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Microfluidic Platforms for Single-Cell Analysis

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chemical cytometry, microchip, fluorescence, capillary electrophoresis, cell-cell variation, genetic analysis

Abstract

Microfluidics, the study and control of the fluidic behavior in microstructures, has emerged as an important enabling tool for single-cell chemical analysis. The complex procedures for chemical cytometry experiments can be integrated into a single microfabricated device. The capability of handling a volume of liquid as small as picoliters can be utilized to manipulate cells, perform controlled cell lysis and chemical reactions, and efficiently minimize sample dilution after lysis. The separation modalities such as chromatography and electrophoresis within microchannels are incorporated to analyze various types of intracellular components quantitatively. The microfluidic approach offers a rapid, accurate, and cost-effective tool for single-cell biology. We present an overview of the recent developments in microfluidic technology for chemical-content analysis of individual cells.

Contents

INTRODUCTION

Ever since the existence of the biological cell was visualized via microscopy, both the commonality and the individuality of cells have been recognized. It was not until recently, however, that cellular heterogeneity was considered significant in hypothesis construction and investigated quantitatively in experiments. We have many intriguing examples demonstrating cell-cell variability and its relevance to biological phenomena. For example, phenotypic diversity in an isogenic microbial population appears to constitute a more efficient survival strategy, as shown in the cases of persister *Escherichia coli* cells with antibiotic resistance and competence development in *Bacillus subtilis* (1). The transcription events in mammalian cells are observed to be subject to random fluctuations, leading to large variations in mRNA copy numbers (2–3). In a clonal population of mouse multipotent progenitor cells, the cell-cell heterogeneity is found to be connected with cell-fate decisions (4). These examples clearly show the importance of cellular individuality.

Successful development of single-cell biology owes much to the progress in measurement science. Obviously, accurate and reliable quantification of a cell's various chemical components that exist in limited amounts is one of the biggest challenges in this field. Flow cytometry and fluorescence microscopy are commonly employed to perform single-cell measurements with distinct advantages of high-throughput content and high-information content, respectively. These methods are indispensible tools for observing behavioral characteristics of live cells. Their use for chemical analysis of single cells, however, is limited to monitoring a few species that can be labeled specifically and differentiated spectrally.

A more direct strategy for quantitative cellular analysis involves breakage of a cell and subsequent extraction and identification of its contents, which allows detection of chemical components that are difficult to label in an intact cell and separation of a complex mixture of species for multicomponent analysis. This alternative approach is, in a sense, an improved, unicellular version of the traditional biochemical assays in which the molecules, obtained by lysing a multitude of cells and purifying the cell lysate, are analyzed. Chemical cytometry, a term coined by Dovichi and collaborators (5) to describe such an approach, was initially realized by Jorgenson and colleagues (6) using microseparation techniques such as capillary electrophoresis (CE) or open tubular liquid chromatography in 1989. Since then, the combination of high-resolution separation techniques and high-sensitivity detection methods has been applied to single-cell analysis of various types of cells and analytes thereof. Excellent review articles (7–10) summarizing the history and the current state of the CE-based technique have been published recently.

CE: capillary electrophoresis

Microfluidics, which refers to fluidic behavior and the control of it within structures of micrometer dimensions, is a field of study closely related to chemical cytometry (11–13). Microfabricated devices that integrate the multistep procedures of cell manipulation, lysis, and chemical analysis have emerged as an enabling platform for single-cell analysis. The capability of handling an extremely small volume of liquid containing cells or reagents, typically in the range of picoliters to nanoliters, can be utilized to minimize sample dilution during lysis and to perform necessary chemical reactions. The separation modalities such as chromatography and electrophoresis within microchannels can be incorporated into the same chip to perform high-resolution analysis after efficient sample transfer. Miniaturization can greatly reduce reagent costs, and automation can prevent measurement errors arising from human operations. These advantages, in conjunction with the possibility of parallelization, facilitate rapid, accurate, and cost-effective analyses of single cells.

PDMS: poly(dimethylsiloxane)

In this review, we focus on the application of microfluidic technology for chemical-content analysis of individual cells and highlight novel techniques that are thought to be important for future development and improvement of the technology. It needs to be mentioned that several review articles have been written on the topic with different scopes and emphases (14–16). Before discussing the applications, we summarize essential components of the microfluidic devices for single-cell analysis.

