, organism, or beyond, systems biology is an approach to studying the complexities of a coordinated whole rather than its individual components. It often involves a multidisciplinary approach to biological studies using large datasets, computational modeling, and bioinformatics with the expertise of biologists, chemists, physicists, bioinformaticists, mathematicians, and more [1].

Advances in biological research, especially in the realm of multi-omics, over the past few decades led to expansion of systems biology. As research techniques improve, so do data generation and the need for effective analysis and application of data. For example, discovery of DNA in 1953 was followed by development of genetic engineering and genome sequencing, which created an immense pool of genetic data [2]. The Human Genome Project further advanced the field of genetics by mapping the entire human genome, which consists of over 20,000 protein-coding genes, producing comprehensive genomics data to be analyzed and used for studying human biology and diseases [3].

Like genetics, the fields of molecular and cell biology have also seen a dramatic increase in data generation over the last few decades due to development and streamlining of technology; these enabled the study of cells with increasing levels of detail and precision, and on scales never before seen. Techniques like mass spectrometry, immunoassays, and x-ray crystallography have facilitated generation of large metabolomic and proteomic datasets [4]. Utilization of this data is instrumental to further advance biological research.

This chapter outlines how to apply systems biology in basic science research, focusing on how to find existing large-scale databases and design high-throughput experiments to generate extensive, high quality data.

Systems Biology Resources

Much of basic science research is based on the fundamental discipline of cell biology and the Central Dogma of biology: a cell's genetic information in the form of DNA is modified and transcribed into RNA and subsequently translated to proteins, which

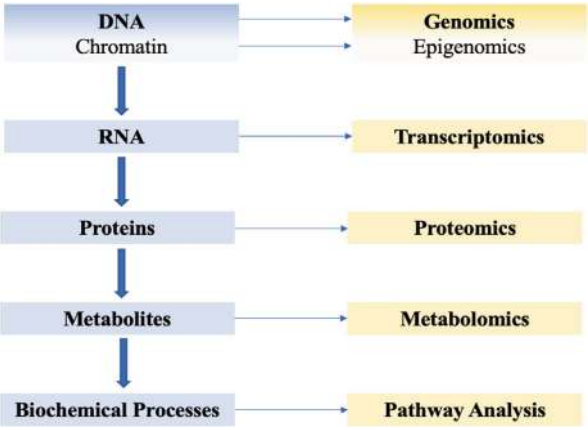

Fig. 19.1 Biological pathways in the cell can be broken down into components, and each of these components can be studied in a large-scale model to generate "omics" data

interact with metabolites that contribute to biochemical processes of the cell. One of the goals of systems biology is to study this pathway in a comprehensive and coordinated manner by analyzing and integrating "omics" data generated from genomic, epigenomic, transcriptomic, proteomic, and metabolomic experiments (Fig. 19.1).

Many bioinformatics databases generated from studies over the past years are available online in the public domain. These databases can be helpful for gathering information, generating hypotheses, and supporting experimental data in basic science and translational research.

Genomic and transcriptomic databases can be useful for finding information about specific genes, comparing expression levels in different cells or tissues, and studying genetic variations and mutations in various stress states. Examples of resources with genomic and transcriptomic information include:

- 1000 Genomes Project (www.internationalgenome.org): comprehensive description of human genetic variations obtained by sequencing genomes from 2504 individuals [5].
- ANTE Oncobox Atlas of Normal Tissue Expression (https://www.nature.com/articles/s41597-019-0043-4 - MOESM1): a collection of transcriptomic profiles of normal tissues for use as reference samples [6].
- ArrayExpress (https://www.ebi.ac.uk/arrayexpress): repository for data from DNA microarray and high-throughput sequencing experiments [7].
- dbSNP (https://www.ncbi.nlm.nih.gov/snp/): database of identified single nucleotide polymorphisms and other genetic variations in humans and other species [8].
- dbVAR (https://www.ncbi.nlm.nih.gov/dbvar/): a collection of more than six million structural variants (inversions, translocations, insertions and deletions), also known as copy number variants [9].
- Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/): repository for microarray, next-generation sequencing, and other forms of high-throughput functional genomics data [10].

