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From Mouth Pipetting to Microfluidics: The Evolution of Technologies for Picking Healthy Single Cells

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Although single-cell omics studies have gained high popularity in recent years, isolation of healthy single cells from primary samples remains challenging and, in many cases, the critical step for a successful experiment. The virtues and limitations of conventional cell sorting strategies, such as manual picking and flow cytometric sorting, are briefly analyzed here and the unique opportunities for microfluidics from the technological perspective are discussed. It is being reasoned that, by combining assessment of healthy features and high-throughput sorting of single cells, microfluidics will play an important role in retrieving specific single cells for highly accurate and precise omics analyses. With further incorporation of functional features, such as microenvironmental stimulations and response readouts at the single-cell level, microfluidics will become an integrative research tool that brings many new possibilities in biomedical studies at the finest resolution.

1. Introduction

Cells, the basic units of life, have been intensively studied at the bulk level, typically with input of thousands to millions of cells. Many fundamental concepts in complex biosystems, such as immunology, development, cancer, neurobiology, and others, have been deduced from ensemble measurements using such large amounts of cells. Interestingly, the results have clearly indicated the existence of cellular heterogeneity among cells, and that such heterogeneity is not only an intrinsic property, but also a functional necessity for these biosystems. It is natural to contemplate that the optimal input for most, if not all, experiments should be an individual cell. However, handling of single cells requires special technologies.

Recent progresses in single-cell studies have embraced many newly developed technologies and measurements, especially single-cell omics (e.g., genomics, transcriptomics, proteomics,[1–3] metabolomics,[4] and/or image-omics[5]) and single-cell manipulation and control (e.g., cell cytometric sorting, patch clamps, etc.). Quantitative assessments of the states, functions, and responses of single cells could lead to better understanding of many details. Furthermore, rewiring of cells to gain specific functions, such as induced pluripotent stem cells and chimeric antigen receptor T cells, has shown promise for not only fundamental sciences, but also clinical applications,[6,7] and such engineering can also happen at the single-cell level.

Although many experimental protocols have been developed by various researchers in the past decade to greatly improve single-cell omics studies, the technical challenges actually start from the first step, comprising the isolation and selection of single cells from primary tissues for investigation. The most mature single-cell omics studies are transcriptomic analyses, i.e., RNA-seq experiments. From this perspective, we will mainly focus on single-cell RNA-seq experiments, but all of our discussions can be naturally applied to other omics studies. Such projects typically require sampling of multiple (a few to a few thousand) single cells for each round of experiments, and need cells to remain in a "healthy" state or stay alive with minimal perturbation from experimental processes. We, and others in the field of single-cell studies, have found that the technical difficulties associated with retrieving healthy single cells can significantly cripple the representativeness and significance of downstream single-cell analyses. Hence, from this perspective, we will focus on the topics of isolation and selection of healthy single primary cells. We will briefly review the conventional practices for manual cell picking and flow cytometric sorting and analyze the virtues and limitations of these approaches. We will also briefly discuss the emerging methods, especially microfluidic-facilitated approaches to capture, manipulate, and process single cells, from the technological perspective. Each of these technologies has been covered in depth by recent reviews, and we thus urge our readers to refer to these reviews and original publications for design principles and experimental details.[8–14]

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2. Perspectives

2.1. Healthy, Barely Alive, or Dead, This is the Question

Single-cell omics studies, especially the identification of cell types and discovery of new cell types through transcriptomic landscapes, are in great demand. Almost all experimental protocols for studying single-cell omics have been developed and

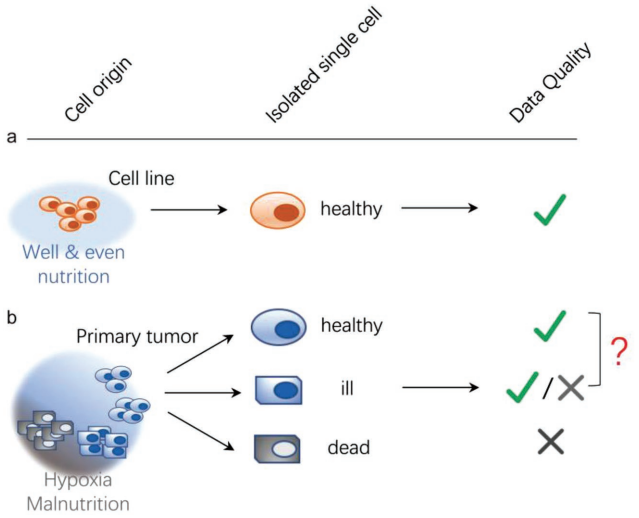


Figure 1. Lack of criteria to distinguish healthy cells from unhealthy cells. Environmental nutrition differences define cell health. a) Under sufficient nutrition, single cells from cultured cell lines are generally healthy. b) Under harsh environments (hypoxia or malnutrition), primary tumor cells can be healthy, unhealthy, or dead in vivo. Cell health is reflected in quality of downstream data, such as single-cell RNA-seq. The ticks and crosses represent good and poor data quality, respectively. The question mark represents the problem that although live and dead cells can be discriminated by cell viability dyes, distinction of healthy cells from unhealthy cells is not addressed.

