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Review

Recent advances in single-cell analysis using capillary electrophoresis and microfluidic devices☆

Wei-Hua Huang*, Feng Ai, Zong-Li Wang, Jie-Ke Cheng

College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China

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Abstract

Cells are the fundamental unit of life, and studies on cell contribute to reveal the mystery of life. However, since variability exists between individual cells even in the same kind of cells, increased emphasis has been put on the analysis of individual cells for getting better understanding on the organism functions. During the past two decades, various techniques have been developed for single-cell analysis. Capillary electrophoresis is an excellent technique for identifying and quantifying the contents of single cells. The microfluidic devices afford a versatile platform for single-cell analysis owing to their unique characteristics. This article provides a review on recent advances in single-cell analysis using capillary electrophoresis and microfluidic devices; focus areas to be covered include sampling techniques, detection methods and main applications in capillary electrophoresis, and cell culture, cell manipulation, chemical cytometry and cellular physiology on microfluidic devices.

Keywords: Single-cell analysis; Capillary electrophoresis; Microfluidic devices; Chemical cytometry; Cellular physiology

Contents

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* Corresponding author. Fax: +86 27 68754067.
E-mail address: whhuang@whu.edu.cn (W.-H. Huang).

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1. Introduction

Most cell-based biological assays are performed with bulk experiments based on analysis of cell populations, and the averaged data can satisfy the demand for many biological researches. However, it is well known that individual cells, even those identical in appearance, differ in numerous characteristics, such as the concentration of a critical metabolite and particular gene expression, cell-based assays data averaged across cell population signal sometimes give misleading information. Furthermore, biochemical processes often occur on short timescales or non-synchronously, compounds inside cell involve fast and kinetic change, taking a population average will not lead a proper understanding of how the cellular chemistry occurs, analysis of the molecular events on single-cell level can only give the accurate information of the cellular chemistry. Additionally, many diseases like cancer start with a single cell, early diagnosis of rare mutations from the population cells that herald the inception of a disease necessitate individual cell analysis. Therefore, single-cell analysis has become a "hot topic", and increased emphasis is now being placed on developing high throughput and robust techniques for single-cell analysis.

Numerous methods including capillary electrophoresis (CE), microfluidics, microarray, microelectrodes as well as various imaging techniques have been developed for single-cell analysis, and many excellent review papers have also been published to address such a "hot topic" during the past few years [1–10], which covered the main developments have been made in this crucial area. In this review, we restrict our focus from 2004 to July 2007 on the capillary electrophoresis for single-cell analysis and its applications, and microfluidic-based single-cell analysis including cell culture, cell manipulation, chemical cytometric analysis and cellular physiological studies. Even with this restriction, only these representative publications were selected since there were still massive papers have been published in this timescale, and much important work was not included.

2. Capillary electrophoresis

CE involves injecting a single-cell or subcellular fractions into the capillary, lysing the cell via chemical, electrical or optical methods inside the capillary and followed by separation and detection of the contents in the lysates by means of various detection techniques. CE has played a crucial role for the cytometric analysis for single cells benefited from its characteristics of small sample volumes, high speed, high efficiency and capability of coupling to high sensitive detectors such as laser-induced fluorescence (LIF), electrochemical, chemiluminescence, mass spectroscopy, etc. This section focuses on the current development on cell sampling techniques, detection methods and recent applications of single-cell analysis with capillary electrophoresis, and the microchip-based CE will be presented in the next section.

2.1. Sampling techniques

Conventional sampling for single-cell analysis is performed by electrokinetic or siphon injection of the whole single cell under a microscope with the aid of a micromanipulator that controls the inlet of the capillary adjacent to the individual cells. Though this sampling technique is still widely used, the analysis was restricted at low-throughput. Chen and Lillard [11] have developed a two-capillary scheme in which the two capillaries were jointed by lysis junction for automated and continuous introduction of single cells increased the throughput.