KEY COMPONENTS OF MICROFLUIDIC CELLULAR ANALYSIS

Microvalves

The first step in single-cell analysis using a microfluidic device is to move a cell to a desired location on the chip and isolate it in a chamber for further treatments. The microvalve fabricated with the multilayer soft-lithography technique (17–18) that exploits the elastomeric property of poly(dimethylsiloxane) (PDMS) offers the most straightforward control over a cell and the fluid around it. Researchers can operate this valve by applying pressure to the valve layer to collapse the thin membrane layer in between, thereby closing the channel layer (**Figure 1*a***). One can achieve isolation volumes ranging from tens of picoliters to tens of nanoliters by using a pair of microvalve structures. Relatively easy fabrication and operation as well as small dead volumes have made this type of valve more popular in usage than other microvalves, a thorough review of which can be found in Reference 19.

These valves are often combined to create active elements such as peristaltic pumps. Such micropumps can be used not only to propel cells in a buffer solution but also to enhance mixing of reagents within a microchannel (20–22), where efficient mixing is a challenging task because of the property of a laminar flow.

The development of a three-state valve (**Figure 1*b***) was crucial for chemical cytometry on a microfluidic device (23). It allows isolated single-cell lysate to be derivatized further via chemical reactions. This design element opens the possibility of analyzing intracellular compounds that are difficult to label in vivo with high-sensitivity tags such as fluorophores or immunospecific markers.

Cell Lysis

In chemical cytometry experiments, cell lysis is a crucial step upon which the efficiency of subsequent chemical treatment and analysis of extracted intracellular components depends. Many traditional, macroscale lysis protocols such as bead beating or use of the French press seem to be