optimized through cells isolated from cultured cell lines. When the techniques are applied to single cells isolated from primary tissues, the far more interesting research topics and the real situations that connect clinical implications, operations, and results become more complicated.

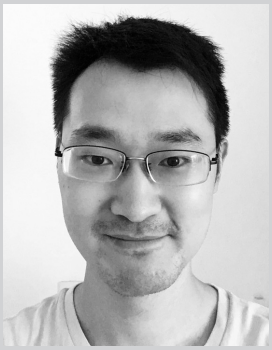
Primary cells are different from cell lines in many aspects.[15] First, nutrition for cell line cultures is typically far more enriched and homogeneous than that for primary cells in vivo, and in turn the metabolic states and transcriptome landscapes at the single-cell level are vastly diverse in vivo. Second, different types and subsets of cells interact, constructively and/or destructively, in vivo, whereas cell lines generally originate from the same cell type and have completely different intercellular interactions. Ironically, it is still difficult to define a simple and effective criterion to select healthy individual cells from a given population of cells. For example, tumor cell lines are perfect model systems to prove technical principles, whereas primary cells from hypoxic malnourished solid tumors are barely alive, as reflected by RNA/DNA degradations or odd patterns of transcriptome/metabolism data (**Figure 1**a).[16] It is easy to discriminate live cells from dead cells, but difficult to identify healthy cells from dying cells, as the differences are often obscure (Figure 1b).

2.2. Mouth Pipetting, the Cliché

Because cell viability has a critical impact on the quality of the single-cell transcriptome landscape, picking healthy single cells is the first step toward a successful project. In a 2016 market survey of the methods for single-cell isolation,[17] manual cell picking, particularly mouth pipetting, surprisingly held 12% of all responses and ranked third after flow cytometry (41%) and microfluidics (29%). The workflow for manual picking contains three operations: morphological evaluation, removal of debris/doublets/clusters, and single-cell manipulation. Although laborious and skill-dependent, this ancient approach is still irreplaceable in many laboratories, even in those equipped with automated instruments. Why?

The first reason is that recognition of healthy cells by trained eyes remains highly accurate and efficient. For trained researchers, smooth-edged transparent healthy cells are too obvious to miss under a microscope. When using a high-throughput flow cytometer, identification of live/dead cells is easily achieved by staining. However, the phenotypic characteristics of healthy cells, such as smoothness of the cell surface, are typically ignored.

The second reason is that for many projects aiming to generate high-quality RNA-seq data for individual single cells, ensuring that the majority of input cells are in good condition



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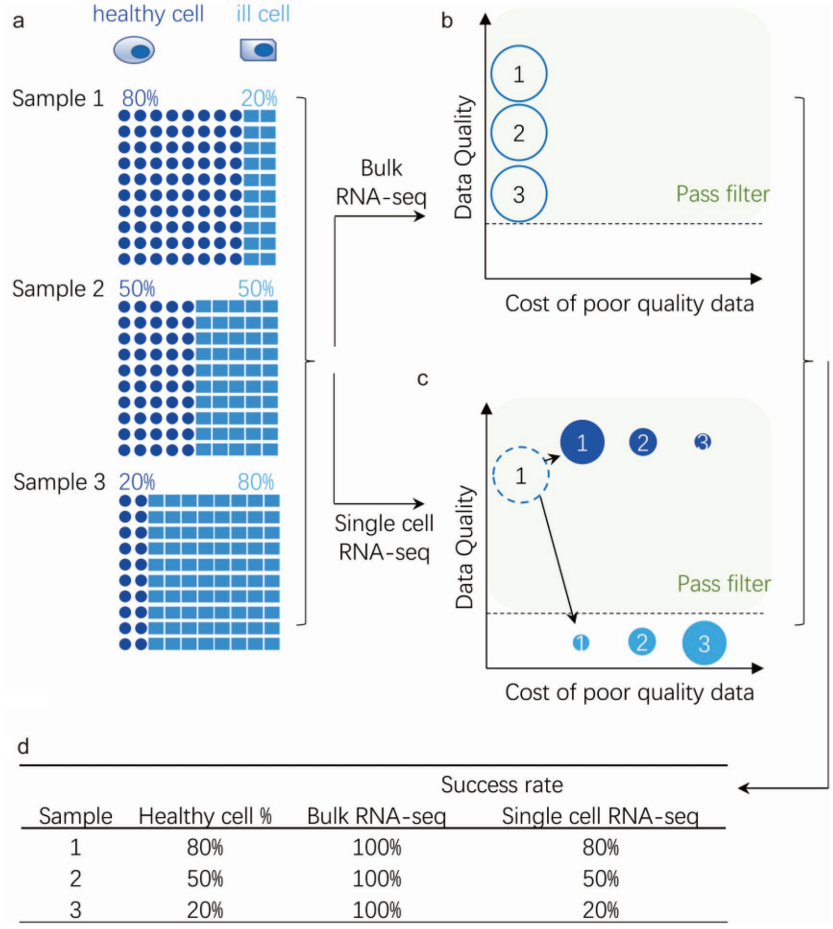

