Continuous Micro-/Nanofluidic Devices for Single-Cell Analysis

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Abstract Micro- and nanofluidic devices are revolutionizing the fields of single-cell analysis, and benefiting related efforts in life science research, agricultural industry, and clinical medicine. These miniaturized devices introduce much desired capabilities in accurate cell and fluid handling, and thus enable quantitative multiparameter and high-throughput approaches to analyze single cells in large numbers, advancing our understanding on how the complex normal and diseased behavior of ensembles of cells emerges from the behavior of each cell or only a few dominating rare cells. The content of this chapter is broadly divided into two parts —single-cell manipulation (SCM) and single-cell analysis (SCA). The first part of the chapter presents state-of-the-art techniques developed to handle single cells, including counting, sorting, positioning, and culturing, which are essential steps in many biological and medical assays. These manipulation techniques are frequently combined with other stimulating and sensing techniques for the observation and characterization of single cells, which are described in the second part of the chapter. Major approaches to probe either intact or lysed single cells, with a special attention on the integration of fluidics and sensor technology, are reviewed. Various operation principles are explained along with pivotal examples demonstrating their applications and perspectives. Droplet-based techniques, although very exciting, are not discussed here due to different sets of technical considerations and performance metrics involved. Techniques providing the access to the intracellular content for sampling or injection of additional compounds are not included here and are covered in Chaps. 3 and 4 of this book, respectively.

Keywords Single-cell · Microfluidic devices · Nanofluidics devices · Cell sorting · Cell manipulation · Flow focusing · Cell count · Cell culture · Cell probing · Sensors

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F.-G. Tseng and T.S. Santra (eds.), *Essentials of Single-Cell Analysis*, Series in BioEngineering, DOI 10.1007/978-3-662-49118-8_7

1 General Introduction

The cell is the basic unit of living organisms. Studying of biological processes at the single-cell level at high throughput is important because cells are inherently variable biochemical reactors. Cellular heterogeneity, present even when genetic and environmental differences among cells are rigorously reduced, is a fundamental principle of cell biology [9, 25, 32, 43, 81]. New discoveries derived from cellular heterogeneity hold great potential to transform systems biology, regenerative medicine, and cancer biology. To understand fully the cellular specificity and complex of tissue microenvironments, it is necessary to quantify molecular and cellular behaviors at the single-cell level. Additionally, rich statistics are required both for capturing the distribution of physiological responses and for detecting rare cells with abnormal or unique phenotypes. Conventional techniques that measure the properties of large ensembles of cells or probe physiology at the cellular scale (i.e., biomarkers, microscopy, micropipettes, etc.) are not well suited for most high-throughput single-cell analyses. Recent advances in micro- and nanofluidic technologies can not only permit accurate handling of cells and small volumes of fluids, but can also perform the task with low cost, high-spatiotemporal resolution, throughput, and hence provide more statistically significant results, as illustrated by several excellent review articles [1, 2, 58, 77, 97]. Here, we review state-of-the-art micro- and nanofluidic approaches utilized for single-cell manipulation (SCM) and single-cell analysis (SCA), including advances in cell counting, sorting, positioning, high-throughput molecular readouts as well as continuous, noninvasive observation of cell behaviors over time. Droplet-based techniques are not discussed here due to different sets of technical considerations and performance metrics involved. Readers are referred to several excellent reviews [33, 46, 71].

Two commonly employed methods for single-cell analysis (SCA) are microscopy and flow cytometry. Microscopy is well suited for studying spatial localization of fluorescence within or between cells and time-dependent behaviors of either fixed or live cells [75, 91]. However, limitations of microscopy often involve throughput, multiparametric assays, and image analysis [20, 80]. On the contrary, cells in suspension can be analyzed in flow cytometry at high throughput ($\sim$100,000 cell/s). Current high-dimensional cytometry is capable of analyzing 100 unique parameters on single cells [42]. However, traditional flow cytometry suffers from several drawbacks, including expensive and non-aseptic hardware and operation, low cell viability, and inability to provide the kinetic and spatial information on the distribution of cellular or subcellular components. For example, the spatial information of the cell–cell arrangement is inevitably lost during sample preparation and it is not possible to track the same single cell over time. These two methods, although successful and well-adopted, are almost complementing to each other and are not designed for handling, manipulation, and dynamic analysis of single cells. Micro- and nanofluidic devices that are linked to conventional methods or employ different working principles provide increased benefits to SCA, as shall be seen in the following sections.

