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Essentials of Single-Cell Analysis

Concepts, Applications and Future Prospects

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Preface

Cells play a significant role in our daily lives. However the intercellular interactions, intracellular behaviors, and environmental responses of cellular organelles are still not fully understood and the ensemble-averaged measurement of millions of cells together is not able to provide detailed information at the single-cell level. For example, the genome, epigenome, and transcriptome analyses of bulk cell populations are informative, however, they cannot reveal the heterogeneity and molecular dynamics within a certain cell population. Also, these analyses cannot provide any information about an underrepresented cell subpopulation that could have a differential or crucial function in a specific biological context, such as stem cells or tumor-initiating cells. In contrast, single cell sequencing (SCS) is able to empirically infer the driver mutations and map the sequential mutation events during cancer development. The integration of genomics and transcriptomics in single cancer cells will also provide valuable information about the functional consequences of mutations and the copy number variations in these cells. In the past few years, significant advances have been made in isolation of single cells, whole-genome or whole-transcriptome amplification and genome-wide analysis platforms, which not only allow high resolution genome and transcriptome analysis, but also have the potential to reveal the epigenome map of the target single cells. Undoubtedly, these novel approaches will produce profound health benefits, such as a more efficient treatment strategy for patients affected by genetic disorders, which can be realized at the single-cell level.

Apart from the considerable power of single-cell analysis (SCA), the huge amounts of data generated from the SCA process have emerged as a challenging issue. In recent years, bioinformatics techniques have been employed to study the "big data" from large ensembles of single cell data. Thus interestingly, some unresolved questions in the past can be answered now thanks to the unique information obtained from single-cell analysis, such as whether any two single cells are really the same if we are able to measure adequate parameters with sufficient accuracy. Are there two cells which have similar biological functions and predictable outcomes when treated with the same drugs or environmental factors?

SCA, without doubt, is an efficient and valuable approach to understand the fundamental biology in embryonic development, to provide detailed knowledge of the cell lineage trees in higher organisms, to dissect the tumor heterogeneity and disease, etc.

To analyze the cellular function, SCA can be performed by combining capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection, electrochemical detection (ED), flow cytometry or mass spectrometry, etc. Recently, with the development of microelectromechanical systems (MEMS) technology and its integration with chemical engineering, biomedical engineering, chemistry, material science, and life science, Bio-MEMS (sometimes considered synonymous with lab-on-a-chip (LOC) or micro total analysis systems (μTAS)) has emerged as a powerful tool for more complex manipulations of chemical and biological agents in micro/nano fluidic environments. Micro/nanofluidic devices with the power to manipulate and detect bio-samples, reagents, or biomolecules at the micro/nanoscale can well fulfill the requirements of single-cell analysis. Thus, they are not only useful for cell manipulation, isolation, separation, and lysis, but are also able to easily control the biochemical, electrical, and mechanical parameters in SCA with precisely controlled dosage, spatial resolution, or temporal pace.

This book includes 15 chapters, covering a wide spectrum of the essential aspects of single-cell analysis. Contributed by experts in their own fields, these chapters provide technical tips based on valuable experience and knowledge. Potential problems and challenges as well as possible solutions are also discussed with an emphasis on the future prospects. "Single-Cell Behavioral Assays for Heterogeneity Studies" describes single cell behavioral assay for heterogeneity study using single cell isolation and tracking to investigate cell proliferation, differentiation, and lineage. There are two platforms being discussed that include the cell migration platform to measure cell motility, deformation, and invasiveness and the cell–cell interaction platform to study the alteration of cell behaviors caused by reciprocal interactions among cells. "Systems Biology in Single Cells" presents single-cell analysis in systems biology. It covers technologies that enable the isolation of individual cells in a form that accommodates systomics studies, the biological methods deployed on such isolated cells to generate system-level information, and the bioinformatics technique that is specifically directed toward single-cell studies. "Electroporation for Single-Cell Analysis" introduces advanced single cell electroporation techniques for cellular delivery and analysis, which might be potentially applicable to cell therapy, clinical diagnosis, drug screening, etc. "Microinjection for Single-Cell Analysis" is devoted to the single cell microinjection technique. The basic knowledge of this technique, its advantages and disadvantages, its development and applications, its basic instrumentation, and modifications are discussed thoroughly in this chapter. "Optical Tools for Single-Cell Manipulation and Analysis" demonstrates the optical tools for single-cell analysis ranging from optical trapping systems which provide a contact-free technique for manipulation of micron-scale objects, through to a selection of different optically-mediated cell membrane disruption methods available for lysis and/or delivery of material. "Optoelectrokinetic Manipulation for Cell Analysis" explores two newly developed optoelectrokinetic techniques termed rapid