2.1.1. Multiple sampling

A single cell is lysed and its contents of interest are separated and detected after the injection of the cell, but this method only represents a snapshot information of the cellular contents, especially for the kinetic process such as the enzyme activities. Shoemaker et al. developed a novel multiple sampling for assaying enzymes from a single cell. Individual cells were isolated using a micromanipulator and placed in nanoliter-scale reaction vessels, the cells were lysed and enzyme activity was assayed by removing 5-nl aliquots from the reaction vessel with a nanopipettor connected to a capillary for separation [12]. Although labor-intensive, this technique was capable of repeat sampling of a single-cell enzyme reaction, and will possibly find the specific application in single-cell metabolic studies in which multiple sampling could potentially allow the correlation of an enzyme's expression level to its specific metabolic products.

2.1.2. Subcellular sampling

Although whole analysis by CE provides valuable information about the content and behavior of single cells, the spatial and temporal information regarding cellular contents inside a single cell could be only obtained by probing subcellular compartments. In the nervous system, individual neurons directly communicate with hundreds of other cells using processes, knowledge of the distribution pattern of the neurotransmitters in a single neuron could be used to develop models for neurotransmitters synthesis, transportation, storage and release. Sweedler's group reported a unique sampling protocol using glycerol to stabilize the neuron during isolation, and demonstrated the chiral separation and quantitative measurement of D-Asp contents in specific subcellular regions of a single *Aplysia californica* neuron. The results showed that the percentage of D-Asp in

