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About the Editors

Buwei Yu is a Professor at the Department of Anesthesiology, Shanghai Ruijin Hospital, Shanghai Jiaotong University School of Medicine, and President of the Chinese Medical Doctor Association and the Society of Anaesthesiologists.

Jiaqiang Zhang is a Professor and Director of the Department of Anaesthesiology and Perioperative Medicine, Henan Provincial People’s Hospital, and a member of the Chinese Medical Association’s Anaesthesia Branch, and the National Committee of the Anaesthesia Branch of the Chinese Medical Association. He is also Vice Chairman of the Anaesthesia Branch of the Henan Medical Association.

His main research focuses include investigating the mechanisms of postoperative cognitive dysfunction, clinical bioinformatics, anesthesia and neurodevelopment, and the physiology of pain. He is the author of more than 150 scientific publications.
Yiming Zeng is a Professor of Respiratory Medicine, Chairman of the Academic Committee of the Second Affiliated Hospital of Fujian Medical University, State Council Expert for Special Allowance, and Director of Fujian Province’s Sleep Medicine Key Laboratory. He was named a middle-aged expert with outstanding contributions from the National Health and Family Planning Commission of the People’s Republic of China (NHFPC). His main research focuses include clinical interventional pulmonology; sleep-breathing disorders; and non-invasive mechanical ventilation. He is the author of more than 150 scientific publications.

Li Li is the Director of Department of Science Research and Discipline Construction, and Principle Investigator of the Clinical Centre of Single-Cell Biomedicine, Henan Provincial People’s Hospital. She is a member of clinical research group of the Chinese Medical Association’s Scientific Research Management Branch, and of the Standing Committee of the Chinese Medical Association’s Henan Research and Management Branch. Further, she is Vice-chairman of Henan Discipline Management Branch of the Chinese Hospital Management Society. She has been engaged in the management of medical scientific research for 30 years, with a focus on big data, laboratory biosafety and medical ethics. She published more than 20 scientific papers.

Xiangdong Wang is a Distinguished Professor of Medicine, Director of Shanghai Institute of Clinical Bioinformatics, Executive Director of Clinical Science Institute of Fudan University Zhongshan Hospital, Director of Fudan University Center of Clinical Bioinformatics, and Deputy Director of Shanghai Respiratory Research Institute. He is also a Visiting Professor at King’s College London. His main research focuses on clinical bioinformatics, disease-specific biomarkers, chronic lung diseases, cancer immunology, and molecular and cellular therapies. He is the author of more than 300 scientific publications.
Understanding Diseases from Single-Cell Sequencing and Methylation

Buwei Yu, Li Li, Jiaqiang Zhang, Xiangdong Wang, and Yiming Zeng

Abstract

Clinical single-cell biomedicine has become a new emerging discipline, which integrates single-cell RNA and DNA sequencing, proteomics, and functions with clinical phenomes, therapeutic responses, and prognosis. It is of great value to discover disease-, phenome-, and therapy-specific diagnostic biomarkers and therapeutic targets on the basis of the principle of clinical single-cell biomedicine. This book reviews the roles of single-cell sequencing and methylation in diseases and explores disease-specific alterations of single-cell sequencing and methylation, especially focusing on potential applications of methodologies on human single-cell sequencing and methylation, on potential correlations between those changes with pulmonary diseases, and on potential roles of signaling pathways that cause heterogeneous cellular responses during treatment. This book also emphasizes the importance of methodologies in clinical practice and application, the potential of perspectives, challenges and solutions, and the significance of single-cell preparation standardization. Alterations of DNA and RNA methylation, demethylation in lung diseases, and a deep knowledge about the regulation and function of target gene methylation for diagnosing and treating diseases at the early stage are also provided. Importantly, this book aims to apply the measurement of single-cell sequencing and methylation for clinical diagnosis and treatment and to understand clinical values of those parameters and to headline and foresee the potential values of the application of single-cell sequencing in non-cancer diseases.
Keywords
Clinical single-cell biomedicine · Single-cell sequencing and methylation · Pulmonary diseases · Methodologies · Clinical diagnosis and treatment

With the rapid development of single-cell biology and sequencing, clinical single-cell biomedicine is defined a new merging discipline to integrate single-cell RNA and DNA sequencing, proteomics, and functions with clinical phenomes, responses to therapies, and prognosis. Several hospitals start a new independent practice to perform clinical single-cell biomedicine, although there are still many challenges to be faced and solved. Clinical single-cell biomedicine is more expected to dynamically monitor cell–cell variations and communications, drug efficacy and resistances, discovery and development of therapeutic targets, and genealogic phenotypes of cells during disease progression [1–3]. Clinical single-cell biomedicine will analyze inter- and intra-cellular heterogeneity, new cell category, dysfunctional regulatory networks, microbes, and disease evolution. In addition to understanding molecular mechanisms using single-cell sequencing and measurements, it is more important to discover disease-, phenome-, and therapy-specific diagnostic biomarkers and therapeutic targets on the basis of the principle of clinical single-cell biomedicine. As the part of clinical single-cell biomedicine, we demonstrated important roles of single-cell sequencing in systems immunology in our previous book entitled “Single Cell Sequencing and Systems Immunology” [4], e.g., as a tool to deeply understanding the development and regulation of systems immunology. In this book, we furthermore overviewed the roles of single-cell sequencing and methylation in diseases and explored disease-specific alterations of single-cell sequencing and methylation. This book specially focuses on potential applications of methodologies on human single-cell sequencing and methylation, on potential correlations between those changes with pulmonary diseases, e.g., lung cancer, chronic lung diseases, and allergic lung diseases, and on potential roles of signaling pathways that cause heterogeneous cellular responses during treatment.