1.1 Microfluidics and Nanofluidics

Microfluidics is the field that studies and exploits the behavior of fluids confined to micrometer dimensions, such as microchannels, droplets, jets, and thin water films, etc. At this small scale, most fluids behave in nonintuitive ways because capillary forces and viscous forces that are usually negligible on a larger scale become the predominant forces. Microfluidic devices that integrate multiple procedures of cell manipulation, lysis, and detection provide enabling platforms for single-cell analysis. They confer advantages, including quantitative predictions of the fluidic environment, similar scales to the size of cells, high throughput with lower cost per assay, smaller reagents and sample consumption, and amiability to be automated and portable. These features make microfluidic devices very suitable for single-cell manipulation and analysis. For example, microfluidic technologies, capable of precise control of nutrient concentrations and the number of adjacent cells, enable the decoupling of confounding factors (e.g., environment and genotype) that can contribute to cellular heterogeneity [15].

When the fluid volume under study has a dimension less than 1 μm, it enters the realm of nanofluidics, at which scale new physical phenomena can be exploited [6, 79]. For instance, electrokinetic effects, like electroosmosis and ion polarization, are strongly exemplified, especially when concentrations of ions in the solution are low. The ability to manipulate fluids, particles, and molecules at nanometer scale provides innovative techniques to single cell analysis, such as protein separation and preconcentration [16], electrostatic trapping of submicrometer-sized particles [52].

2 Single-Cell Manipulation (SCM)

Isolating, sorting, counting, and positioning of cells are essential single-cell manipulation (SCM) steps in preparing for high-performance downstream single-cell analysis. There are currently no standardized techniques for single-cell manipulation. Microfluidic-based techniques can be broadly classified into two categories, passive and active methods, depending on whether an external force field in additional to forces generated by the flow is applied. Passive SCM devices, utilizing the intrinsic properties of cells, fluids, and device geometry for cell manipulation, are in general simpler to fabricate and operate [48]. In contrast, hybrid materials are often required to provide the additional force field in active SCM devices, in which forces such as electric or magnetic force fields may be employed for more versatile manipulation of cells. Another parameter to consider in determining the strategy for SCM is the cell type. Prokaryotes, such as bacteria, are typically smaller than eukaryotes, such as yeasts and mammalian cells. In addition, prokaryotes are in general more resilient to environmental conditions, such as temperature, osmolality, pH, and oxygen levels. In contrast, eukaryotes are more sensitive. Gentle and consistent handling of samples cannot be overemphasized for

high-quality SCA, especially when live mammalian cells are manipulated and analyzed. In the following sections, we will introduce examples and techniques to achieve SCM.

2.1 Cell Isolation

Isolation of cells of interest is commonly the first and critical step in single-cell analysis, as the volume of a typical mammalian cell is only ~4 pL, which is approximately 9 orders of magnitude smaller than common cell culture volume (~mL). Currently isolation of single cells is often achieved stochastically by serial dilutions or by using pipettes when cells are in suspension, or deterministically by using laser capture microdissection to select cells when they still remain adherent. Microfluidic devices have been developed for accurate, automatic, and unbiased isolation of single cells. For example, collecting rate tumor cells [82] and fetal cells in peripheral blood samples [47] are essential for early cancer diagnosis and prenatal screening, respectively (Fig. 1). However, detachment of cells is required for