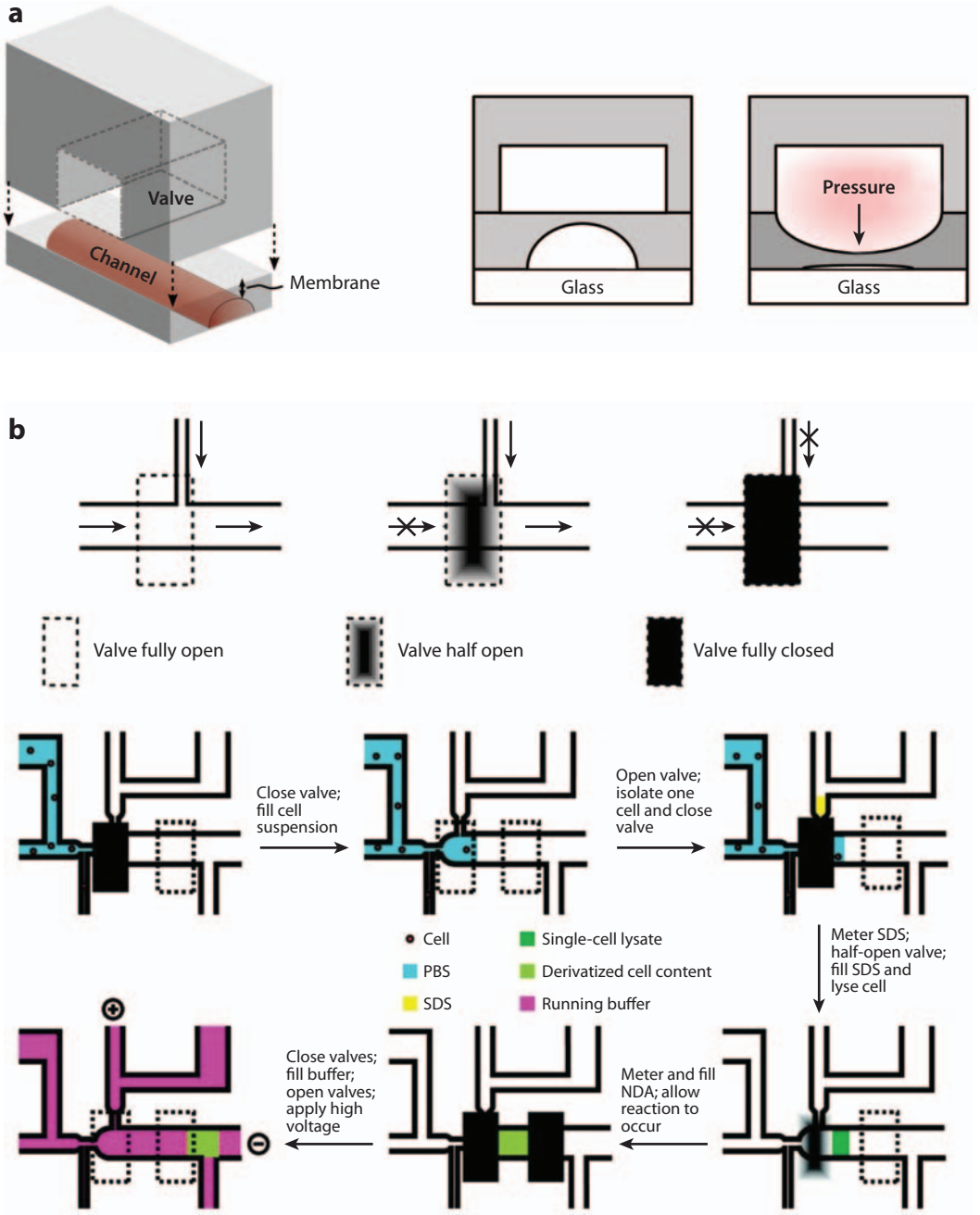

Figure 1

(*a*) Three-dimensional view of the valve, channel, and membrane layers and pneumatic actuation of a poly(dimethylsiloxane) (PDMS) microvalve. (*b*) Schematic illustration of a three-state valve and its application for single-cell lysis and derivatization. Abbreviations: NDA, naphthalene-2,3-dicarboxaldehyde; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate. Reprinted with permission from Reference 23. Copyright 2004 National Academy of Sciences, U.S.A.

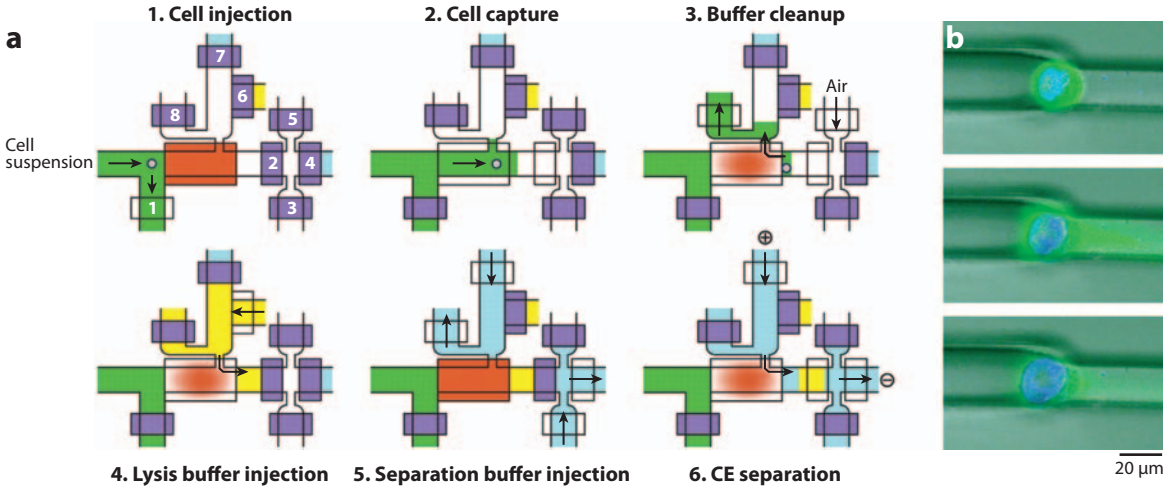

Figure 2

(*a*) Chemical lysis of an individual cell after capture with microvalves. CE, capillary electrophoresis. From Reference 31. Reprinted with permission from AAAS. (*b*) Electromechanical lysis of a single cell stained with calcein (*green*) and Hoechst (*blue*). Reprinted with permission from Reference 29. Copyright 2004 American Chemical Society.

incompatible with microfluidic devices. Therefore, lysis methods that can be scaled down without losing their performance are usually selected.

Di Carlo et al. (24) described mechanical lysis of mammalian cells using sharp nanostructures fabricated inside microchannels. Although most of the cells appeared to be lysed according to the trypan-blue staining assay, less than 10% of the total protein was released into solution. There are other examples of mechanical lysis systems realized in microfluidic devices (25–26), but they are usually developed for the analysis of multiple cells.

Cells placed under sufficiently high electric field undergo electroporation and are lysed eventually (27). Such an electrical lysis process can occur rapidly (on the order of milliseconds). One difficulty in interfacing electrical lysis methods with a microfluidic approach is the formation of gas bubbles under a high electric field. McClain et al. (28) used ac electric fields with dc offsets to circumvent this problem and achieved cell lysis within 33 ms. Munce et al. (29) combined electrical lysis with mechanical stress conferred by a narrow entrance to a separation channel to induce cell lysis at a lower electric-field strength.