The first part of the book emphasizes the importance of methodologies in clinical practice and application, the potential of perspectives, challenges and solutions, and the significance of single-cell preparation standardization. Pensold and Zimmer-Bensch [5] headlined the importance of accurate and reliable cell capturing in single-cell sequencing, overviewed the current state of single-cell isolation methods, and addressed key parameters like sample compatibility, viability, purity, throughput, and isolation efficiency. Gupta et al. [6] systematically described the value of single-cell sequencing in the investigation of T cell receptors and their transcriptional profiles and firstly prospected the importance of the technological development in translational and clinical application. This is an example to apply the single-cell sequencing for special target clusters in a special cell population and illustrate the translational strategy how the single-cell sequencing is developed for clinical application. The single-cell sequencing of T cell receptors has the great value to benefit immune-therapy for cancer and autoimmune diseases.

The methylation and demethylation of cytosine in promoter regions play an important role in the control and regulation of gene expression by the modulation of translation by modifying tRNA-bases or silencing. The process of the methylation within cells can be influenced by their environment or for the development of complex organisms, especially for organs/tissues which are exposed and connected directly to the environment, e.g., lung. This book discusses alterations of DNA and RNA methylation and demethylation in lung diseases and provides the deep knowledge about the regulation and function of target gene methylation for diagnosing and treating diseases at the early stage. Zhou et al. [7] demonstrated global methylation pattern and specific gene methylation status of associated genes in the development of pulmonary fibrosis and methylation patterns and severities of the promoter regions of Thy-1, COX-2, p14ARF, and PTGER2 genes as disease-specific biomarkers to
predict the occurrence and development of the disease. Using bioinformatics, Liu et al. [8] addressed that altered methylations of inflammatory cells downregulated the gene expression of inflammatory mediators and initiated the occurrence of lung diseases. The combination of expression quantitative trait loci and genome-wide association studies was suggested as a new strategy to identify alterations of target gene methylation in chronic lung diseases, e.g., lung fibrosis [9] or chronic obstructive pulmonary diseases [10]. Of many target genes, DNA methylation of RAS-association domain family 1 was proposed as a lung cancer biomarker for new therapeutic strategies and for monitoring the reliability and sensitivity of DNA methylation [11].

One of the important issues in this book is to apply the measurement of single-cell sequencing and methylation for clinical diagnosis and treatment and to understand clinical values of those parameters. Wu et al. clearly reported the urgent need to optimize and standardize the workflow and protocol as well as standard operation performance, the comprehensive single-cell database and knowledgebase, and the design of clinical studies among various hospitals during clinical application [12]. The importance of target gene methylation and expression phenomes, e.g., Aplasia Ras homologue member I [13], P16 gene [14], and related molecular mechanisms of tumorigenesis and progression in various types of cancers, is obvious. Of those, single-cell RNA sequencing can be utilized to identify subtypes of pancreatic cancer [15] and genitourinary malignancies [16] and to improve the quality, efficiency, and specificity of cancer diagnostics [17]. In addition, new therapeutic targets and strategies can be discovered and developed with the improvement of methodologies and knowledge on single-cell sequencing and methylation. Duncan et al. offered an example of PI3K inhibitors and a frontline view of biological effects of the PI3K pathway and multiple isoforms of PI3K, mutations found in the PI3K isoforms in many different types of cancer, and new strategy of combination therapies between PI3K inhibitors and other target-driven therapies [18].

One of advances in this book is to headline and foresee the potential values of the application of single-cell sequencing in non-cancer diseases, which will be the frontline science and need more efforts to be explored. Garcia et al. provided the comprehensive understanding of single-cell RNA sequencing in human renal, pancreatic, and viral diseases [19]. This is an important and expecting review to discuss the specific application of single-cell sequencing in cellular compositions, heterogeneity and uncovering clues of viral infections and diseases of the kidney and pancreas for the development of targeted and personalized therapies. Singh specially emphasized the importance of single-cell sequencing in the discovery of the drug resistance clone, intercellular variation and communication, mutations and transcriptional profiles of a pathogen across different stages of human genital infections [20]. Rajan and Dall’Acqua addressed the potentials of those advanced technologies in the discovery and development of antibody-based humanized therapies [21]. Single B cell sequencing will provide a new approach and emerging strategy for antibody-based therapy. Chang et al. summarized the potential application and values of single-cell sequencing in the development of neurological cells and microglia as well as single-cell changes during brain injury [22].

This book is one of initiatives to deeply understand the importance and value of single-cell sequencing and methylation measurement for clinical application, although there are still many challenges and obstacles to be broken through. It is also highly expected to translate the simultaneous measurement of both single-cell sequencing and methylation in a human cell, e.g., parallel single-cell genome-wide methylome and transcriptome sequencing. There is a rapid growth in the development and improvement of single-cell methylation and sequencing, e.g., single-cell bisulfite sequencing for genome-wide base-resolution mapping of single-cell DNA methylation, random displacement amplification sequencing for the first full-length single-cell RNA-sequencing method, single-cell and single-base resolution DNA methylation analysis based on reduced-representation bisulfite sequencing,
and single-cell, locus-specific bisulfite sequencing for cell-to-cell variability and the pathogenic history. Complete DNA CpG methylomes at the single cell can be screened and compared comprehensively through whole genome bisulfite sequencing, reduced-representation bisulfite sequencing, and enrichment-based methods such as MeDIP-seq, MBD-seq, and MRE-seq. At the end, we as co-editors of this special book would like to take this special opportunity to deeply appreciate all authors and contributors for the intensive and hard works to make this book possible for publication. We are especially grateful for those experts to review and comment chapters in order to maintain the high quality and look forward to working with all of you in future.

References


Buwei Yu is a professor in the Department of Anesthesiology at Shanghai Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine and is President of the Chinese Medical Doctor Association and the Society of Anesthesiologists.