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Sample	Healthy cell %	Success rate: Bulk RNA-seq	Success rate: Single cell RNA-seq
1	80%	100%	80%
2	50%	100%	50%
3	20%	100%	20%

Figure 2. Picking healthy single cells is critical for further analysis when working on a complex biosystem. a) The technological advantages of manual cell picking are illustrated by a hypothetical experiment. The ranks of the healthy cell proportions are Sample 1 > Sample 2 > Sample 3. b) Consideration of data quality and cost for poor-quality data in classical bulk RNA-seq. Poor-quality data from unhealthy cells are averaged in bulk sequencing. The blue circles represent bulk RNA-seq data. The black dashed line represents the quality control criteria. The green area represents data sufficient to pass the quality control filter. c) Data quality and cost of poor-quality data in single-cell RNA-seq. Each cell is controlled for quality, and thus poor-quality data from unhealthy cells are filtered out. The dark blue circles represent RNA-seq data for healthy single cells, and the light blue circles represent RNA-seq data for unhealthy single cells. The areas of the circles are correlated with the cell proportions. d) In the end, the cost of poor-quality data is significantly higher in single-cell RNA-seq compared with bulk RNA-seq, emphasizing the crucial role for healthy single-cell isolation.

will significantly improve the experimental efficiency, and hence reduce the total cost. Let us hypothetically consider an experiment (**Figure 2**), in which the same sample sets have been used for classical bulk RNA-seq and single-cell RNA-seq back-to-back (Figure 2a). In the bulk measurement, a higher ratio of healthy cells results in higher average quality of the data. Conventional practice is to include a simple quality control that can filter out bad-quality data sets before further analysis (Figure 2b). However, in single-cell experiments, every cell is sequenced and scrutinized, and only the healthy single cells provide data with fine quality for further analysis (Figure 2c). Too few healthy cells in the population can result in the majority of cells having insufficient genes detected, i.e., *garbage in, garbage out* (Figure 2d).

Furthermore, for precious samples containing only a small number of the target cells, manual picking is probably the only choice that can guarantee healthy single-cell retrieval without wasting samples. For example, most early-stage developmental biology studies still rely on this method. It seems that, even with careful training and extensive practice, the throughput of manual picking hardly exceeds 100 cells h^{-1}, or 1000 cells d^{-1}, which then becomes the real bottleneck of the experimental design.

2.3. Virtues of Flow Cytometry

Despite the lack of morphological information, conventional flow cytometric sorters excel in several ways (**Table 1**). Probably the most significant advantage of flow cytometry is that the investigation can be performed at extremely high speed, up to ≈10^5 cell s^{-1}. Cells are captured by small liquid droplets, jetted into the air, and accurately deflected into receiving vessels at the single-cell level. With proper combination of excitation and emission wavelengths using multiple lasers and specific filters, a single machine can detect up to about 50 parameters. Modern instrumentation allows single-cell sorting directly into microtiter plates at the speed of a few minutes per plate, and these standardized supplies can be seamlessly integrated into liquid-handling robots for high-throughput automation. Moreover, flow cytometric sorters can accurately detect cell doublets or clusters and exclude them from collection. This function is especially critical for single-cell studies.