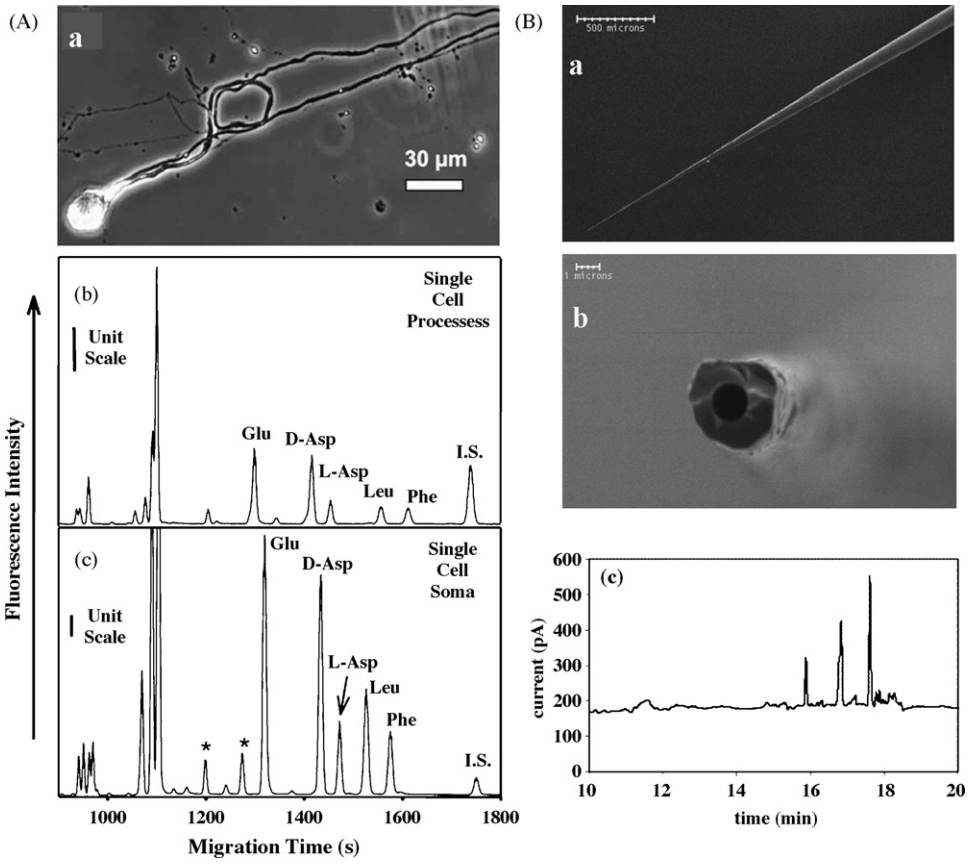

Fig. 1. (A) Detection of D-Asp in the processes of sensory neurons. (a) Image of an isolated sensory neuron acquired using variable relief contrast. Electropherograms of D-Asp in (b) the processes and (c) the soma from a single pleural sensory neuron. Peak identities: Glu, glutamate, with a comigrating compound; D-Asp, D-aspartate; L-Asp, L-aspartate; Leu, leucine; Phe, phenylalanine; I.S., internal standard, L-cysteic acid; *, unidentified peaks. (B) (a) SEM images of a microinjector at a magnification of 50. (b) SEM images of the tip of microinjector at a magnification of 7500. (c) Electropherogram of a 280-fL sample injected from a 20-μm diameter PC12 cell obtained using a 770-nm i.d. capillary. (A) and (B) are reproduced, with permission, from Refs. [13,14], respectively.

the processes from different types of neurons varied significantly; however, morphologically distinct regions of the same neuron exhibited similar ratios of D-Asp, despite differences in the amounts observed (Fig. 1A) [13].

Ewing's group fabricated microinjectors from 770 nm i.d., 150 μm o.d. capillaries, with tips narrowing to 2.5 μm o.d., combined with the electroporation technique that created transient pores with 1–240 nm in diameter in the cell membrane. They demonstrated a relatively nondestructive sampling of the subcellular samples from intact single mammalian cells, separation of cytoplasmic samples (as little as 8% of the total cell volume) taken from PC12 cells have been achieved, and dopamine has been identified and quantified in individual PC12 cells using this technique (Fig. 1B) [14,15].

2.1.3. Organelle sampling and separations

The capability to release intact and organelles from a single cell followed by CE analysis is a powerful approach to describe the chemical content of individual organelles and to further develop other techniques based on individual organelle. Arriaga's group have reported the analysis of organelles, including mitochondria, lysosomes, and nuclei by CE–LIF [16], but the organelles were obtained by bulk fractionation based on mechanical homogenization or nitrogen cavitation approaches that are not compatible with single-cell analysis. In their later articles, they described the use of digitonin to partially disrupt the plasma membrane of single cells on-column and the intact nuclei were released from single cells for CE analysis, prior to injection, the nuclei were targeted with a fluorescent nuclear-tagged protein that was expressed from a plasmid for LIF detection [17]. Presently, the similar technique was used by their group to on-column release mitochondria from single cells, the intact mitochondria that tagged via the expression of the fluorescent protein DsRed 2 were separated and analyzed with CE–LIF detection [18].

2.2. Detection methods

2.2.1. Fluorescence

Due to the high sensitivity, laser-induced fluorescence (LIF) detection has become one of the most important techniques for analyzing biomolecules inside cells with the development of derivatization agents and optical detection instruments, a variety of components including amino acids, peptide, protein, nucleic acid inside a single cell have been detected with LIF at low concentrations. Many biomolecules have native fluorescence, and laser-induced native fluorescence (LINF) detection can be adopted directly to investigate these molecules. CE

supplies a very powerful tool to separate the analytes of interest especially those in low content inside a single cell from endogenous autofluorescence components. The separation and detection of green fluorescence protein (GFP) expressed in a single bacterium was firstly presented by Dovichi's group [19]. CE separated GFP from native cellular autofluorescent components, reducing the background signal and improving detection limits to 100 ymol (60 copies) for GFP. The expressed GFP in a single *Deinococcus radiodurans* bacterium under the control of the *recA* promoter was detected as $\sim 2 \times 10^4$ EGFP molecules/cell.

A multichannel native fluorescence detection system for CE has recently been developed by Sweedler's group to obtain more information. In their instrument, a series of dichroic beam-splitters were used to spectrally distribute the emitted fluorescence into three wavelength channels: 250–310 nm, 310–400 nm, and >400 nm, and the fluorescence in each of the three wavelength ranges was detected by a separate photomultiplier tube. The instrument provided more information than a single-channel system, without the complexity associated with a spectrograph/CCD based detector. With this instrument, analytes could be separated and identified not only on the basis of their electrophoretic migration time but also on the basis of their multichannel signature, which consisted of the ratios of relative fluorescence intensity detected in each wavelength channel. The instrument was used to detect and identify the neurotransmitters in serotonergic LPeD1 and dopaminergic RPeD1 neurons from *Lymnaea stagnalis* [20].