Li Li is director of the Department of Science Research and Discipline Construction and Principal Investigator of Clinical Centre of Single-Cell Biomedicine, Henan Provincial People’s Hospital. She is a member of clinical research group of Chinese Medical Association’s Scientific Research Management Branch, Standing Committee member of Chinese Medical Association’s Henan Research and Management Branch, and vice-chairman of Henan Discipline Management Branch of Chinese Hospital Management Society. She has engaged in the management of medical scientific research for 30 years, and her main research is focused on health management scientific research big data, laboratory biosafety, and medical ethics. She has published more than 20 scientific papers.

Jiaqiang Zhang is professor and director of the Department of Anesthesiology and Perioperative Medicine at Henan Provincial People’s Hospital and is a member of Chinese Medical Association Anesthesia Branch and the National Committee of the Anesthesia Branch of the Chinese Medical Association. He is vice-chairman of the Anesthesia Branch of the Henan Medical Association. His main research is focused on investigating the mechanism of postoperative cognitive dysfunction, clinical bioinformatics, anesthesia and neurodevelopment, and physiology of pain. He is the author of more than 150 scientific publications.
Xiaodong Wang is a distinguished professor of medicine, director of Shanghai Institute of Clinical Bioinformatics, executive director of Clinical Science Institute of Fudan University Zhongshan Hospital, director of Fudan University Center of Clinical Bioinformatics, deputy director of Shanghai Respiratory Research Institute, and visiting professor of King’s College London. His main research is focused on clinical bioinformatics, disease-specific biomarkers, lung chronic diseases, cancer immunology, and molecular and cellular therapies. He is the author of more than 300 scientific publications with the impact factor about 900, citation number about 6920, h-index 48, i10-index 221, and cited journal impact factor about 8000.

Yiming Zeng is a professor of Respiratory Medicine, chairman of Academic Committee of the Second Affiliated Hospital of Fujian Medical University, State Council Expert for Special Allowance, and director of Sleep Medicine Key Laboratory of Fujian Province. He achieved the Outstanding Contribution of Middle-aged Expert of National Health and Family Planning Commission of the People’s Republic of China (NHFPC). His main research is focused on clinical, interventional pulmonology, sleep-breathing disorders, and noninvasive mechanical ventilation. He is the author of more than 150 scientific publications.
Methods for Single-Cell Isolation and Preparation

Daniel Pensold and Geraldine Zimmer-Bensch

Abstract

Within the last decade, single-cell analysis has revolutionized our understanding of cellular processes and heterogeneity across all disciplines of life science. As the transcriptome, genome, or epigenome of individual cells can nowadays be analyzed at low cost and in high-throughput within a few days by modern techniques, tremendous improvements in disease diagnosis on the one hand and the investigation of disease-relevant mechanisms on the other were achieved so far. This relies on the parallel development of reliable cell capturing and single-cell sequencing approaches that have paved the way for comprehensive single-cell studies. Apart from single-cell isolation methods in high-throughput, a variety of methods with distinct specializations were developed, allowing for correlation of transcriptomics with cellular parameters like electrophysiology or morphology.

For all single-cell-based approaches, accurate and reliable isolation with proper quality controls is prerequisite, whereby different options exist dependent on sample type and tissue properties. Careful consideration of an appropriate method is required to avoid incorrect or biased data that may lead to misinterpretations.

In this chapter, we will provide a broad overview of the current state of the art in matters of single-cell isolation methods mostly applied for sequencing-based downstream analysis, and their respective advantages and drawbacks. Distinct technologies will be discussed in detail addressing key parameters like sample compatibility, viability, purity, throughput, and isolation efficiency.

Keywords

Single-cell isolation · Tissue dissociation · Dilution · Micromanipulation · Laser microdissection · FACS · Microfluidic

In recent years, rapid technological development and improvements have been achieved in the field of high-throughput sequencing enabling diverse applications due to significant drop of costs. Together with methodological advances especially in the field of single-cell isolation, this has paved the way for reliable single-cell-based analysis in high-throughput. Still, tissue dissociation, single-cell separation and isolation represent arguably the greatest source of technical variation, contamination, and batch effects in any single-cell study [1], and hence represent the key determinants for a successful experimental design. There are diverse approaches described to isolate material for single-cell omics with
different advantages and limitations for protein, RNA, or DNA analysis [2–4]. In this chapter, we will mainly discuss methods aiming at RNA and DNA isolation for sequencing-based analysis.

According to a survey about the German market carried out in 2014 [2], the most frequently used approaches for single-cell isolation were fluorescence-activated cell sorting (FACS, 33%), manual micromanipulation (17%), laser microdissection (17%), random seeding or serial limiting dilution (15%), and microfluidics/lab-on-a-chip methods (12%). Other technologies including optical tweezers, dielectrophoresis, or non-contact depending methods were less frequently reported (in total 6%). The requirements for technologies to separate and isolate single cells from specimens of different nature are as diverse as the purpose for which the cells are used in downstream processing and analysis. So far, no available method suits all demands. When only considering the isolation process in matters of efficiency and cell viability already numerous factors including cell type, sample preparation, device calibration, sorting mode, and substrate are of high relevance, in addition to other factors that are rather hard to quantify (e.g., operator skills, Fig. 2.1). The following paragraph seeks to cover the most general requirements for many of the approaches discussed in more detail in the following chapters, helping to choose the method of choice.

2.1 Parameters to Be Considered

Typically, specimen type and origin define to a great extent which technology is best applicable for sample preparation. Most methods described here require cells in suspension for separation and isolation procedures. Thus, all samples easily dissociable, or liquid materials like immunological organs such as peripheral blood, spleen, or lymph nodes called liquid biopsies, are straightforward to handle often requiring only concentration of the material. However, commonly samples originate from solid and complex tissues requiring chemical or enzymatic as well as mechanical dissociation of the cells.