– RefSeq (https://www.ncbi.nlm.nih.gov/refseq/about/): a comprehensive set of DNA, transcript, and protein sequences that functions as a reference for gene annotations and analyses [11].

In addition to genomic data, elucidating the cellular proteome is an important cornerstone for a more complete understanding of cell biology and disease as it pertains to systems biology. Advancement in techniques like mass spectrometry and protein microarray has enabled high-throughput studies of proteins and generation of large datasets. Proteomic databases can provide information on protein structures, functions, and interactions:

– Human Protein Atlas (https://www.proteinatlas.org): proteomic database generated from immunohistochemistry and immunocytochemistry experiments [12].
– IntAct (https://www.ebi.ac.uk/intact): database of protein-protein interactions [13].
– PRIDE (https://www.ebi.ac.uk/pride/archive): one of the largest international repositories of mass spectrometry-based proteomic data [14].
– PROSITE (https://prosite.expasy.org): database of protein domains, families, functional sites, and associated patterns and profiles for identification [15].
– Protein Data Bank (http://www.wwpdb.org/): archive for three-dimensional structural data of biological macromolecules including proteins and nucleic acids [16].
– UniProt (www.uniprot.org): database of protein sequence and function [17].

Exploration of metabolite structures, functions, and interactions can also provide unique insight into systems biology. Studying the cellular metabolome is a crucial part of biomedical research as numerous cellular processes involve small molecule metabolites and their complex interactions. Many metabolomics databases generated from mass spectrometry experiments can provide information on metabolites and help analysis of metabolomic data:

– METLIN (https://metlin.scripps.edu/): database of metabolites and other small molecules generated from mass spectrometry experiments with information on each molecule, including their chemical and biological activities [18].
– Small Molecule Pathway Database (www.smpdb.ca): database of small molecule interactions including metabolic pathways, metabolic diseases, metabolite signaling, and drug action pathways [19].
– Human Metabolome Database (www.hmdb.ca): information on small molecule metabolites including compound description, structure, disease associations, pathway information, and gene sequence data [20].

There are also portals that contain many different types of databases that can be cross-referenced. These resources can often be a good starting point for researching a specific topic and guide the development of experiments to test specific

hypotheses. They can also be helpful for integrating different datasets to make connections between different omics data:

- ExPASy (https://www.expasy.org): bioinformatics resource portal that provide various databases and software tools that can be used to research genomics, proteomics, structure analysis, systems biology, evolutionary biology, population genetics, transcriptomics, and medicinal chemistry [21].
- Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/): collection of genomic and metabolic databases with pathway maps for interactions and networks of cellular processes and human diseases [22].
- National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov): collection of databases on DNA, RNA, proteins, and metabolites with information on molecular structures, expressions, maps, variations, etc.

Biomedical research involves a combination of basic science, translational, and clinical research. Applying translational and clinical data can help guide basic science research toward clinically relevant goals. Resources with clinical data on human diseases and drug interactions can be useful for this purpose.

- ChEMBL (https://www.ebi.ac.uk/chembldb): database of drug-like bioactive compounds from published literature and FDA-approved drugs with detailed information on their bioactivity [23].
- Clinicaltrials.gov (https://clinicaltrials.gov): list of clinical trials being conducted around the world.
- DrugBank (https://www.drugbank.ca): database of detailed drug data including comprehensive drug target and drug action information [24].
- Online Mendelian Inheritance in Man (www.omim.org): database of human genes and their genetic phenotypes associated with diseases and disorders [25].

There are many more bioinformatics resources than those listed above that can help guide research, plan experiments, and perform quality control checks. Many portals even allow upload of experimental data by labs to encourage expansion of shared data and knowledge. These resources can provide abundant information and often decrease or eliminate the burden of producing omics data, which can be cost- and labor-intensive.