Improved sensitivity is always achieved by fluorescent derivatization with a fluorophore, and derivatization is very significant and widely used in single-cell and subcellular analysis, given the small volumes of the samples and the small amount of materials being analyzed. But the non-specific and incomplete derivatization may limit its application, so developing more specific and sensitive derivatization agent as well as the label techniques will facilitate the CE–LIF for single-cell analysis. Fluorescence combining with immunoreaction has been used to detect specific proteins. Jin's group described the determination of different forms of human interferon-γ (IFN-γ) in single natural killer cells by CE with on-capillary immunoreaction and LIF detection. One single cell and the monoclonal antibody labeled with fluorescein isothiocyanate of IFN-γ were successively (in turn) electrokinetically introduced into the front end of a separation capillary that acted as a microreactor to allow different forms of IFN-γ from the lysate of the cell to process the immunoreaction with their labeled antibody. The complexes of different forms of IFN-γ with their labeled antibody were separated and detected by CE with LIF detection with a limit of detection of zeptomoles [21]. To overcome the diffusion of the analytes along the longitudinal axis of the capillary during the on-capillary immunoreaction with antibody in the capillary microreactor and improve the separation efficiency, an improved labeling technique—intracellular immunoreaction was later developed in their group by introducing FITC labeled anti-IFN-γ monoclonal antibody into NK cells by electroporation. The immunoreaction occurred inside a single cell before being electrokinetically injected into the capillary, the cell was then lysed and different forms of IFN-γ in the cell were separated and detected with a great improvement in width at the peak half-height ($W_{1/2}$), numbers of theoretical plates (N) and resolution (R) as compared with those using the on-capillary immunoreaction (Fig. 2a) [22].

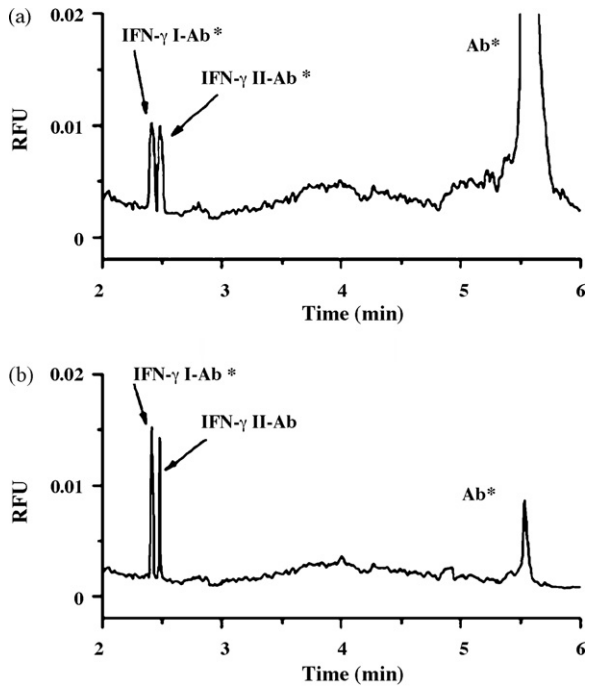

Fig. 2. Electropherograms of IFN-γ in single NK cells using (a) intracellular immunoreaction and (b) on-capillary immunoreaction. Reproduced, with permission, from Ref. [22].

Xiao et al. described the characterization of the P-glycoprotein (PGP), a transmembrane efflux pump in multidrug resistance (MDR) research, by using two antibodies mouse-raised JSB-1 antibody and goat anti-mouse IgG-FITC (GAMIF) for immunoreaction and LIF detection. A permeable intact cell after the immunoassay binding with fluorescence labeling antibody was injected into the capillary and directly separated without lysis, and the PGP amount on the cell could be outlined and calculated, it was found that the PGP amount on K562 MDR cell line was 3.88 times higher than that on K562 sensitive cell line [23].