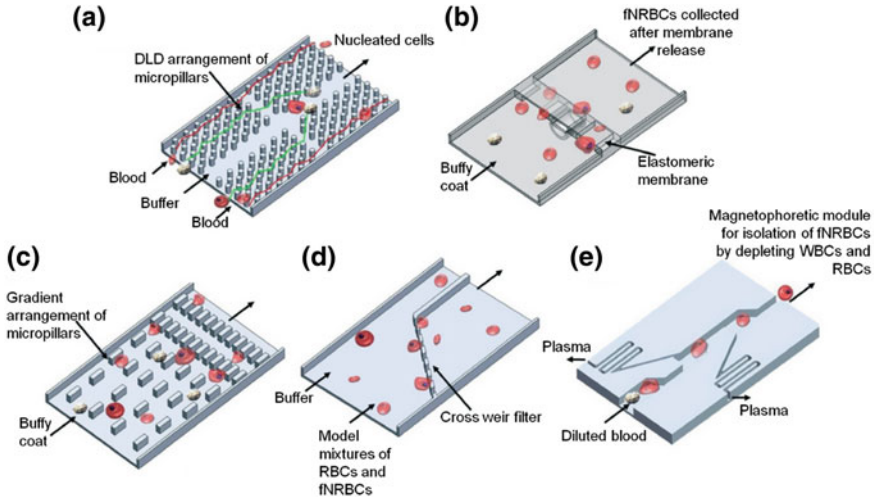

Fig. 1 Various passive microfluidic approaches for the isolation of cells. **a** Cells are separated by size using deterministic lateral displacement (DLD). Small cells tend to follow the direction of the fluid flow, whereas large cells continue to get displaced laterally by the asymmetrically placed micropillars. **b** Fetal nucleus red blood cells (fNRBCs) are concentrated at the microgap and later released after the underneath diaphragm is deflected. **c** Cells that are small and deform readily can squeeze through gaps, while others are retained. **d** Adult red blood cells (RBSs) are smaller than fNRBCs and can pass through the cross weir filter, whereas fNRBCs cannot pass and are diverted to the other collecting channel. **e** At a bifurcation, cells tend to migrate into the center of the channel of the higher flow rate, while cell-free plasma exits the branches of lower flow rates, known as the Zweifach–Fung effect. Reproduced from Ref. [47] by permission of The Royal Society of Chemistry

adherent cells biopsied from tissues or cultured within the microdevice, which may cause perturbation to the phenotype of the cell due to culture conditions and stimuli introduced during the detaching and analysis procedures.

2.2 Cell Counting

Cell counting is one of the fundamental procedures in cell biology research. It can be accomplished using optical, electrical, or magnetic means. Microscopy and flow cytometry are two of the most widely used optical techniques for single-cell analysis. The hemocytometer, originally designed for the counting of blood cells, is frequently used for assessing the concentration of various types of cells. It consists of a chamber defined by a grid-patterned bottom glass microscope slide with raised wedges that hold the top coverslip at a fixed distance off. Many disposable plastic hemocytometers have been marketed. They eliminate the need to wash for reuse of the glass hemocytometer and are especially advantageous when infectious or hazardous materials are involved.

Flow cytometry is an optical technique that is capable of counting cells at the single-cell resolution. It is also the gold standard, most widely used cell sorting technology. In flow cytometry, cells are "shot" through a capillary past an intersecting light beam, causing scattering of light; thus each cell is "read" as a signal of scattered or fluorescent light intensity by means of a light detector. To ensure that all cells pass through the same observation point and in a single-cell file, the cell suspension is injected by means of a glass capillary into a "sheath" flow, which focuses the capillary flow into a thin, single-cell wide flow. Flow cytometry is a powerful tool and can be capable of sorting of cells when equipped with a downstream sorting device that distributes the cell into different reservoirs based on the information relayed by the detector and rules determined by the user. Miniaturized flow cytometers have been successfully demonstrated [17, 18, 40, 100]. They allow for implementing microvalves, micropumps, or integrated microoptical stimulation/detection. In addition to cost reduction, major advantages of microfluidic flow cytometry include a much lower number of cells required and the potential for integration with multiple functionalities, both of which are of particular values for studying rare cells or clinical samples. The low throughput typically associated with early microfluidic flow cytometers have been greatly improved up to $\sim 10^6$ cell/s utilizing inertial effects (Fig. 2) [40]. A recent advance is the integration of inexpensive optical detectors or imaging modules [29, 99], which makes the flow cytometer much affordable and user friendly.