Chemical lysis is based on the disruption of the cell membrane, often with detergent solutions (see **Figure 2**). The lysis of bacterial cells is more difficult than the lysis of mammalian cells because of the presence of cell-wall structures and because bacterial cells usually require pretreatment with enzymes to digest the cell walls. It is important to consider in the microchip design the volume change caused by the addition of lysis reagents to minimize dilution of analytes. Picoliter-scale isolation chambers controlled with microvalves were used to achieve single-cell capture and lysis (23, 30–31). A similar approach was extended to perform cell lysis in an array of capture chambers (32), although the simplified chip design limited the scope of postlysis analysis to the detection of specific proteins. Another factor for consideration is whether the lysis condition induces denaturation of analytes and/or reporting molecules such as fluorescently labeled antibodies and thus interferes with the detection strategy. For example, a careful optimization of reagents and their concentrations was necessary for single-cell enzymatic activity assays (33).

PCR: polymerase chain reaction

Laser lysis is a recently developed technique that seems quite promising in terms of simplifying the incorporation of the lysis protocol into a microfluidic chip. Plasma formation induced by a pulsed laser beam creates shock waves and cavitation bubbles that can cause cell rupture (34–35). Chiu and colleagues (36) demonstrated laser-induced lysis of single cells within droplets generated by a microfluidic device. Quinto-Su et al. (37) examined the performance of the technique within a microchannel using time-resolved microscope imaging and observed reconcentration of released cell contents, which is a characteristic advantageous for chemical cytometry.

PURIFICATION AND SEPARATION

Once the cell contents are extracted by lysis, it is often necessary to purify the target molecules (especially in the case of nucleic acids) in order to prevent degradation or cross-contamination. For easier transfer to microdevices, DNA purification has been performed mainly via solid-phase extraction instead of the conventional, liquid-liquid extraction methods (38). A microchannel packed with silica beads or sol-gel phases was successfully used to extract standard DNA samples with 60–70% efficiencies (39). Austin and collaborators used different types of DNA extraction methods such as dielectrophoretic trapping (40) and continuous-flow post arrays (41) to lyse *E. coli* cells and isolate chromosomes from cell debris. Santiago and colleagues (42) demonstrated the use of isotachophoresis to purify nucleic acids from 10 to 25 blood cells.

Purification of mRNA has been achieved via columns packed with microbeads (43–45) or porous polymer monoliths (46) that are functionalized with oligo-dTs. It should be noted, however, that the purification with microbeads or polymer monoliths based on the poly-A tail of mRNA is limited to eukaryotic cells because only 60% of bacterial mRNA is known to have poly-A tails (47). For the purpose of isolating total RNA from mammalian cells, Irimia et al. (48) used silica columns in a microfluidic device for extracting nucleic acids from 150 cells and then removed DNA enzymatically. The quality of extracted RNA faithfully reported the gene expression pattern as compared with GeneChip (complementary DNA microarray). A 96-well format device with microposts for solid-phase extraction made of photoactivated polycarbonate was also used to extract total RNA from *E. coli* with high efficiency (49).

Nucleic acids are usually amplified via polymerase chain reaction (PCR), after which they can be sequenced or hybridized to probes in macroscale experiments for identification. In contrast, proteins and other metabolites extracted from cells cannot be chemically amplified and require direct identification and quantification on the microfluidic chip. Therefore, the analysis of nonamplifiable analytes in a single cell has been performed mostly on microfluidic devices that integrate the entire procedure for sample preparation, separation, and identification. Among other separation techniques, microchip CE has proven to be the most useful toward this end. It can be conveniently integrated with other chip elements, and it allows for rapid, high-resolution separation of chemical species based on their differences in electrophoretic mobilities, which are largely determined by mass-to-charge ratios. Various operational modes and applications of microchip CE have been reviewed elsewhere (50–53).

Our laboratory has developed a series of microchip CE techniques for analyzing amino acids and proteins. The key feature is using a mixture of ionic and nonionic detergents as a surfactant to PDMS chips. The hydrocarbon chains of the nonionic detergent, *n*-dodecyl-D-maltoside, adsorb onto the hydrophobic surface of PDMS and cause the apparent surface property to become hydrophilic, thus preventing nonspecific adsorption of analytes (54). Either by varying the concentration of the ionic detergent, sodium dodecyl sulfate (55), or by embedding negatively charged functional groups into the PDMS polymer (56), researchers can control the surface charge and consequently the electroosmotic flow to optimize CE performance. This mixed micelle system has

been successfully used to separate simple dye mixtures, protein charge ladders, immunocomplexes, and phycobiliprotein complexes (55, 57).