2.2.2. Amperometry

Amperometry, as the most widely used electrochemical detection method for CE, is based on the oxidation or reduction of the electroactive compounds on the working electrodes, and the oxidation or reduction current can be directly correlated with analyte concentration. Comparing with other detection method, amperometric method needs low-cost detectors and instrumentations while offers remarkable sensitivity. Amperometry is also selective since only electroactive compounds that undergo either oxidation or reduction on the electrodes can be detected. Developments of microelectrodes fabrication techniques and different electrode material can improve both the sensitivity and selectivity, which facilitated the widespread application of amperometric detection in CE-single-cell analysis. The improvements of the sensitivity could be also achieved

by using extremely smaller electrode or bundle electrode according to the inner diameter of the capillary used for separation. An etched carbon fiber microelectrode had been coupled with CE in 770 nm i.d. capillaries for the detection of dopamine from the cytoplasmic samples [14,15]. Amperometric detection at a carbon fiber microdisc bundle electrode was used to investigate the reaction product of a single fibroblast cell of mouse bone marrow exposed to disodium phenyl phosphate as enzyme substrate in the running buffer for 30 min on-column prior to CE [24].

2.2.3. Chemiluminescence

Chemiluminescence (CL) is characterized by simple and low-cost optical systems requiring no light sources, avoiding the effects of stray light and the instability of light source, and thus providing low background with excellent sensitivity. Our group developed a capillary electrophoresis immunoassay (CEIA) method based on enhanced CL detection to detect bone morphogenic protein-2 (BMP-2) in rat vascular smooth muscle (VSM) cells. Horseradish peroxidase (HRP) was firstly linked to BMP-2 in VSM cells with noncompetitive format, HRP-Ab_2-mAb-BMP-2 complex and free HRP were baseline separated and detected owing to the catalysis of HRP on the luminol/H_2O_2/*p*-iodophenol chemiluminescence reaction. The BMP-2 was detected with a detection limit of 6.2 pM and the technique was successfully applied to arteriosclerosis development mechanistic study by investigating the change of BMP-2 content in VSM cells stimulated by angiotensin II [25]. Based on the catalytic effect of hemoglobin on the luminol/H_2O_2 reaction, Zhi et al. described the determination of the hemoglobin in single human blood cells and the statistical result of the average content of hemoglobin in 26 human red blood cells was 23.6 pg [26].

2.3. Applications

2.3.1. Protein

The increasing interest has been focused on the study of the proteome since the genome is sequenced. Dovichi's group is developing tools to study the proteome in single cells with two-dimensional CE and LIF detection by which they want to investigate the protein expression in single cells and to determine how protein expression changes across a cellular population during cancer progression and development of an embryo. They developed a two-dimensional capillary electrophoresis method for the study of protein expression in single mammalian cells. Capillary sieving electrophoresis (CSE) combined with micellar electrokinetic capillary chromatography (MEKC) was adopted to adequately resolve the large number of proteins. The separated fractions from the first CSE capillary were transferred to the second capillary for the subsequent MEKC separation, over 100 transfers and second-dimension separations were performed over a ~3.5-h period. They demonstrated the generating protein fingerprints from single native MC3T3-E1 osteoprogenitor cells and MC3T3-E1 cells transfected with the human transcription regulator TWIST (Fig. 3), and also presented single-cell protein fingerprints from MCF-7 breast cancer cells before and after the treatment to induce apoptosis [27]. In their later work, the protein expressed in single-cell embryo from *Hsf1* gene knockout mice was characterized by 2D SDS-MEKC separation to obtain protein expression fingerprints, and over 100 components were resolved with a spot capacity of about 380 in a 1-h 2D-CE separation [28]. To improve the throughput, they recently demonstrated a multiplexed 2D-CE system by which samples were injected into five first-dimension capillaries and fractions were transferred across an interface to five second-dimension capillaries [29].