A Coulter counter detects cells upon their transfer through a pore, and is an electrical sensing zone method of counting cells, bacteria, and virus particles [8, 28, 62]. This method is relatively fast, real-time, label-free, viscosity-independent, and does not require large sample volumes. When an electric potential is applied across a pore contained within an insulating membrane, a transmembrane ionic current is established. When a particle less conductive than the electrolyte solution travels

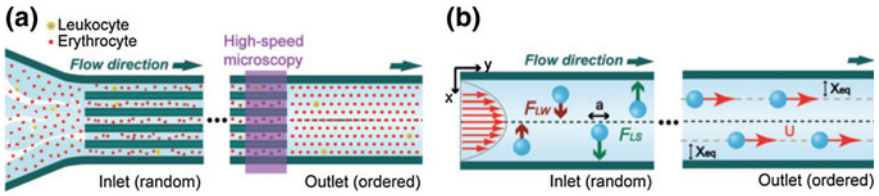

Fig. 2 A high-throughput microfluidic flow cytometer. **a** Schematics illustrate randomly distributed cells at the inlet become ordered at the downstream channel. **b** Cells are focused to specific lateral equilibrium positions, *Xeq*, where the wall effect lift force, F_{LW}, and shear-gradient lift force, F_{LS}, balance each other. Reproduced from Ref. [40] by permission of The Royal Society of Chemistry

through the pore, the same volume of the electrolyte solution is replaced by the particle, resulting a current blockade, or resistive pulse. The duration of this resistive pulse can be used to assess the surface charges carried by the particle [51], while the frequency of resistive pulses reflects the particle concentration [21, 73, 93]. Micro- and nanofluidics resistive pulse sensors advance over the traditional Coulter counter with a lower cost and a higher sensitivity. On-chip electronic sensing systems can be integrated with the fluidic network to achieve a better signal-to-noise ratio [78]. Alternatively, cells tagged with magnetic beads in the presence of abundant non-tagged cells can be counted accurately utilizing the Hall effect [44].

2.3 Cell Sorting

Microfluidic devices based on various cell sorting principles have been designed. Cells may be sorted based on biomarkers and antigens [18]. Alternatively, label-free cell separation can be achieved by exploiting intrinsic physical characteristics of the cell, such as its size [5, 19, 60, 87], deformability [30, 38], density [35], electric [10, 63, 67, 69], acoustic [27], and magnetic properties [44]. Here we introduce a few cell sorting approaches developed in recent years. Interested readers may also check out the excellent and comprehensive review papers [3, 4, 13, 31].

Capture molecules, recognizing and binding to molecules on the surface of the target cell, have been used to sort cell populations successfully for various cell types. Targeted cells are immobilized on the surface of a substrate or a magnetic bead coated with capture molecules, while the other cells are rinsed away. One of the important parameters in designing such a system is to maximize the chance of the target cell to explore the surface and hence be captured. Different approaches have been developed to achieve this goal, including incorporating micropillars [65], herringbone structures [82], or nanostructures [14, 66] inside the device, or using fibrous materials [11], etc. Antibodies have been the most popular choice as capture molecules. They can be highly sensitive and specific to targets. However, expensive

and lengthy processes associated with the antibody production hamper the biomarker discovery using antibodies. Aptamers, which can be single-stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) molecules folding into unique three-dimensional (3D) structures, can function similarly as antibodies and have the potential to accelerate the exploring appropriate biomarkers for specific cell types. Target-specific aptamers have been screened using microfluidic-based systematic evolution of ligands by exponential enrichment (SELEX) and phage display technology, which process is automated and greatly reduces the time and expertise required [36].