Ultrasensitive detection of separated analytes is required for enabling quantitative measurements of intracellular components from single cells on a microfluidic device. Laser-induced fluorescence (LIF) in a variety of configurations is the most widely used detection method because of its extreme sensitivity and the availability of fluorophore conjugation strategies (31, 58–59). Absorption measurements are less sensitive than fluorescence detection but can be applied to unlabeled, general chemical species (60–62). Another label-free detection method is based on electrochemical detection (63–65).

LIF: laser-induced fluorescence

MDA: multiple-displacement amplification

APPLICATIONS IN SINGLE-CELL ANALYSIS

DNA

It may seem unnecessary to analyze genomic DNA at the single-cell level considering that the genetic material can be biologically amplified to a sufficient amount in an isogenic culture. When generating such a pure culture is nontrivial, however, the genomes should be characterized cell by cell. A representative example involves microbial communities found under various environmental conditions. Culture-independent methods are highly desired for investigating individual contributors in these heterogeneous populations, in view of the low fraction (~1%) of culturable bacterial species that exist in nature (66–67).

For genetic analysis, Quake and coworkers (68) used a microchip that can perform multiplex PCR with individual bacterial cells in parallel (see **Figure 3**). Single-cell isolation was achieved probabilistically; cell density was adjusted for less than 33% of the 1176 reaction chambers (6.25 nL each) to be occupied. Specifically designed primers and fluorescent probes were used to detect the amplification of the 16S rRNA gene, which is a phylogenetic marker, and the formyltetrahydrofolate synthetase (FTHFS) gene, which is an important gene related to bacterial metabolism. Once amplification patterns were identified with fluorescence imaging, PCR products of interest were retrieved from microchambers and sequenced for further analysis. This scheme revealed a novel clustering of rRNA gene sequences that may represent the ribotypes of the FTHFS-encoding cells, which had never before been cultured in the microbial community.

The same group (69–70) developed an integrated microfluidic device for the purpose of whole-genome amplification from a single bacterium via multiple-displacement amplification (MDA). The key feature of the chip is the utilization of gas permeability of PDMS to measure nanoliter volumes of liquids as defined by the channel dimensions. Testing the chip with *E. coli*, the researchers found that the miniaturized version of the commercial MDA protocol significantly reduced amplification bias (69). Although the sequence specificity of the amplification products from microfluidic MDA experiments was very high (>95%), the genome coverage was reported to be 33% when compared with the *E. coli* reference genome. The chip was also used to analyze rare bacterial cells collected from the human mouth. The study showed that the previously unexplored phylum can be accessed through the simple microscopic selection based on morphology and that single-cell genome amplification can provide rich genetic information relevant to microbial ecology.

Microfluidic devices were also used to analyze genomic DNA without amplification. Klepárník & Horký (71) performed microchip electrophoresis to assess the effect of doxorubicin, a cytostatic, on the apoptosis of single cardiomyocyte cells. A lab-on-a-CD platform (72) was used to load and lyse a cell, and fluorescently labeled DNA was separated on-chip through the use of a 2% linear polyacrylamide (LPA) solution as a sieving matrix. DNA fragmentation patterns, which report on