electrokinetic patterning (REP) and optoelectronic tweezers (OETs). Both the fundamental knowledge and their applications in cell-related research are covered in this chapter. "Continuous Micro-/Nanofluidic Devices for Single-Cell Analysis" discusses continuous micro/nanofluidic devices for single-cell analysis in two parts. The first part presents state-of-the-art techniques developed to handle single cells, including counting, sorting, positioning, and culturing. The second part describes the manipulation techniques combined with other stimulating and sensing techniques for the observation and characterization of single cells. "Single-Cell Mechanical Properties: Label-Free Biomarkers for Cell Status Evaluation" emphasizes the microfluidic approaches including microfluidic constriction channels, microfluidic optical stretchers, and microfluidic hydrodynamic stretchers, which are being developed as next-generation, automated, and high-throughput techniques for characterization of the mechanical properties of single cells. "Cytometry of Single-Cells for Biology and Biomedicine" presents flow cytometry, scanning image cytometry, and microfluidic cytometry with fluorescent probes used for single-cell analysis in biology and biomedicine. It also discusses the advantages of combining different approaches in integrated instruments that could perform both flow cytometry and image analysis on single cells as well as examining the internal contents of each single cell. "Single-Cell Genomics and Epigenomics" discusses the role of single-cell analysis in genomics and epigenomics, where it describes the major technological developments achieved in single cell "omics," the technical challenges to overcome, the potential applications, as well as future developments and breakthroughs. "Single-Cell Metabolomics" focuses on the single cell metabolomics in systems biology, where the recent improvement of analytical tools to unravel single cell metabolomics and their specificity, the limitations and challenges alongside the future prospects are discussed. "Applications of Cell-Based Drug Delivery Systems: Use of Single Cell Assay" presents different types of cell-based drug delivery systems to facilitate treatments for infectious and noninfectious diseases. Potential and limitations of single cell assay in this type of drug delivery systems is reviewed along with the clinical aspects. "Applications of Single Cell Sequencing in Cancer" describes the methodologies of single cell sequencing, as well as its existing and potential applications in reconstructing the evolutionary history of cancer progression and in profiling cancer transcriptome. "Single-Cell Characterization of Microalgal Lipid Contents with Confocal Raman Microscopy" highlights the recent advances in confocal Raman microscopy and its application in single cell characterization of microalgal lipid contents, which demonstrates cell-to-cell variation in structural features of expressed lipids among the screened *C. reinhardtii* mutants. "Single Differentiated Neurons from Pluripotent Embryonic Stem Cells: Motor Protein Modeling and Neurodegenerative Disease" illustrates how cross-field techniques, including the use of P19 neurons, single-cell DNA delivery devices, microchannel platforms, and kymograph data analysis for physical modeling, can enable the characterization of fundamental properties of neurodegenerative disease mechanisms.

We hope this book can be enjoyable reading material and at the same time a useful resource for scientists in academia and professionals in industry working on different aspects of SCA.

Fan-Gang Tseng
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Contents

Single-Cell Behavioral Assays for Heterogeneity Studies

Yu-Chih Chen, Patrick Ingram, Yi Luan and Euisik Yoon

Abstract Cell heterogeneity is an emerging challenge in cell biology and cancer therapies. Each cell in heterogeneous populations has its own unique behavior, and thus responds differently to the same reagent or drug, making analysis or treatment difficult and complicated. Therefore, it is important to understand the heterogeneity characteristics of cells in phenotypic behavior assays. Although conventional fluorescence-activated cell sorting can separate cells based on markers, most assays simply present the average behavior in a batch of cells sorted by markers. It is important to have a capability to fully characterize individual cell behaviors in a miniaturized, high-throughput platform, distinguishing their heterogeneity and tracking their responses over time. This chapter introduces the technological innovations enabling these assays and the associated biological experiments, including (1) single-cell isolation and tracking schemes to investigate the cell proliferation, cell differentiation and lineage, (2) cell migration platforms to measure cell motility, deformation, and invasiveness, and (3) cell–cell interaction platforms to study the alteration of cell behaviors caused by reciprocal interactions among cells.