Endogenous physical traits of cells, independent of surface markers, provide another aspect for the sorting of cells. Cells have been sorted based on their size, deformability, density, electric, acoustic, and magnetic properties. Different dependences of various forces on cells' physical properties are exploited to drive cells differently in a designed direction or to a location where forces balance out, and hence the sorting of cells. For instance, the interaction of the lift force with Dean flow drag force leads to the focus of cells to defined size-dependent equilibrium positions inside the microfluidic channel [22]. Cancer cells and leukocytes can be separated based on differences in their sizes and electrical properties (Fig. 3) [37].

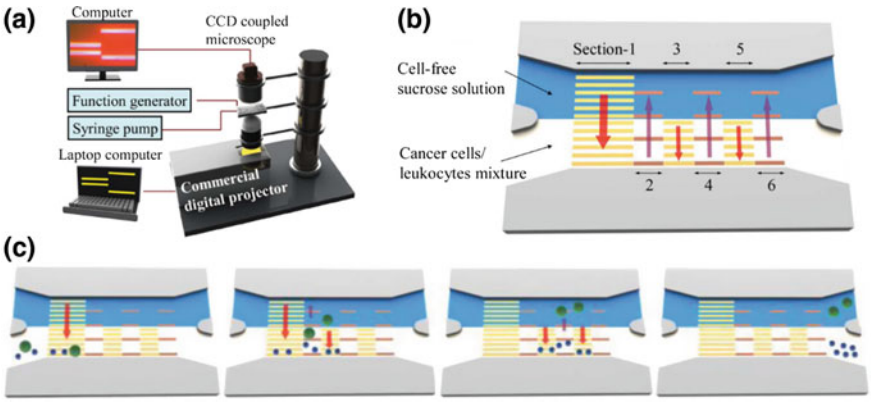

Fig. 3 Cell sorting using optically induced dielectrophoretic force (ODEP). **a** Photoconductive bottom electrodes of the microfluidic device can be reconfigured using a projector to generate programmable DEP forces. **b** Cells suspended in sucrose solution are introduced into the device featured with six sections of moving optically induced bottom electrodes. **c** Cells are lined up close to the edge of the channel in Sect. 1. Cells of larger size and higher permittivity are dragged across the boundary of two fluids by the DEP force and carried away by the cell-free sucrose solution in Sects. 2, 4, and 6, whereas other cells are pushed back toward the edge of the channel in Sects. 3 and 5. Reproduced from Ref. [37] by permission of The Royal Society of Chemistry

2.4 Cell Positioning

One of the essential steps in single-cell manipulation is moving a cell to a desired location for subsequent observation and treatments. Parameters, such as the type and number of single cells to be positioned, duration of monitoring, and target readouts, affect the decision on which approaches are suitable for achieving experimental goals. One of the pioneering papers in microfluidics, presented in 1997 by Jed Harrison's group at the University of Alberta, Canada, demonstrated the electrokinetic routing and on-chip lysing of cells [55]. Since then, a variety of alternatives have been proposed due to concerns of the high voltages involved in electrokinetic valving. Here we discuss technologies for positioning cells using hydrodynamic trapping and several others mechanisms.

In hydrodynamic trapping, cells may be delivered and positioned to stagnant or low-flow locations created in designed channel geometry or inside the induced vortex [56]. Microwells are simple cell trapping devices [72, 96]. Cells are captured by gravity into a microwell array for imaging or subsequent analysis on its content such as RNA or DNA [24, 94]. It is not easy for fluid to dislodge the trapped cell. Often repeated cell seeding procedures are employed to increase the microwell occupancy rates. Cells can be also delivered actively by fluid to the cell trap consisting of a constriction that is smaller than the size of the cell [12]. Once a cell is inside the trap, occluding the constriction, fluid is diverted and delivers cells to other unoccupied cell traps (Fig. 4) [23, 50]. Alternatively, cells may be trapped