Moreover, downstream analysis largely dictates the method of choice for sample preparation. Independent of the targeted information level ranging from genomics, epigenomics, transcriptomics to proteomics, cell integrity and viability has to be ensured, to avoid early degradation of DNA, RNA, or proteins, respectively. Stress factors like mechanical forces, radiation, chemical changes in the cellular environment, etc. may alter the intrinsic cellular states, e.g., by inducing differentiation or apoptosis [5–7]. Further relevant considerations refer to the purity of the isolated single cells to avoid potential contamination with cell fragments, free DNA or RNA molecules. For some experiments, cultivation after cell separation is intended, requesting sterile operational conditions, which can be achieved in some approaches by using disposable components (e.g., microfluidic chips).

Throughput in terms of single cells isolated per second and the total number of single cells are further crucial parameters. Low-throughput applications in the range of one to several hundreds of cells are usually performed manually or half-automated using systems like Fluidigm C1 (South San Francisco, CA, USA). Thereby, most manual approaches such as micromanipulation, optical or acoustic tweezers work with high precision, care, or high efficiency. Low-throughput methods apply to samples evident in low cell numbers, which require small sample volumes and low dead volumes as critical parameters.

High-throughput approaches enable capturing of cells to a range of several thousands. Such approaches may also be applied to detect under-represented cell types like CTCs (circulating tumor cells) as a rare cell population within billions of different blood cells [8]. High-throughput methods like FACS often rely on strong sample dilution with the final sample volume being usually large, which impacts downstream processing. Another key issue is the separation yield, cell-capture or isolation efficiency in regard to the total input. Especially for low-abundant samples or rare biopsies, a maximum in capturing rate is essential [2, 9, 10]. Actually, most of the common high-throughput technologies such as FACS or droplet generation
have their strongest limitation in terms of precision, cell-capture efficiency, and vitality of cells after isolation.

The economical aspect including all costs from sample acquisition, dissociation, separation, isolation to amplification and library preparation for next-generation sequencing [11] represents another issue influencing the method of choice. Of course, analysis of low numbers of particularly selected cells will be in total cheaper than analyzing thousands of randomly chosen ones. However, costs per cell decline with throughput, which significantly dropped within the last years and which is expected to progress. The cost per cell nowadays varies depending on the approach and facility between less than $0.30 for some customized microfluidic systems up to $1 to $2 for early-indexing plate-based 3′-RNA sequencing methods. Manual methods and late-indexing full-length transcriptome profiling often require separate sample processing, consequently increasing costs (starting about $8–12 per cell) and time.

An overview of the different parameters that determine the choice of a particular approach is summarized in Table 2.1. All these and other parameters force operators to choose a certain compromise for the experimental design to match the goal of each individual study. As follows, we will discuss specific features, applications, and limitations of a broad spectrum of single-cell isolation methods.
2.2 Cell Dissociation and Enrichment

First, the protocol for dissociation and isolation needs to be carefully optimized to ensure reliable and valid data, aiming to keep original conditions to the greatest possible extent (Fig. 2.1). Tissues vary significantly in extracellular matrix (ECM) composition, cellularity, and stiffness, which influences the requirements for dissociation protocols. Dissociation is often achieved by enzymatic digestion and/or mechanical dissociation, which both can lead to activation of stress-related genes [5]. General prerequisites for a successful and mild dissociation with minimized procedure-induced intrinsic changes include a low time scale, working on ice whenever possible, keeping pipetting and centrifugation to a minimum, as well as using calcium and magnesium-free but bovine serum albumin-containing buffers and media. Cell lysis-induced extracellular DNA can be diminished by DNase I application during cell separation.

Enzymatic digestion is commonly conducted by trypsin, collagenase, protease, acutase, elastase, or dispase treatment as well as by commercial enzymatic mixtures such as TrypLE Express and Liberase Blendzyme 3, depending on the composition of the extracellular matrix and cell–cell connections in the different tissues. The application of cold active enzymes like proteases from Bacillus licheniformis thereby helps to reduce heat stress caused by enzymatic digestion at 37 °C [12]. The careful adjustment of enzymatic incubation duration for each sample type is crucial, as extended incubation times may induce cell lysis of fragile cell types. Short incubation in turn, bears the risk of incomplete cell separation and the exclusion of large cell clusters during subsequent filter steps, potentially leading to bias in cell composition [13]. Of note, all enzymatic treatments may affect the transcriptome of single cells through cellular uptake or by altering intercellular communications [14, 15].

For mechanical dissociation fire-polished glass capillaries with cell size and tissue input-adjusted tip sizes are usually applied. The frequency of pipetting represents another critical parameter strongly impacting cell viability and dissociation effectiveness. The avoidance of air bubbles during all pipetting steps is essential as oxidative stress is one of the major sources for cell death. The extracellular matrix and also damaged or dead cells are the largest source for contamination, e.g., by inducing free RNA and DNA, which affect subsequent downstream processing, especially for sequencing approaches. Including a density gradient centrifugation may help to reduce debris as well as free RNA and DNA molecules but may simultaneously result in biased recovery of the remaining cell populations. Another way to remove clumps and debris from suspensions is to filter with appropriately sized cell strainers. However, the produced suspension should be processed as soon as possible, ideally within 30 min after dissociation, to avoid re-aggregation of cells or induction of transcriptional changes. Otherwise, loss of cellular functions, cell–cell interactions, and the tissue architecture may lead to transcriptional adaptation [16].