For any large-scale single-cell studies with throughputs ranging from a few hundred to a few thousand cells, flow cytometric sorting is probably the best choice based on two considerations. First, using microtiter plates, researchers can perform most available single-cell RNA-seq protocols, thus suiting a wide range of research purposes. Second, the cost of such operations, including the consumption of reagents and supplies, remains manageable. The balance between throughput and sensitivity is always a major concern for the design of a single-cell project. Basically, if one wants to profile more genes, especially those with low abundance, in each single cell, or discover subtle expression differences between cells, specific protocols are required and more sequencing reads are needed per cell. Currently most other methods that can scale up to a few thousand or more cells (eg. Dropseq) suffer from low sensitivity of gene detection, which thus limits downstream analyses.

Cell appearance reflects the cell status. If cellular images of individual single cells can be captured in situ during cytometric

Table 1. Comparison of conventional imaging flow cytometry.

		Conventional flow cytometry	Imaging flow cytometry
Cell detection	Morphology information	–	+
	Fast detection	+	+
	Multiparameter	+	+
Cell sorting	Fast drop formation	+	–
	Single droplet manipulation	+	–
	Fast bulk cell sorting	+	–
	Single cell index sorting	+	–
Contamination control	Live/dead	+	+
	Doublets exclusion	+	+

sorting, better assessment of each cell's condition can be achieved based on a high-content measurement. Recently, imaging flow cytometry has quickly evolved to capture the spatial information of cells, rather than a single value of intensity. Adapted with an optical microscope and a line-scanning camera, an imaging cytometer can take single-cell multicolor snapshots at the speed of about 1000 cells s^{-1}. Fast imaging allows for precise determination of healthy cells in the population, by identifying both the overall morphologies (e.g., shape and intactness) and the distinct subcellular distributions of cells at different statuses (e.g., autophagy and apoptosis).[18,19] To date, however, imaging flow cytometry has not yet incorporated sorting functions successfully, thus jeopardizing its value for biomedical applications (Table 1).

With the increasing needs for single-cell handling, simplification of flow cytometry instruments is becoming a new trend. Through acoustic focusing of cells, hydrodynamic focusing can be replaced by a sheath-free sample-only mode.[20,21] Meanwhile, labeling and detection modalities can also be non-optical. CyTOF, a mass spectrometry-based cytometry approach, has gained much attention. By replacing fluorophores with metal isotopes, CyTOF can demonstrate a significantly reduced spectrum spillover of optical detection, and in turn enhanced parameter space.[22] Again, however, neither of these two new methods currently has any sorting capability.

To date, flow cytometric sorters are probably the most handy and robust automated solutions for single-cell isolation, high-throughput analysis, and single-cell investigation. The rapid development of imaging flow cytometry, novel cell sorting mechanisms, and non-optical detection may facilitate the differentiation of healthy cells from other cells, and further improve single-cell studies with better quality of omics data and better representation of the real inner life of individual single cells.

2.4. Microfluidics, the New Challenger for the Task

Conceived in the 1970s and commercialized in the 1980s, flow cytometry has triumphed in cell identification and isolation for four decades. In the past few years, microfluidics has shown the capability to challenge this situation. Although the original concept of microfluidics was proposed only a short time after flow cytometry, mature applications of microfluidics to single-cell studies have remained rare until recently. In a sense, flow cytometry works at the scale of "macrofluidics," because its fundamental concept and engineering are essentially interchangeable with those of microfluidics. Therefore, to identify or isolate cells, almost every virtue of flow cytometry has been successfully transferred into microfluidics, including hydrodynamic focusing, acoustic focusing, multiparameter cell detection, piezo-based droplet formation, and sorting.[23]

It is worth noting that a simpler, easier, and probably also better microfluidics-based sorting flow cell has been well incorporated into flow cytometers, and is becoming more popular in single-cell studies partially through the reduction in cross-contamination. In another approach, a simple yet efficient acoustic microfluidics system has been incorporated into cytometric readers. More similar solutions are expected.

However, we would like to argue that, rather than being an evolutionary clone of conventional flow cytometry, microfluidics or "lab-on-a-chip" approaches are a revolutionary direction, especially for the identification and isolation of healthy primary single cells. Three fundamental characteristics are embedded in microfluidics: compactness, integration, and controllability. The synergistic combination of these characteristics will create new functions that only microfluidic devices or systems can provide.

First, microfluidics typically deals with liquid in nanoliter or even smaller volumes, which is suitable for single cells that have characteristic volumes in picoliters. The microfluidic chambers or channels can offer a precise and accurate controllable microenvironment for single cells, and act as a perfect vessel for processing single cells or monitoring their responses.[24] Well-designed microfluidic devices can provide a unique platform to continuously record the active life of a single cell in situ, for example, the secreted vesicles released from a particular cell, and even sort out these exosomes for further analysis.[25] Such functions seem natural when using microfluidic devices, but would be difficult by modifying the working protocols of conventional flow cytometric sorters.