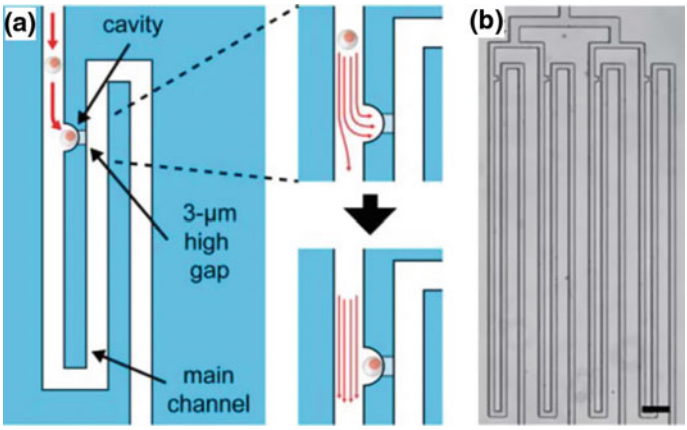

Fig. 4 A self-regulating hydrodynamic single-cell trapping device. **a** Schematic depicts a cell is delivered by the flow and trapped at the cavity connecting to a 3 μm high gap. The *top* insert illustrates the majority of the flow goes through the gap that has less fluidic resistance than the main channel. Once the cavity is occupied, the flow is redirected to deliver cells to downstream cell traps along the main channel (*bottom* insert). **b** An array of single-cell traps are incorporated in the device. Four cell traps are shown (*scale bar* is 100 μm). Reproduced from Ref. [50] by permission of The Royal Society of Chemistry

hydrodynamically within a vortex induced by modified channel geometry [39], acoustic streaming [34], or electroosmosis [59].

Arrays of trapped single cells have also been created by exploiting the differences in various properties between cells and the surrounding media. For instance, small coils fabricated using CMOS (complementary metal–oxide semiconductor) processes can trap cells labeled with magnetic beads [53]. Dielectrophoretic (DEP) traps represent an attractive solution for selectively trapping and releasing single cells; each trap is created by a set of electrodes (in a microfluidic chamber) that generate a nonuniform electric field and hence electrodynamic forces acting on the cell when the suitable electric fields, usually in AC, are applied to the electrodes [86]. However, subtle adverse effects on cells due to the typical electric fields applied in DEP limit its applicability for long-term clasp and culture of cells [41, 61].

Cells can be trapped chemically using antibodies or extracellular matrix molecules. Various techniques have been developed to pattern chemicals, biomolecules, or cells on a surface. Interested readers may check out excellent reviews [58, 74].

One recent trend to position cells is to print cells directly. Surface printing of cells has been demonstrated using a microfluidic 'pen' [45]. 3D printing of cells and biocompatible materials has shown promising progress, while advances in increased biocompatibility, print resolution and speed are needed [64].

3 Single-Cell Analysis (SCA)

Progress in microscale and nanoscale technologies is revealing new insights into single cell biology. Analysis of single cells can be monitoring a few parameters of intact cells or directly identifying its contents after cell lysis. Spatial and temporal information of a few labeled species or detectable parameters can be performed through observing intact cells, while inaccessible for labeling or complex mixture of species may be identified in cell lysate. However, separate samples are needed for each individual experimental condition if cells are to be lysed for measurements.

3.1 Intact Cells

Many assays used in molecular or cellular biology and drug development target the selection or screening of cells based on complex phenotypes or behaviors such as morphology, migration, or growth rates. Although detection of (usually fluorescent) markers for a specific gene or enzymatic activity may serve as reporters on the behavior of interest, this is a nonoptimal approach because the behavior is not screened directly, which can result in false positives or false negatives if the marker also reports on other biochemical pathways or the behavior involves many other pathways (as is often the case). Salient features of microfluidic systems, such as the

precise temporal and spatial control of the fluid and substrate at the micrometer scale allow the precise regulation and modulation of the cellular microenvironment. Many microfluidic technologies have been developed for high-throughput sorting of cells based on complex cell behaviors. Experimenters are often presented with an additional set of challenges when the measurement or question requires the interrogated sample to be kept alive.