Recently, innovative microfluidic cell dissociation devices have been fabricated that may drastically change the way solid tissue samples are processed into single cells, allowing for automated processes [17]. Such new technologies avoid inter-assay variation occurring from differences in handling of the tissue. Included microfluidic structures have been optimized for straightforward tissue digestion, cell dissociation, filtering, and polishing, mainly by passing the tissue sequentially through progressively smaller size scales. Tissue-specific kits can be designed to integrate differences in ECM and interconnectivity of cells, improving reproducibility and efficiency of single-cell preparations [18, 19].

Even if performed automatically, tissue dissociation accounts as a major source of variation in single-cell analysis, for which applicable methods using preserved samples [20–22] or nuclear RNA/DNA [23–26] represent an attractive alternative. Nuclear RNA enrichment based on cellular membrane disruption [23–26] has been shown to be sufficient to capture cell type information [26]. However, the overall resolution per cell is
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Limiting dilution</th>
<th>Micromanipulation</th>
<th>Automated (partially microfluidic)</th>
<th>LCM</th>
<th>FACS</th>
<th>Microfluidic</th>
<th>Hydrodynamic</th>
<th>Droplet</th>
<th>Dielectrophoresis</th>
<th>Optical and acoustic tweezers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample type</td>
<td>Living</td>
<td>Living</td>
<td>Living</td>
<td>Living or fixed (e.g., INTACT)</td>
<td>Living</td>
<td>Living</td>
<td>Living</td>
<td>Living</td>
<td>Living</td>
<td>Living</td>
</tr>
<tr>
<td>Sample requirements</td>
<td>D or LB</td>
<td>D or LB</td>
<td>Tissue, D or LB, homogeneous or heterogeneous</td>
<td>Tissue</td>
<td>D or LB, heterogeneous</td>
<td>D or LB, homogeneous</td>
<td>D or LB, homogeneous</td>
<td>D or LB, homogeneous</td>
<td>D or LB, homogeneous</td>
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<tr>
<td>Input amount</td>
<td>Large</td>
<td>Small</td>
<td>Small</td>
<td>Moderate</td>
<td>Large</td>
<td>Large</td>
<td>Large</td>
<td>Large</td>
<td>Moderate</td>
<td></td>
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<tr>
<td>Cell stress</td>
<td>Gentle</td>
<td>Gentle to moderate</td>
<td>Gentle to moderate</td>
<td>Often impaired</td>
<td>Often impaired</td>
<td>Moderate</td>
<td>Diverse</td>
<td>Moderate</td>
<td>Moderate</td>
<td></td>
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<td>Enrichment bias</td>
<td>No</td>
<td>User selected</td>
<td>User selected</td>
<td>Diverse</td>
<td>User selected</td>
<td>Cell size and shape</td>
<td>Cell size and shape</td>
<td>Cell size and shape</td>
<td>Yes, user selected</td>
<td></td>
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<tr>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes/No</td>
<td>Typically not</td>
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<td></td>
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<td>Moderate</td>
<td>Very low</td>
<td>Very low</td>
<td>Low to high</td>
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<td>High</td>
<td>Low to high</td>
<td>Moderate</td>
<td>Very high</td>
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<tr>
<td>Precision, Accuracy</td>
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<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Yes/No</td>
<td>Yes/No</td>
<td>NA (Poisson)</td>
<td>Moderate to high</td>
<td></td>
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<td>Doublet Rate</td>
<td>Low</td>
<td>NA</td>
<td>Low</td>
<td>2–3%</td>
<td>2–40%</td>
<td>3–30%</td>
<td>4–12%</td>
<td>Up to 50%</td>
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<td>Singlet capture rate</td>
<td>NA</td>
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<td>High</td>
<td>NA &gt;80%</td>
<td>2–40%</td>
<td>Cross contamination by free RNA/DNA</td>
<td>Cross contamination by free RNA/DNA</td>
<td>Cross contamination by free RNA/DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separation yield, cell-capture or isolation efficiency</td>
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<td>High</td>
<td>High</td>
<td>Moderate to high</td>
<td>High</td>
<td>Low to moderate</td>
<td>Moderate</td>
<td>Low to moderate</td>
<td>Moderate</td>
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<tr>
<td>Purity/ Contamination risk</td>
<td>High false positive rate</td>
<td>Mechanical shearing damage</td>
<td>Diverse, require high user skills</td>
<td>Potential mechanical shearing damage</td>
<td>Co-isolation of surrounding material</td>
<td>Potential cell damage</td>
<td>Cross contamination by free RNA/DNA</td>
<td>Cross contamination by free RNA/DNA</td>
<td>Cross contamination by free RNA/DNA</td>
<td></td>
</tr>
<tr>
<td>Automation</td>
<td>Yes and No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes and No</td>
<td>Yes</td>
<td>Yes and No</td>
<td></td>
</tr>
<tr>
<td>Commercial</td>
<td>CellenONE, WOLF CellSorter, CellSelector, Single-Cell Printer</td>
<td>Diverse</td>
<td>Diverse</td>
<td>C1, MicrowellChip, SeqWell, Nanogrid, BD Rhapsody</td>
<td>Chromium, Dolomite Nadia, ddSeq</td>
<td>DEPArray</td>
<td></td>
<td></td>
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<tr>
<td>References</td>
<td>[2] [45, 47, 49]</td>
<td>[45, 63]</td>
<td>[2, 45, 97]</td>
<td>[75] [102]</td>
<td>[2, 97, 117]</td>
<td>[2, 97]</td>
<td>[2, 27, 116, 117]</td>
<td>[120]</td>
<td>[138]</td>
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reduced and the RNA is biased towards the nuclear fraction. This approach has been extensively applied for epigenetic and transcriptomic profiling of differentiated neurons, as adult neurons are difficult to dissociate reliably due to their axonal and dendritic processes, which are damaged or destroyed during the dissociation process [27, 28]. A major advantage of single nucleus RNA sequencing is that it can be applied to frozen and fixed tissue [20, 21, 29–32], enabling simultaneous processing of samples acquired at different time points, thereby minimizing technically induced batch effects.