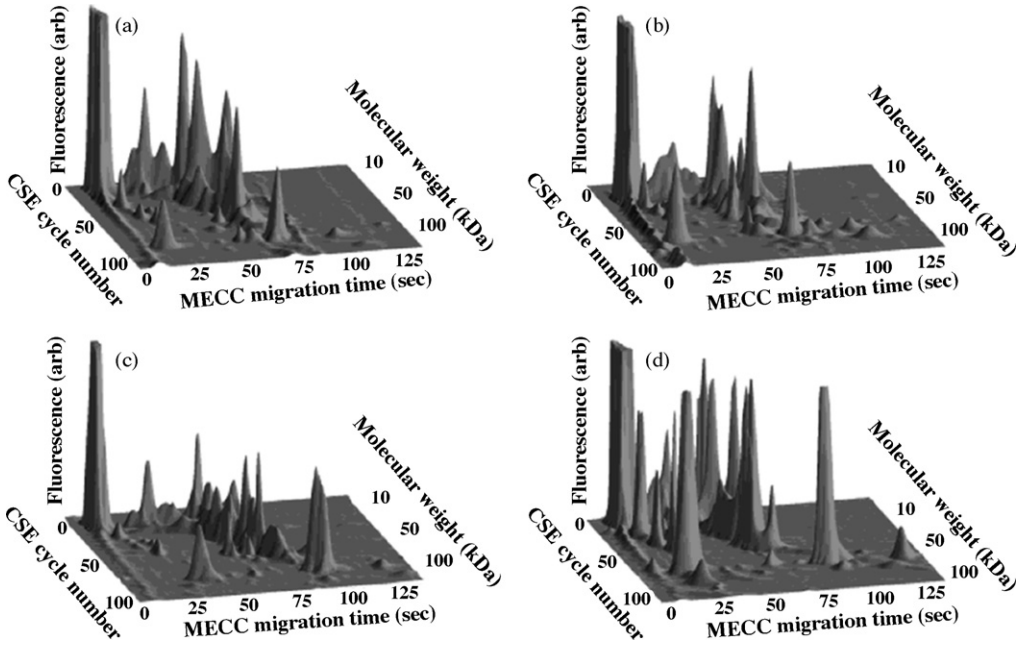

Fig. 3. Protein landscape images from single MC3T3-E1 cells. Landscapes a–c were generated from untransfected cells. Landscape d was generated from a single MC3T3-E1 cell that had been transfected with the transcription regulator TWIST. Reproduced, with permission, from Ref. [27].

2.3.2. Neuroscience

Neuroscience research on single-cell level can afford precisely temporal and spatial information of the neurotransmitters distribution as well as the intercellular communication for the better understanding of neuronal function and dysfunction. Single-cell CE has played important roles in neuroscience research since its earliest applications on snail neurons with initial cytoplasmic sampling from *Planorbis corneus* [30] and the whole cell injection of *Helix aspersia* [31]. Since then, numerous works have been performed to determine the neurotransmitters inside single neurons.

The peptides and proteins present in animals are almost exclusively made up of L-amino acids. Surprisingly, D-amino acid-containing peptides have been found in several animals. Sweedler's group demonstrated the determination of D-amino acid-containing peptides in single neurons using CE for the first time, by adding cyclodextrin, the D-Trp-containing peptide NdWFa and the L-Trp-containing peptide NWFa in individual neurons from *A. californica* were separated and individually quantified, and the results showed that the D enantiomer presented in a dominant concentration while little NWFa compared to NdWFa in the peptidergic ventral neurons [32]. Their later work on D-Asp using CE–LIF combined with subcellular sample revealed that D-Asp/L-Asp ratio in the processes from same neurons was similar, but varied significantly for different types [13].