Second, the concept of "lab-on-a-chip," which takes multiple functions together through elegant integration, has always been one of the major pursuits in microfluidics. Multiple physics approaches (e.g., mechanical force,[26] electronics, magnetics, imaging,[27,28] thermal control, droplet sorting,[29] cell printing) and other biochemical assays have been successfully integrated with planar chip–based microfluidic devices to perform complex operations for biomedical studies and clinical applications, such as cell culture,[30] antibody–antigen reactions,[31] and single-cell sequencing,[32] and we have also contributed to these approaches.[33] Experiences and investments in microfabrication through decades of prosperous microelectronics have also facilitated microfluidics, offering high-quality and low-cost manufacturing of tiny features and robust coupling between different materials and parts. Compact and multifunctional microfluidic devices can certainly be more user-friendly and more capable than flow cytometers when dealing with single cells.

Third, each aspect of microfluidics can be stringently controlled. Microfluidic devices are flexible in terms of

functional design and technical complexity, but the foundation of this flexibility is efficient and accurate control of each component and function. For example, every fluidic channel can be controlled for open/shut options with densely addressable microvalves through a multilayer polydimethylsiloxane architecture, which enables multistep biochemistry reactions with high precision.[34] Meanwhile, in conventional flow cytometry, fluidics are simplified into one stream, without parallel capacity.

Microfluidics has great potential for single-cell studies, and has become a potent challenger in the field. However, we like to point out that, as with any other technology, microfluidics is still far from perfect. It naturally has at least two drawbacks for cell isolation applications. First, compared with flow cytometers, the flow rate of microfluidic devices is low, which does reduce the shear stimulation or damage of cells, but inevitably limits the throughput for cell identification and sorting ($\approx 10^3$ vs $\approx 10^5$ in commercial flow cytometers). This problem may be resolved by using parallel fluidics.[35] Second, microscale fluidics is intrinsically much more sensitive to environmental perturbations than macroscale fluidics. Proper choices of materials for devices may help to stabilize the flow and hence improve the robustness of the whole experiment.

2.5. Microfluidics to Fulfill Healthy Cell Isolation

When we return to a focus on healthy status assessment of single cells, can microfluidics be a paradigm shift? To date, two quantifiable methods may be reasonable candidates to combine with microfluidics: bioelectrical impedance and cell morphology.

Impedance measurement is not new to cell investigations. The method has been applied to cell counting, cell subset discrimination, and cell toxicity studies. Sensitive measurements for subtle events, such as cell protrusion, have also been described.[36] Furthermore, a proof-of-concept study for impedance incorporation into flow cytometry and microfluidics sorters was reported.[37] It seems that impedance signals are highly related to cellular contents and cell integrity, suggesting a strong possibility that such measurements can provide greater contributions than other modalities to single-cell omics studies as a gating criterion to filter "garbage" cells.

Morphologic features are another approach with high potential for reasonable integration into microfluidic devices. On the one hand, a simple and efficient microfluidic sorter was recently built by single droplet-cell printing, although it lacked morphology information compared with conventional flow cytometers.[38] However, such a method is a good way to solve the "chip-to-world" problem. On the other hand, imaging-based computer vision was recently demonstrated for microfluidic devices, with and without a slow sorting strategy.[39,40] In this manner, the picking process can be operated on demand. We thus have strong confidence that a novel healthy cell microfluidic sorter will come onto the market sometime soon, with a WYSIWG (what you see is what you get) workflow to serve the most critical single-cell analyses.

3. Summary

In this perspective, we have briefly reviewed the prevailing methods for single-cell analysis and discussed the critical need to assess the healthy status of cells in single-cell omics studies. To prevent "garbage-in-garbage-out" fiascos, robust and accurate picking of proper single cells for bias-free and cost-effective studies is necessary, but challenging. Manual picking does not meet the required throughput for single-cell studies, which is growing by leaps and bounds. Meanwhile, flow cytometry does not provide sufficient power to gauge the needed cells, with limited capability to accept a small amount of precious single cells. We thus propose that, with the current resonance of microfluidics for single-cell RNA-seq, a more versatile microfluidic approach that can combine a novel modality for cell evaluation will become a "killer app" in the field.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cell status, lab on a chip, microfluidics, omics, single cell sequencing

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