For many applications, the enrichment of particular cell types after dissociation is beneficial or even essential for downstream analysis (Fig. 2.1). Sedimentation or density centrifugation during the dissociation process separates cells based on cell size and density. Further, mechanical filtering, hydrodynamic, electrokinetic or acoustophoresis approaches can be applied [33, 34]. Very common are immune-capture methods like magnetic bead-based immune selection (MACS) or immunofluorescence using FACS, facilitating rare cell populations to be analyzed at single-cell level without the necessity to sequence several thousands of cells. This was for example applied to enrich and analyze hematopoietic stem cells from red bone marrow [35, 36]. Still, an a priori immune selection of living cells for FACS or MACS is commonly based on cell surface receptor labeling that may induce intracellular signaling upon antibody or ligand binding [37, 38]. Most of these methods are capable of positive enrichment of cell types of interest or negative depletion of unwanted cells. Apoptosis markers and fluorescent DNA intercalating agents are applied to label and sort out damaged cells. A specific enrichment can also be achieved by induced expression of fluorescent reporter genes, which enables the identification of a particular cell type without immune-labeling. Reporter gene expression can either be driven by lineage-specific promoters, or engineered to be co-expressed with any protein of interest. A more advanced approach identifies individual cells based on the microanatomical location. For that, fluorescent reporters being either photoactivatable [39–41] or photoconvertible [42–44] are used to precisely mark cells of interest by two-photon microscopy. By linking such reporters to cell type-specific expression, cells can be precisely identified based on expression markers and additionally based on specific microanatomical locations within a tissue prior to dissociation. Recently, this method was applied to perform NICHE-seq, systematically characterizing the cellular composition of the spleen among other immune niches [40]. However, such enrichment approaches come with the disadvantage of additional steps and time, which in turn influence the intrinsic cellular state and survival.

Due to the overall cost and time-intensive process of single-cell sequencing experiments, careful quality control and monitoring is required (Fig. 2.1). The performance of the different approaches can be assessed by the use of several readouts presented in the following paragraphs. Although most of the relevant parameters for quality control are related to sequencing results and will be discussed elsewhere, quality control can and needs to be included already during isolation to minimize the proportion of low-quality cells. Most importantly, attention should be paid to the avoidance of cell duplets or even multiple cells, as well as dead cells in the cell capture step, as they remain difficult to assess in downstream analysis. Imaging-based approaches are valuable tools to control for the successful isolation and viability of single cells. Flow cytometry like FACS is particularly useful to measure several critical metrics simultaneously, such as cell viability, and rates of doublets and small cell clusters. The introduction of artificial spike-in RNAs during the isolation process (External RNA Controls Consortium (ERCC) standards [45] or Sequin standards [46]) helps to calibrate measurements and account for technical variability during subsequent sequencing data analysis.

In summary, single-cell preparation requires numerous considerations and careful optimization, to choose the most appropriate protocol (Fig. 2.1). Enrichment of certain cell types is frequently required but also comes with
drawbacks that need to be considered. In the following paragraphs, we will discuss the limitations and advantages of particular single-cell isolation methods in more detail.

### 2.3 Dilution-Based Single-Cell Isolation

Laboratories and companies all over the world have used serial limited dilution for decades to isolate single cells (Table 2.1). Nowadays, this method experiences a renaissance being implemented in automated pipetting robots that tremendously reduce personnel workload. In principle, many technologies, even the microfluidic traps rely on the simple fact that due to statistical distribution of cells in a suspension, the number of cells in a highly diluted sample will be at some point so low that individual cells will be present in a targeted small volume (Fig. 2.2a).

Serial limited dilutions are indeed easy to carry out with standard laboratory inventory, and with the help of automation by pipetting robots it is a simple, reproducible and relative cost-efficient method. Therefore, many pharmaceutical companies still rely on fully automated serial dilution solutions. However, due to the lack of control, it is not possible to isolate specific cell types with this method per se, but it can be combined with upstream sorting or enrichment techniques. It further has to be considered that serial limited dilutions are prone to high false positive rates and to exclusion of cells of interest. Hence, it is widely applied to microbial samples, but less applicable for isolation of cells from complex or rare specimens [47].

### 2.4 Isolation by Micromanipulation

In the past, manual micromanipulation was one of the mostly applied isolation approaches, nowadays still being useful for particular applications. Albeit time consuming and labor intensive, this method provides clear advantages in matters of isolation precision (Table 2.1) [48–53]. Two of the first single-cell DNA or RNA isolation methods used glass capillaries to harvest either the whole cell, which assures complete isolation and minimizes loss of cell material [54–57], or the cytoplasm by patch clamping [58, 59].

A common setup for manual cell isolation typically consists of an inverted microscope equipped with micropipettes that are coupled to a micromanipulator, controlled by motorized mechanical stages (Fig. 2.2b). Piston systems coupled to glass capillaries enable reliable and precise respiration and dispensation [54, 56]. The tip opening diameter of the glass capillaries is adjusted by electrode pullers and sharp edges are polished by heat-induced melting to avoid mechanical shearing of cells. Silanization of the micropipette prevents sticking of cells, or RNA and DNA molecules to the glass capillary [49, 51].

Cells are usually provided in suspension under a microscope allowing the operator to select for specific target cells according to optical parameters like size, shape and granularity, reporter expression or cell surface labeling (Fig. 2.2b) [49]. Such targeted isolation of a particular cell under visual control represent a crucial advantage of this approach. The target cell is aspirated into the micropipette and transferred to a new reaction tube, being released by dispensation together with the aspirated liquid volume. However, the comparatively high volume of suspension needed for cell harvesting represents a major drawback [56], as it might contain contaminants of extracellular RNA and DNA resulting from cell damage [56]. Many protocols therefore suggest several washing steps prior to cell lysis to ensure contamination-free isolation [49–51].