Keywords Single cell · Behavioral assay · Cellular heterogeneity · Hydrodynamic capture · Suspension culture · Cell isolation · Cell migration · Chemotaxis · Cell–cell interaction · High throughput

1 Introduction

The cell is a fundamental building block of our body. To probe the properties of this basic unit, there are two different methodologies: One is genotypic analysis, in which we typically analyze genomes, DNA methylations, and mRNA expressions using micro-arrays, RTq-PCR, or even next-generation sequencing (NGS); and the

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other is phenotypic (behavioral) analysis, in which we study viability, migration, proliferation rates, cytokine secretions, and protein activities. One key advantage of genotypic analysis is that it can be done without any in vitro cell culture, which may alter the properties of primary samples. Since the genotypic analysis is basically to measure markers in molecular biology, the mechanism can be interpreted directly based on the measurements. Although the genotypic analysis has many merits, the expression of the same biomarker may be different for different diseases or even different patients. For example, let us illustrate the limitation of genotypic analyses in cancer cells. There is considerable evidence that supports the presence of cancer stem-like cells (CSCs), which are only a small subset of cells but retain the ability to initiate new tumors or metastasize [1–4]. However, due to the heterogeneity and plasticity of CSCs, (1) the markers for "stem-ness" are not consistent within subtypes of a specific cancer or across different types of cancer; (2) current markers enrich in CSCs but also include non-stem tumor cells; and (3) it is unclear whether these markers can identify the same or separate CSC populations [5–7]. Compared to the complicated marker-based analyses, the behavior-based phenotypic assays are relatively straightforward and are not limited by the availability of given markers. The malignancy of a cancer sample can be estimated using the results from drug screening and metastasis assays regardless of whether it has a given CSC marker or not, allowing for marker-free assays. In this chapter, we discuss phenotypic assays examining cell behaviors.

Conventionally, behavioral assays can be performed using either in vivo mouse models or dish-based assays. The mouse model can closely mimic physiological processes in vivo, but it has drawbacks of long assay time (can easily go up to weeks) and high cost. Although it can be a good tool for late stage validation, it is impractical to perform large-scale screening in the early stage of research. Compared to mouse in vivo experiments, dish-based assays have advantages of low cost and short turnaround time (typically a few days). Using automatic systems, high-throughput drug/stimulus screening can be easily performed. However, dish-based assays suffer from two issues: (1) The condition in a dish is quite different from the in vivo environment, so the result is less physiologically relevant. (2) Dish-based assays are designed for a large number of cells, so it is difficult and unreliable to perform single-cell assays. There is a fundamental trade-off between physiological relevance and throughput (Fig. 1). In vivo models are physiologically relevant but low throughput, while dish-based assays are high throughput but less physiologically accurate.

Microfluidic assays can provide a solution to this fundamental dilemma. Using miniaturized microfluidic devices, more experiments can be done using less cells and reagents than conventional dish-based assays. Meanwhile, better microenvironment control can be achieved in miniaturized microchambers, so that the microfluidic assays can be physiologically closer to in vivo conditions. In addition to higher throughput and better microenvironmental control, the use of microfluidics enables reliable single-cell assays, which are critical for understanding cell heterogeneity. In this chapter, we introduce various microfluidic devices developed for single-cell behavioral assays, providing four key advantages as illustrated in Fig. 2: (1) ability to

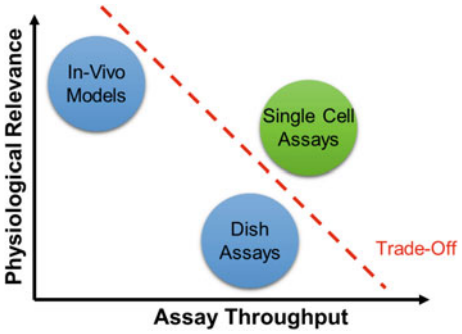
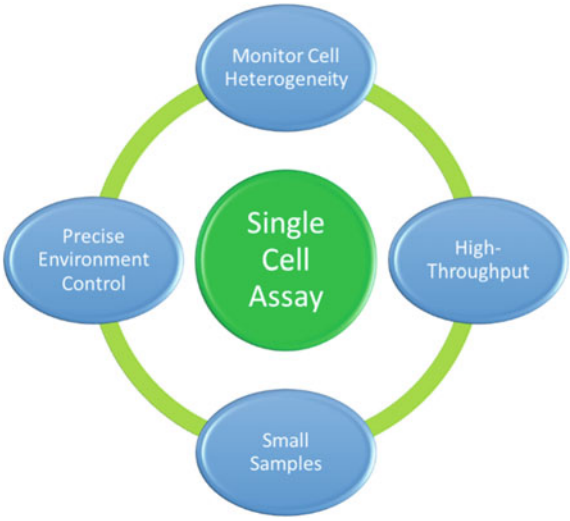