The capability of miniaturized devices to position cells and to create well-defined physical and chemical microenvironments provides unique opportunities to study cell biology and screen drugs [95]. Different types of cells may be cocultured. In addition, large numbers of single cells may be trapped and clonally expanded. Drugs may be tested on cells grown into 3D aggregates. Mounting evidences have revealed cells in conventional monolayer culture differ from cells in 3D environment in various cellular activities such as proliferation rate, cytotoxicity and viability, cellular functions and structure, morphology and differentiation efficacy [26, 89]. 3D cell culture is potentially a powerful tool to mimic physiological tissue environment and hence confers a high degree of physiological relevance of cell-based assays and advances the quantitative modeling of biological systems from cells to organisms. Various microfluidic devices have been designed to increase uniformity and efficiency of formed 3D cell cultures, to provide better controlled cellular environment, high-throughput screening (HTS), and to simplify handling procedure [49, 54, 76, 85]. It is, however, important to note that care has to be taken while designing on-chip cell culture systems and interpreting results. Differences between experimental conditions, including the transport and spatiotemporal gradients of gases, ions, nutrients, waste products, and factors released from cells and devices, need to be carefully considered. 3D cell cultures, such as cancer spheroids and embryoid bodies, can be dissociated into single cells to characterize cellular heterogeneity and identify rare cells. Disaggregation of cells is usually accomplished by enzymatic digestions, which can be inefficient, inconsistent, and lead to cell damage. Mechanical dissociation of cells has been demonstrated using microfluidic devices incorporating constrictions to shear cell aggregates into single cells with a better consistency and cell viability (Fig. 5) [57, 70].

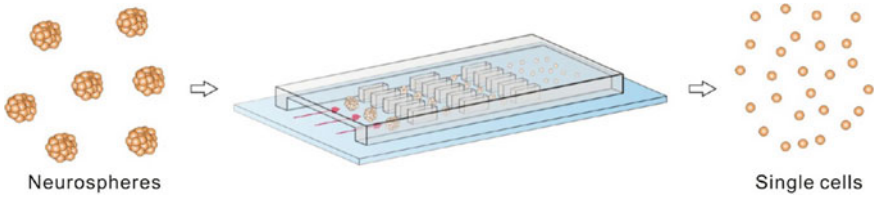

Fig. 5 Enzyme-free dissociation of neurospheres into single cells. Exogenous contamination is reduced using flow and microstructures to mechanically dissociate neurospheres with high yields of single cells and viabilities. Reprinted with the permission from ref. [57]. Copyright 2013 American Chemical Society

3.2 Cell Lysate

Breakage of a cell reveals its content for direct extraction, separation, and identification. However, standard biochemical techniques, while successful in many applications, often lyse a multitude of cells and introduce a more than a millionfold dilution of the cell content into microliter volume, risking the sensitivity and reliability of single-cell assays. High-resolution separation and high-sensitivity detection methods have been instrumental to the analysis of lysate from single cells. In general, there are two approaches to analyze single-cell lysate. One aims to integrate micro- or nanosensors with the fluidic system. The other focuses on the miniaturization of analytical chemical methods. Parameters to be considered include techniques to lyse cells, properties of target molecules, sample separation, and coupling to outside instruments, etc.

Microfluidic devices have recently proved to be very convenient and useful tools for single-cell genomics, transcriptomics, proteomics, and metabolomics [7, 88, 98]. Microfluidic devices provide a very small growth environment, comparable to the dimensions of the environment that surrounds a cell, in order to analyze cells under conditions that are similar to those that occur naturally. Also, these tools provide a controlled area for monitoring the small changes in a single cell [83, 84]. Waters et al. had demonstrated the cell lysis, polymerase chain reaction (PCR), and electrophoretic analysis of single-cell DNA on a microfluidic device in 1998 [90]. A bit more than a decade later, White et al. demonstrated high-precision reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays on hundreds of single cells in parallel using a fully integrated microfluidic platform in 2011 (Fig. 6) [92]. The pace of progress has been remarkable. However, it remains technologically challenging to distinguish cell heterogeneity from the technical variation that is intrinsic to the detection method, such as PCR, when the amount of sample from a single cell is minute.

3.3 Integrated Sensors

Biochemical microsensors have been successfully demonstrated for the detection of biomolecules, chemicals, and gases with high sensitivity [68]. They can be included on-chip to characterize the molecular contents of the sample in real time. Integration techniques that are biocompatible while conserve or even improve the performance of biosensors are being developed.