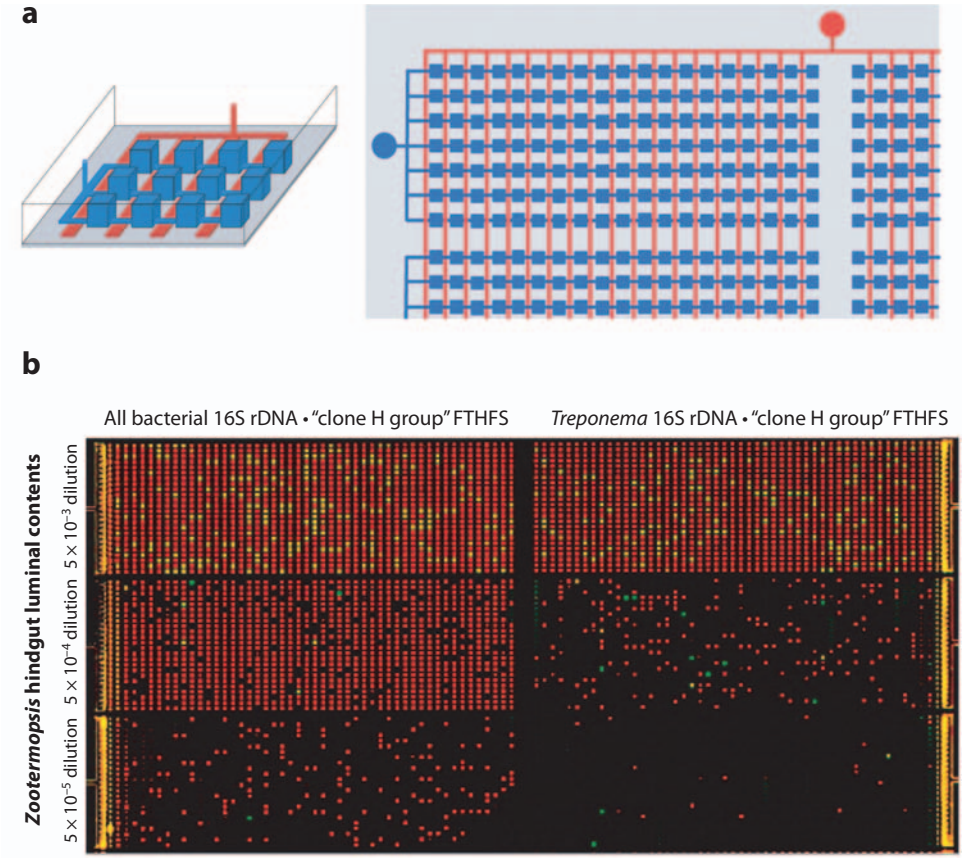

Figure 3

(*a*) Schematic diagram showing a microfluidic digital polymerase chain reaction (PCR) chip. (*b*) Multiplex microfluidic digital PCR of single bacteria cells in environmental samples. FTHFS, formyltetrahydrofolate synthetase. From Reference 68. Reprinted with permission from AAAS.

whether the cell is apoptotic or necrotic, were determined directly from the electropherograms. The authors found that cells exposed to doxorubicin for 24 h exhibited onset of necrosis, which might induce heart failures in patients treated with the drug for an extended period of time.

RNA

Researchers usually measure the quantity of RNA at the single-cell level by miniaturizing the standard protocol of first amplifying RNA molecules into complementary DNA (cDNA) via reverse transcription (RT) and then analyzing the DNA. Microfluidic devices were used for more efficient sample handling and in vitro transcription (IVT) reactions. Bontoux et al. (73) miniaturized the RT process down to nanoliter volumes using a rotary mixer with microvalves. When single-cell amounts of RNA from mouse brain tissues (10 pg) were used as the starting material, microliter- and nanoliter-scale reactions yielded gene expression coverage of 4% and 74%, respectively, which was determined by the comparison of DNA microarray results with the control experiment (15 μg of RNA). The authors also analyzed RNA extracted from single neural progenitors and reported a

high degree of cell-cell variation in gene expression profiles, although only four cells were observed in total.

Marcus et al. (44) used a more sophisticated microfluidic device that incorporated a purification step using beads coated with poly-T nucleotides to isolate mRNA from a single fibroblast cell and to synthesize cDNA. The authors created the bead column by utilizing sieve valves, which close partially when actuated. Zhong et al. (74) improved a similar type of device by increasing the number of cells that can be simultaneously handled from 4 to 20 and used it to measure mRNA copy numbers in single human embryonic stem cells. Instead of using sieve valves, Liu et al. (75) fabricated microcoil structures in addition to the PDMS flow channels, which were used to generate a magnetic field for capturing magnetic beads and to raise the temperature within the channel via Joule heating in order to activate the RT process. Kralj et al. (76) examined the microfluidic IVT approach using bead-packed columns by comparing microarray results from on-chip and off-chip protocols. They obtained good correlation ($R^2 = 0.90$) with 20 pg of RNA, which approximates to an amount from a few cells.

Proteins

Although proteins usually exist in higher quantities than DNA and mRNA, they are more difficult targets for single-cell analysis because (*a*) unlike nucleic acids, molecular amplification is not readily available, and (*b*) the identification of protein species is not trivial. Nonetheless, quantitative measurement of proteins, the functional workhorses of the cell, is crucial for understanding cellular behaviors and mechanisms. Integrated microfluidic systems enable protein analysis by minimizing the sample dilution (and therefore maximizing the effective limit of detection) and by offering separation capabilities for differentiating protein species according to their physical and chemical properties.