Although being a very flexible approach in regard to cell types and substrates, the manual process of obtaining single cells by micromanipulation limits the overall throughput (3–8 cells/h) rising the risk of intrinsic changes like transcriptomic profiles caused by the extended handling time [2, 16, 60]. In addition, the low throughput limits the applicability to studies of cellular heterogeneity requiring numerous single cells. There are some commercial technologies...
that automated the isolation to some degree and hence, increased the throughput. The ALS CellSelector is a freely configurable pipetting robot for automated transfer of single cells out of solution into a well-plate format (e.g., 96-well plate). Other automated micromanipulation systems allowing for higher throughput in single-cell isolation are based on acoustic microdispensing systems (CellenONE, Cellenion) or piezoelectric approaches (WOLF Cell Sorter, NanoCellect Biomedical or Single-Cell Printer, Cytena). The CellenONE system, integrating the microcapillary dispersion technology with constant imaging of the target cells, offers a new, innovative approach to isolate cells gently, rapidly and with high precision, applicable for downstream single-cell sequencing. Systems based on piezoelectric actuation utilize a hydrodynamic pressure pulse within a microchannel to manipulate single cells [61]. Commercialized systems like the WOLF Cell Sorter combine piezoelectric actuation with laser detection system comparable to FACS on a microfluidic scale [62]. With a response time of ~0.1 ms, more than 1000 cells can be sorted per second, being collected in 96- to 384-well plates. The Single-Cell Printer (Cytena) includes an automated image analysis system for detection and generates droplets containing a single cell using a microfluidic drop-on-demand dispenser chip [2]. The piezoelectric actuator raises the pressure inside the chip to generate a single droplet, thereby ejecting droplets through a nozzle encapsulating single cells [63], whereby unwanted droplets are sorted out enabled by image analysis. The system was shown to operate with >80% efficiency and 90% viability at optimal sample concentration of $6.2 \times 10^5$ cells/mL.

Alike whole cell harvesting, manual isolation of cellular material by patch-clamp approaches is time consuming and limited in throughput (Fig. 2.2b) [64–66]. It is frequently combined with electrophysiological recordings coming with the great advantage of known position and electrophysiological properties of cells. The tip of the glass capillary used for recording as well as isolation of the cell’s material is much smaller and normally produces a tight sealing in a gigaohm range between the cell membrane and the tip. This ensures that even small currents across the membrane are captured while creating a barrier between the extracellular fluids and the cytoplasm. Upon finishing the electrophysiological recordings, a sub-atmospheric pressure is applied inside the glass capillary to rupture the cell membrane in the patch allowing for harvesting of the cytoplasm. The well-trained operator needs to collect as much cytoplasmic content as possible without destroying the cell. This approach comes with high sample-to-sample variation hampering quantitative analysis. Similar to whole cell harvesting, the micropipettes are often silanized to avoid loss of sample and contamination from surrounding cells and ECM [56].
For both described manual approaches, the monitoring of the successful transfer of the isolated material to the final reaction tubes is rather difficult, as this step requires leaving the focus plane with the glass capillary. Recent technologies improved on that by fully automated isolation and placement of single cells assisted by video systems and image processing algorithms [67].

Despite the mentioned limitations, applications of these methods are diverse, ranging from bacterial analysis [68, 69] to reproductive medicine [70], forensics [71], endosymbionts from termite gut analysis [72], and crenarchaeota from soil [73], and becoming especially attractive for samples with limited cell numbers or fragile cells types. Manual approaches can easily be combined with pre-enrichment methods or fluorescent labeling, like it was applied by Ramskold et al. [74] to isolate circulating tumor cells from the blood of a melanoma patient.

2.5 Laser Dissection

An alternative method of manual isolation is laser capture microdissection (LCM) or laser microbeam microdissection (LMM), an advanced technique to collect individual cells or cell compartments from usually solid tissue samples under visual control (Fig. 2.3a) [75–77]. This is especially applicable for samples or biopsies being less amenable to single-cell suspension dissociation [78]. Samples are typically provided as formalin-fixed paraffin-embedded or cryo-fixed tissues [79]. The Leica LMD7000 system with Live Cell Cutting (LCC) function even permits dissection of living cells from tissue sections [80–84].

LCM and LMM systems rely on an optical microscope coupled with a coaxial cutting laser and computer assisted control. The operator marks the targets to be cut off automatically with a cutting width of about 1 μm or less. Following laser-based sectioning, different technologies allow for precise harvesting (Fig. 2.3a; reviewed by Hodne and Weltzien [85]). The classical laser capture microdissection (LCM) exploits a contact-based extraction by employing an adhesive inert membrane to the section surface, which is melted locally after target excision by low energy infrared (IR) laser pulses [77]. Hence, samples are extracted via adhesion to the membrane or adhesive tube caps (Fig. 2.3a). For laser microbeam microdissection (LMM) [86], the laser-cut sample is either falling into a reaction tube following gravity, also called contact-free gravity-assisted microdissection (GAM), or it is catapulted against gravity. The first option requires inversely mounted substrates placed about a collection tube (Leica LMD7000, Fig. 2.3a). In contrast, contact-free laser pressure catapulting (LPC) utilizes a local plasma impulse induced beneath the cell by a short, defocused laser pulse to catapult excised samples (or compartment) into a nearby collector container (Zeiss PALM, Fig. 2.3a).