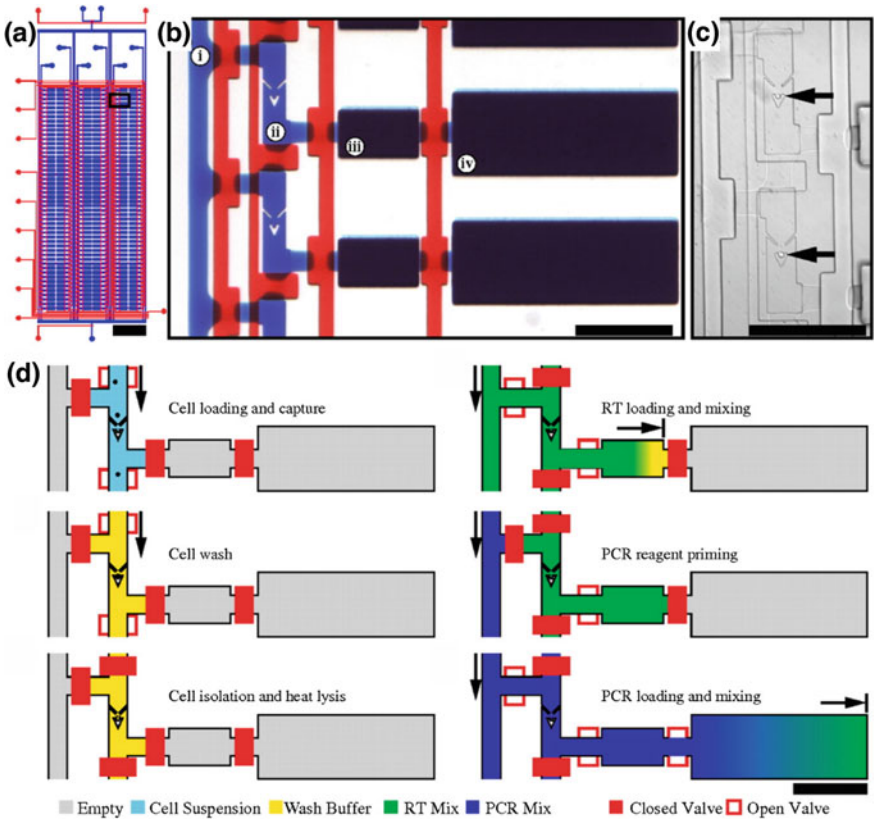

Fig. 6 Microfluidic single-cell RT-qPCR. **a** The microfluidic device contains 6 sample inputs and is capable of performing 300 RT-PCR reactions using ~20 μL of reagents (*scale bar* is 4 mm). **b** A micrograph of an array unit consisting (i) a reagent channel, (ii) a cell capture chamber, (iii) a reverse transcription (RT) chamber, and (iv) a PCR chamber (*scale bar* is 400 μm). **c** A micrograph of two-cell capture chambers with trapped cells indicated by arrows (*scale bar* is 400 μm). **d** The operation of the device. Cells in suspension are introduced into the device and captured. Extracellular RNAs are rinsed away prior to heat lysis of trapped cells. RT and PCR reagents are injected sequentially for single-cell transcriptome analysis (*scale bar* is 400 μm). (Reproduced with permission from White et al. [92])

4 Summary

Micro- and nanotechnologies enable scientists to handle and analyze single live entities (ranging from cells, embryos, to worms) at high throughput in small fluid volumes, and are revolutionizing the fields of molecular biology, biochemistry, and cell biology because they are providing a more quantitative description of cellular heterogeneity—which is crucial in understanding both physiological and patho-physiological phenomena such as differentiation, migration, reproduction and

cancer, among many others. For the same token, this ability is greatly benefitting many related efforts in biotechnology, e.g., in the development of cell analysis chips for PCR, patch clamp electrophysiology, etc. We can expect that the miniaturization trends and integration of analytical components continue to greatly advance the field of single-cell analysis.

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