Generating Big Data

Integrating large-scale databases like those described above using multi-omics approaches can reveal comprehensive insight into complex biological systems including disease mechanisms and drug responses [26]. In order to achieve this, it is necessary to generate appropriate data at multiple levels of cellular processes as described above (Fig. 19.1).

Omics experiments can be resource-intensive as they involve large numbers of comparisons, data points, and statistical analyses. Details of omic-specific data generation are described in the sections below. It is crucial to design the experiments carefully to maximize efficacy and utility. This involves choosing the best assay to compare the most relevant conditions using an appropriate, representative biological model. Samples for omics experiments can be generated from cell culture, organoid, and in vivo models, but each assay and sample type require different sample processing methods which is an important step to ensuring high-quality data. Also, it is crucial to have replicate samples for each experiment to ensure reproducibility of data.

Genomic, Transcriptomic, and Epigenomic Experiments

Genomics is the study of genetic material (DNA)—its structure, function, and variants. While the field kicked off with Sanger chain-termination methods, modern genomics relives heavily on more cost-effective high-throughput methods known broadly as Next-Generation Sequencing (NGS), which includes whole genome sequencing, whole exome sequencing, and targeted sequencing. Illumina Sequencing is perhaps the most well-known platform and uses sequencing-by-synthesis technology to track the addition of labelled nucleotides as the DNA chain is copied. Other platforms like Pacific Biosciences and Oxford Nanopore similarly allow for parallel sequencing and massive generation of high-throughout data [27]. Analysis of genomic data can elucidate genetic markers of disease process by revealing biological pathways and processes that are altered in different disease states. One form of gene expression data can be presented as a matrix to show copy number variations across cell types and even across timepoints; an example is shown in Fig. 19.2a [29]. Here, various amplifications and deletions of regions of chromosomes are depicted and compared between single circulating tumor cells from blood drawn across different timepoints from a single patient with castrate resistant prostate cancer, including periods of stable disease as well as progression.

Transcriptomics analyzes RNA transcripts to inform gene expression in different tissues and conditions. RNA-seq and microarrays are perhaps the most commonly used techniques in this field. RNA-seq involves sequencing mRNA molecules; this method can identify novel transcripts as well as accurately quantify gene expression levels in a sample [27]. Microarray is another high-throughput technology that allows simultaneous measurement of many gene expression levels. It relies fluorescently labelled samples that are hybridized onto plates with thousands of DNA or RNA probes specifically designed to complement genes of interest. The fluorescence is quantified using specialized software to determine the amount of mRNA and gene expression levels. More detail on microarray experiments and their applications can be found in a review article by Kumar [28]. Additionally, transcriptomics can be performed on single cells via single cell RNA sequencing (scRNA-seq), which would provide a whole new dimension of data at the level of the individual cell within a complex system. Protocols are often centered around cell isolation or