Fig. 1 Comparison between in-vivo models and dish-based assays in terms of physiological relevance and assay throughput. The single-cell microfluidic approach is expected to achieve better performance in both aspects than dish assays

Fig. 2 Four key advantages of microfluidic single cell platforms



monitor and track individual cells, (2) control of various microenvironments on-chip for emulation of bioprocesses, (3) accommodation of high-throughput screening, and (4) capability of handling small amount of cells and reagents.

2 Single Cell Isolation and Tracking

2.1 Non-microfluidic Single Cell Experiments

To study cell behaviors, Petri dish culture has been used for more than 100 years. Cells can be cultured in this simple device, and various assays can be performed in a dish to test and verify hypotheses in cellular biology. Although this conventional

tool has advantages of simplicity and robustness, it is limited in its capability to study and analyze single cell heterogeneity. The dish-based method collects data from the average over a large number of cells with an underlying assumption that all cells are identical. However, new discoveries in biology support the existence of heterogeneity within a cell population, meaning that measuring the average behavior only reflects a part of the fact, while cellular heterogeneity may be more important to understand the holistic behavior of cells. Some early trials in single-cell assays still used Petri dish by applying serial dilution to achieve very low concentrations of cells to monitor single-cell behaviors [8]. As the cells are sparse in a dish, each cell can be considered isolated from one another and can be treated as noninteracting single cells. However, there are limitations, including the difficulty to monitor the same cells over time in a large dish, labor intensiveness, and low throughput. In addition, cells typically need autocrine (signal to itself) signaling to survive, so diluting cells in such a low concentration can lead to poor viability, especially for primary samples. To characterize the behavior of each individual cell reliably at high throughput, microfluidics can be an ideal platform to provide precise cell positioning and isolation. We review the recent engineering innovations and platforms to achieve single cell tracking in this section.

2.2 *Hydrodynamic Cell Capture Scheme*

In order to precisely position single cells, hydrodynamic capture has been widely used, utilizing differences in hydrodynamic resistance in microfluidic channels by optimizing the paths. This method can be implemented without using any external pumps, valves, or other complicated instruments [9–12]. As demonstrated in Fig. 3, the scheme utilizes the hydrodynamic resistance difference between the two paths: path A (or the central path) and path B (or the serpentine path) [10]. The

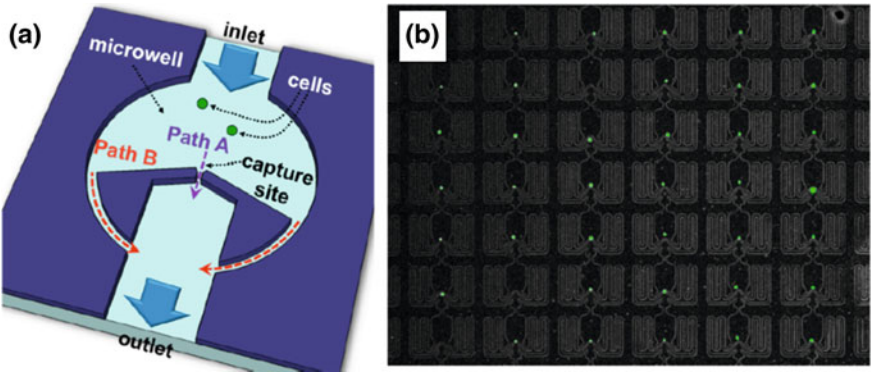


Fig. 3 Hydrodynamic cell capture scheme. **a** Schematic diagram of a unit microwell with hydrodynamic capture scheme. **b** Photograph of captured single green-fluorescent PC3 cells in microwell array. (Figure reprinted with permission from Ref. [10]. Copyright 2011, American Institute of Physics)