Toner and coworkers (30) used 25-pl chambers to capture and lyse a single mammalian cell. They determined the amount of actin indirectly by measuring changes in the extracellular concentrations of actin-binding dye molecules and estimated it to be 1×10^6 molecules per cell. Wu et al. (23) created a three-state microvalve that can close a 70-pl capture chamber for lysis and partially open the chamber to add the labeling reagent, naphthalene-2,3-dicarboxaldehyde (NDA). Using this device, the group obtained the abundance profile of amino acids in a single Jurkat T cell by performing CE with LIF detection and compared the profile with the multiple-cell results.

Hellmich et al. (77) reported the development of a microfluidic system for single-cell capture and subsequent CE analysis. A combination of an optical tweezer and the geometric obstacles fabricated within the microchannel was utilized to lyse an insect cell at a specific location of the chip. The authors tested the LIF detection not only with 488-nm laser excitation but also with 266-nm laser excitation in the ultraviolet (UV) region to allow the label-free detection of protein species utilizing tryptophan autofluorescence. Although UV-LIF detection was performed only with standard protein solutions at a much higher concentration than single-cell quantities, this type of general detection strategy should be an important tool for protein analysis. The same group improved the injection plug size by shortening the electrical lysis time. They demonstrated the CE separation of two proteins from a single cell that were genetically labeled with green fluorescent protein and yellow fluorescent protein (78).

Our laboratory developed integrated microfluidic devices for analyzing low-abundance proteins by direct counting (31) (see **Figure 4**). For proteins that are not naturally fluorescent, a generic method of tagging target proteins with fluorescently labeled antibodies was pursued. A purification step after the completion of the labeling reaction was unnecessary because the excess labeling reagent was baseline-separated from the labeled protein species in the subsequent CE.

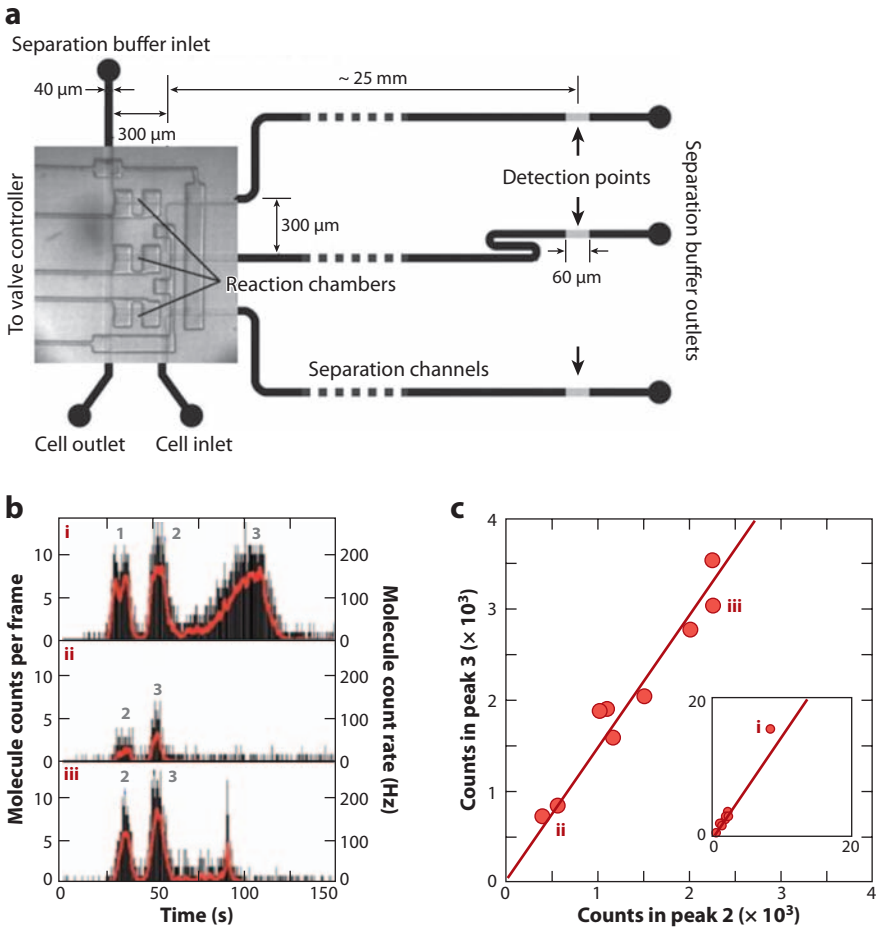

Figure 4

(*a*) Layout of protein-analysis chip for a single cyanobacterium. (*b*) Molecule-counting results of three cells from a nitrogen-depleted culture. (*c*) Phycobiliprotein copy-number distribution of 10 cells from a nitrogen-depleted culture. From Reference 31. Reprinted with permission from AAAS.