One of the main challenges in laser-assisted microdissection is to dissect only the cell or compartment of interest without contamination from neighboring cells or other unspecific fragments. Imprecise cutting or a poorly calibrated system can lead to both, false positive and false negative results. Although the newest generation of systems assist the operator with a high level of user-friendliness and automation, the selection process remains user-based and therefore strongly impacts throughput and reproducibility. Similar to the manual isolation with micromanipulation, it might remain unclear whether the cell was actually transferred and/or whether any contaminants were co-isolated, especially for contact-based cell extraction (adhesive methods) [77].

The integrity of the extracted material is important for reliable downstream analysis of biomolecules such as DNA, RNA, and proteins [87]. Depending on the applied fixation methods, or cryopreservation [88], as well as extraction methods (adhesive methods, GAM, LPC), single-cell integrity might be compromised [89]. In general, LCM/LMM based assays generate low yield of material, particularly for low abundance RNA species [89].

While most of the high-throughput methods relying on dissociated cells, LCM/LMM methods as well as cytosol harvesting with a patch-clamp
pipette can be applied to intact tissue [75]. Moreover, cells are harvested under direct microscopic control providing additional information, like localization within tissue architecture, morphology, or immune-labeling.

In the past years, various single-cell analysis applications based on LCM/LMM isolated cells have been published: Single-cell RT-PCR [90], short tandem repeat analysis (STR) analysis in forensics [91], Western blot and mass spectrophotometry analysis [88]. Analysis of solid tissue sections without prior dissociation is of great interest when investigating heterogeneous specimens regarding their cellular structure as well as physiological and pathological processes [92]. For instance, in solid tumor research linking the molecular information of individual cells to their specific localization or context within a complex tissue has become an important field of research [93].

2.6 Fluorescent-Activated Cell Sorting

First applications of flow cytometry instruments reach back to the early 1970s, and since then numerous patents and methodological advancements have enabled the robust and reliable commercial flow cytometry used today [94–97]. Within the methodological spectrum of flow cytometry, FACS provides the ability to enrich and isolate particular cell types, collecting them in separate target vessels (Fig. 2.3b) [2, 13]. Nowadays, FACS is the accepted worldwide standard in analysis and sorting of cell populations [98], probably also due to the widespread distribution of devices.

By laser excitation and emission FACS devices read out multiple parameters offering various analytical options (Fig. 2.3b). Cellular
properties like relative size and granularity are registered using the forward scatter (FSC) and side scatter (SSC), respectively. This information is further relevant to discriminate between single cells and doublets, or cell cluster, which is highly important to ensure single-cell isolation. Furthermore, fluorescence signals resulting from induced reporter expression or from preceeding labeling of cell surface markers can be gathered to classify subtypes of cells [2, 99].

The suspension is pressure driven through the system and lined up using a sheath flow liquid utilizing the effect of hydrodynamic focus. To ensure single-cell separation, a relatively high liquid flow is established that consequently leads to a strong dilution of cells within the system [85]. The liquid stream is channeled through a small nozzle (typically 60–100 μm diameter) and afterwards disrupted into a continuous line of flying droplets by applying targeted ultrasound vibration. These droplets, of which some include cells, pass the different laser beams and detectors used to measure the physical, chemical, or optical properties. Analysis occurs online and cells are sorted by electrical deflection of droplets into different outlet channels according to the user-set gateings (Fig. 2.3b). In case of single-cell RNA sequencing, target cells can be sorted individually into 96- or 384-well plates. In the past, all collected cells needed to be processed manually before sequencing, causing enormous work-load and thus hampering high-throughput studies. With the help of automation and individual pre-defined sequencing indices in the collection wells, more comprehensive studies are now manageable. With an advanced method called “index sort,” it is even possible to retrospectively correlate scRNA-Seq data and protein expression of cell surface markers measured during isolation, which is of particular interest when cells have been stained and analyzed for multiple markers [100, 101].

Depending on the application, FACS can be carried out in different modes specialized on high-throughput, enrichment or purity, differing in the actual sorting rate (between some hundreds up to several thousands of cells/s) as well as capturing efficiency. The major advantages of FACS are flexibility in terms of cell type, compatibility with standardized substrates and buffers, high levels of accuracy, high-throughput and last but not least the operability and cost efficiency as well as the high prevalence of operating systems (Table 2.1). Still, a proficient operator is required to consistently and accurately sort cells into the center of a well, so that cells are immersed in the lysis buffer [3]. Further, the operator needs to calibrate proper gating for the FSC and SSC to ensure low doublet rates, reported to be as low as 2.3% [102]. Consequently, the flow sheath liquid will be increased to reach such low values augmenting the sample volume for each isolated cell. This causes higher risks of co-isolation of cell debris or free RNA contaminations [51]. The high liquid volumes also limit sorting speed and hence throughput, which will be especially obvious when low-abundant cell types need to be collected from a large number of suspended cells. Nevertheless, FACS is suitable to isolate rare cell populations with less than 1% but it necessitates a comparably large amount of starting material (>10,000 cells). As FACS analysis relies on cell suspensions, dissociation of solid tissue with all the aforementioned disadvantages has to precede (see Sect. 2.2).

Due to the high-pressure flow within the system cell viability may be an issue when applying FACS [103, 104]. Moreover, the osmotic and pressure stress occuring during sorting was shown to induce changes in cell expression profiles [5–7]. Additionally, labeling of cell surface receptors mostly involves their stimulation with antigens potentially leading to intracellular signaling and hence, an altered phenotype, necessitating to keep the time of labeling and sorting procedure as short as possible [85]. As already mentioned in former chapters, FACS is routinely applied to enrich cell populations prior to other approaches of single-cell isolation. Vice versa, pre-enrichment by other approaches like negative selection or depletion of unwanted cells through magnetic-activated cell sorting (MACS) likewise improves FACS results.

Due to its broad applicability, FACS studies cover nearly every cell type ranging from