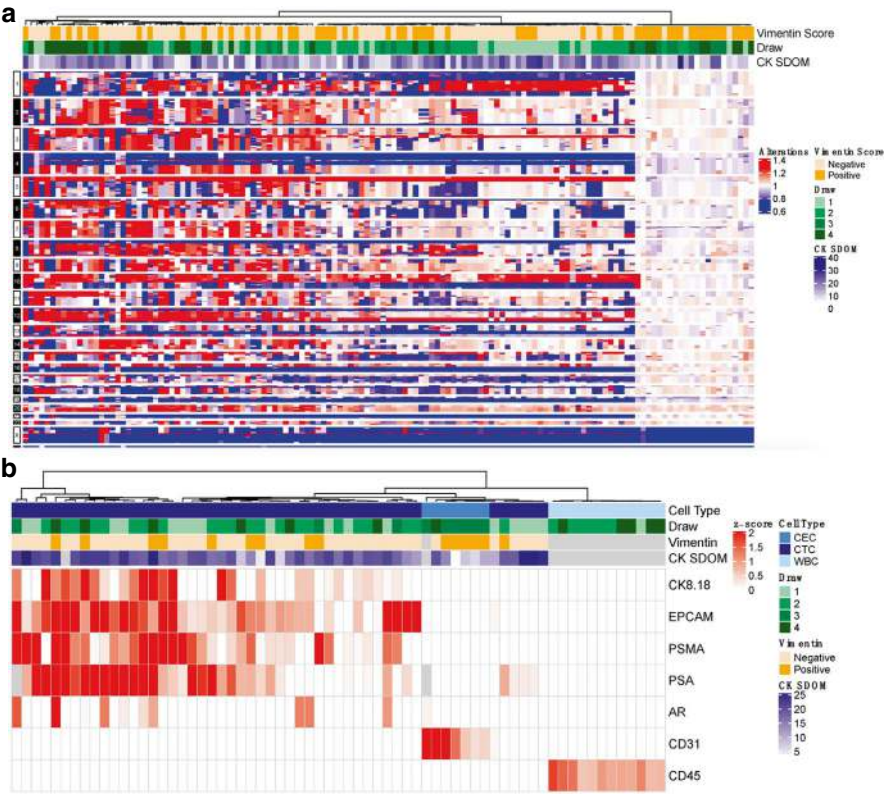

Fig. 19.2 (**a**) Mutational profiling (copy number analysis) from whole-genome sequencing of single cells isolated across multiple timepoints along the clinical course of a metastatic castrate-resistant prostate cancer patient during stable disease and progression of disease. Red indicates amplifications and blue indicates deletions of parts of the indicated chromosomes. [28]. (**b**) Proteomic heatmap of comparing multiplex protein expression of single circulating rare cells and CD45+ white blood cells across four blood draws

capture, cell lysis, reverse transcription, DNA amplification and library preparation [30]. Taken yet a step further with the advent of spatial transcriptomics, gene expression heterogeneity can be investigated within specific cellular environments and distinct tissue. Spatial trancriptomics uses methods like in situ hybridization or spatially resolved RNA sequencing while preserving the spatial coordinates of RNA molecules within a tissue sample. This allows for the mapping of complex tissues like the tumor microenvironment (TME), for example, where differential cell types interact [31]. Of these cell interactions, those involving T-cells, the major contributors of adaptive immunity, are of particular interest. As such, exploring the T-cell receptor (TCR) repertoire can elucidate nuances in the TME during and after treatment and identify immunogenic neoantigens for immunotherapeutic targets. One method to explore this repertoire utilizes TCR sequencing, and this can be performed in bulk or on single cells. Library preparation on these samples most often uses multiplex PCR, with forward primers for V genes and reverse primers for either

J or C genes. Upon completion of the library preparation, the samples can be sequenced, usually using Illumina platforms, which are known to nicely balance reads lengths, depth of analysis and bias profile for most experimental designs [32].

Epigenomics analyzes gene expression by studying the epigenetic modifications of a cell's genome— by DNA methylation, histone modifications, and chromatin remodeling. Epigenetic modifications regulate the packing of chromatin, which directly impacts the function of transcriptional machinery as it interfaces with DNA. This can be studied using array-based techniques that rely on pre-designed microarrays and restriction enzymes that digest only methylate or only unmethylated segments; additional experiments use bisulfite treatments to convert unmethylated cytosines to uracil followed by sequencing, offering single CpG resolution when studying DNA methylation. When studying histone modification, then chromatin immunoprecipitation sequencing (ChIP-seq) is often the method of choice. ChIP-seq is used to identify protein-binding sites on DNA and determine how transcription factors and other DNA-associated proteins affect gene expression. Finally, for studying chromatin accessibility, the main assays include ATAC-seq [33], DNase-seq, and FAIRE-seq, which use open-chromatin digestion modalities to quantify nucleosome positioning to determine the compactness of various genomic regions [34].

Proteomic and Metabolomic Experiments

Both proteome and metabolome serve as important links between the genome and phenotype. High-throughput proteomics and metabolomics reply heavily on the advances made in mass spectrometry technologies. Mass spectrometry can elucidate chemical composition of a sample by using ionization of chemical compounds to generate charged molecules and measure their mass-to-charge ratio. Different techniques can be used to measure amounts of protein, peptides, and metabolites in samples [35]. By studying samples from healthy cells and tissues, as well as diseased and treated specimens, it is possible to elucidate nuances in phenotype in complex biological systems. Although mass spectrometry experiments require specialized equipment and trained experts, many institutions have labs and core facilities that can help plan and perform mass spectrometry experiments. Once experiments are executed, elements that are statistically different in the experimental groups can be presented by generating heat maps (Fig. 19.2b); a review on how to produce heat maps from mass spectrometry data can be found in an article by Key [36].