We determined the amount of protein molecules by monitoring the number of fluorescence burst events generated when they flowed through a small, defined detection volume. The use of a cylindrical lens for laser excitation allowed for high-efficiency collection of fluorescence signals from a rectangular microchannel configuration, which avoids clogging of the channel caused by cell debris in the case of a nanochannel monitored with spherical optics. From this direct-counting result, the average copy number of β_2 adrenergic receptor (β_2AR) bound with Cy5-labeled antibodies was estimated to be 1.8×10^4 molecules per cell, in agreement with ensemble measurements. Naturally fluorescent, light-harvesting protein complexes—named phycobiliproteins (PBPs)—from a cyanobacterium were also analyzed in a minimally parallel fashion (i.e., three cells per analytical round). The protein copy numbers, as low as 400 molecules per cell, were successfully quantified via the single-molecule fluorescence detection scheme. A large cell-cell variation in protein copy numbers was observed for both β_2AR and PBPs. A rare type of cell, which did not undergo the

PBP degradation process under a nutrient-deprivation condition, was also found. This finding demonstrates the power of a single-cell approach to biological problems that cannot be provided by conventional ensemble-averaging techniques.

OUTLOOK AND CONCLUSIONS

We all know that group characteristics are a poor indicator of the actions taken by the individuals within that group. It is no different for cells, but most previous biological studies have had to rely on observations of the collective behavior of thousands to millions of cells. Microfluidic platforms hold the promise of revealing to us the differences among individual cells that compose a group. The new capability that such studies may make possible has important consequences, from gaining a fundamental understanding of life processes to developing new medical treatments for disease states. Yet, single-cell analysis is in its infancy, and we are still learning to make baby steps toward improving this technique. We suggest that the following areas need improvement for advancing single-cell microfluidic analysis to the next level and generating significant impact on biological research.

Currently, there exists a barrier between the two approaches in the field; namely, a simple chip design leads to a high throughput with limited details, and a complex design means many more details but a low throughput. To break down such a dichotomy and gain from both strengths, a more efficient mechanism for accurate cell control seems to be the solution. If a large number of cells can be individually addressed in a relatively simple microchip, both the throughput and the quality of single-cell data will improve. Among other approaches, optoelectronic tweezers (79), image-based optofluidic cell sorting (80), and droplet-based cell manipulation (81) appear promising for this purpose.

The application of MDA as a whole-genome amplification tool has proven to be superior to the conventional approaches based on PCR (82–83). Although the microfluidic version of MDA improved the performance of the technique, the genome coverage needs to be improved further to enable truly culture-independent genomic analysis of novel microorganisms from environmental samples or of a small number of human cells from clinical samples. Examples of such efforts include a recent report of estimated genome coverage exceeding 70% from a single marine bacterium using modified commercial MDA protocols (84) and a preliminary result from our laboratory using a microfluidic MDA chip (85).

The development of more rapid and less invasive lysis protocols is another crucial area that needs improvement. The extent to which the lysis process itself alters cellular behavior is not clear. Particularly, the intracellular molecules such as mRNAs and regulatory proteins may respond to stress conditions rapidly. Faster and more controlled lysis methods will provide a window through which the natural state of dynamic intracellular environments can be studied. Laser-based lysis (37) and strategies that carefully combine chemical and electrical lysis methods by utilizing microenvironmental controls within a device are expected to make important advances.

Novel detection modalities with high sensitivity for general detection of analytes also warrant research efforts. The use of cavity ring-down spectroscopy for absorption measurements in nanoliter volumes (86), the use of thermal lensing detection (87), and various ideas for interfacing a microchip with a macroscale analytical instrument such as a mass spectrometer (88–90) are noteworthy recent developments.

As advances and breakthroughs in these areas accumulate, we can expect to see in the near future the realization of dream experiments such as extracting whole-genome-level information reliably from a single biological cell (91) or performing single-cell experiments with a systems-biology perspective (92–93). Such dreams can be realized only by advances in instrumentation—that is,

the development, testing, and ultimately the commercialization and widespread adoption of new tools for these purposes.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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