Data Analysis

Analysis of experimental results that involve evaluating only a handful of variables and hypotheses is a characteristic of more traditional times. Today, when analyzing omics data, scientists face the challenge of simultaneously needing to test thousands

of variables from different from a multitude of interrelated dimensions and systems (e.g., DNA, RNA, protein, metabolites). To meet this need, statistical methods have been developed to enable extraction of the many variables that are differentially expressed between experimental conditions.

One such method includes principal component analysis, a technique of dimensionality reduction and data visualization. After processing the data to account for background noise and normalizing to similar distributions among variables, principal component analysis can be applied to reduce the complexity of the data. Principal component analysis is a multivariate statistical procedure that converts the values of a set of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. This procedure assigns the largest possible variance to the first principal component with the sum of the first few components approaching the total variance of all the variables in the original data. Replacing the original variables with the first components reduces high-dimensional dataspace into fewer dimensions, effectively making data analysis more manageable [37].

Another set of statistical methods important in omics research is analysis of differential expression, which is used to identify differentially expressed transcripts, genes, and proteins between experimental groups. Commonly used statistical tests here include t-tests, ANOVA, and likelihood ratio tests; the test should be selected based on experimental design and distribution assumptions [38]. Rank Product, which uses an algorithm to rank the significant variables based on degree of fold changes, can also be used. With omics data analysis, type I errors are often accounted for by using false discovery rate, which is adjustable to accommodate for an acceptable false positive rate when testing multiple hypotheses [39].

These analyses require computational modeling which can be done by using statistical softwares (many of which are freely available online) like R or Python. Data analysis can also be outsourced to individuals or companies that specialize in omics data analysis.

Data Integration

While studying a single layer of omics data can demonstrate reactive processes caused by a perturbation, integration of multiple layers of omics data can help elucidate important cellular pathways and networks. One way to integrate multiple omics datasets is identify common drivers across the omics layers using cross-reference database portals like KEGG [40]. Doing so can help identify cellular pathways that are up- or downregulated and pinpoint the level at which the changes occur. For example, a biomarker that is elevated at transcriptomic and proteomic levels may not translate to increase in resulting metabolites due to compensations from other pathways. Outlining these patterns from a multi-omics experiment can elucidate the biological processes altered by perturbations like genetic mutation, disease, or treatment. Significant pathways can be presented in network maps.

Limitations of Big Data

Big data is not necessarily good data. Together, advances in data generation with technological jumps, and leaps in the development of tools of analysis, have expanded our reservoirs of study [41]. As such, there is a need, perhaps now more than ever before, of identifying accurate data. Data quality must be ensured when working with these databases, especially now that many offer sharing and upload capabilities. To avoid using inaccurate, incomplete, or biased data, it is necessary to scrutinize the sources and experiments. Along these lines, it is also necessary to address the challenges of data integration. Data may be generated more efficiently now than ever before, but without the ability to integrate diverse data obtained from various experiments and generated in different formats, analyzing the datasets may pose a significant challenge.

Conclusion

The interwoven relationship between biology and pathology are extremely complex and riddled with numerous nonlinear pathways and interrelated systems. The traditional approach of studying biological disciplines in isolation rather than considering their interactions can be prone to missing crucial interactions and mechanisms. Systems biology helps fill the gaps in knowledge by approaching biomedical research from a big-picture angle. Advancement in high-throughput data generation and analysis has helped systems biology become an integral part of biomedical research, as procuring large amounts of data became more efficient and accessible. Continuing development in high-throughput research techniques, computer science, and bioinformatics will lead to bigger role of systems biology in biomedical research.

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