Nitric oxide (NO), a key messenger molecule involved in the regulation of various physiological and pathological mechanisms, has become a popular neurotransmitter for its roles in neurotransmissions and the regulation of other physiological processes. Study of NO biology on single-cell level would deepen our understanding on the functions of an individual cell and its response to intracellular or intercellular stimulations. Intracellular concentrations of the major nitric oxide synthase (NOS)-related metabolites such as arginine, citrulline, argininosuccinate, NO^{2-} and NO^{3-} in single-identified neurons from the pulmonate mollusc *L. stagnalis* by CE–LIF or CE conductive detection have been performed by Sweedler's group [33]. For the NO detection, 4,5-diaminofluorescein (DAF-2) is the most commonly used indicator, however, the interaction of DAF-2 with intracellular dehydroascorbic acid (DHA) and the impact of ascorbic acid (AA) on the levels of N_2O_3, the intermediate product of the oxidation of NO that reacts with DAF-2 affects the quantification of the NO. Sweeder's demonstrated the detection of NO in single buccal neurons from *L. stagnalis* by using the enzyme ascorbate oxidase (AO) to catalyze the oxidation of AA to DHA and following CE–LIF separating the fluorescent products of the reaction of DAF-2 with NO and DHA. The results showed that significant NO was detected in NOS-positive B2 neurons while no NO detected in NOS-negative B4 neurons [34].

2.3.3. Gene expression

Single-cell reverse transcriptase-polymerase chain reaction (SC-RT-PCR) has recently become an important tool for the determination of specific gene expression in heterogeneous tissues. SC-RT-PCR typically requires a second round of nested PCR. However, Zabzdyr and Lillard recently described the first qualitative analysis of single-cell multiplex products of the RT-PCR required only one round, and the expression of estrogen receptor α (ERα) and β-actin in individual MCF-7 cells was monitored using CE–LIF with fragment sizes of 318 and 838 bp [35]. Liu et al. used CE–LIF combined with SC-RT-PCR to determine *LEAFY* expression at single-cell level from different *Arabidopsis* tissues [36].

2.3.4. Others

Detection of microbial contamination in test samples is a crucial component of safety and quality control in the food, pharmaceutical, and medical industries as well as in the public sector. Therefore, there is a great need for rapid analysis for the presence or complete absence of microorganisms. Armstrong's group described the initial work on the detection of bacteria and fungi by using CE for microbial contamination. This method involved using dilute cationic surfactant buffer to sweep microorganisms out of the sample zone and a small plug of "blocking agent" to negate the cells' mobility and induce aggregation, the nutrient broth media was used as the effective blocking agent, which resulted in a limited sensitivity of 50 cells due to the natural background fluorescence from the nutrient broth [37]. In their recent work, the fluorescence of the blocker plug was reduced by as much as 40× and single cells of bacteria and fungi were detected by substituting caprylyl sulfobetaine in place of the nutrient broth (Fig. 4) [38]. This rapid technique would play potential roles in microbial contamination in "real-world" samples.

Dovichi's described for the first time the analysis of glycosphingolipid metabolism in single cells using CE–LIF, the ganglioside G_{M1} was tagged with the fluorescent dye tetramethylrhodamine and then was taken up and metabolized by a culture of pituitary tumor (AtT-20) cells for 50 h. Eleven components were detected from the lysate of a single cell by CE–LIF detection and the average peak height of these components spanned more than two orders of magnitude, and the average cell took up roughly 2 amol (10^6 copies) of the labeled substrate [39].

3. Microfluidic devices

CE supplies a powerful technique for single-cell analysis especially on the separation and detection of chemicals of interest inside single cells, but many procedures such as cell culture, cell sorting, injection, lysis and separation are performed separately, which makes the whole process of single-cell analysis time-consuming and low-throughput. Furthermore, single-cell analysis at present need a versatile technique that could offer multiplex information more than cellular chemical analysis. It is well known that cells, in their natural environment, are subject to multiple cues that vary in time and space, study on the biochemical behaviors such as movement, differentiation, secretion, endocytosis, and apoptosis, which necessitates developing more versatile platform for single-cell analysis. Microfluidic or lab on a chip (LOC) device, which allows the integration and automation of many manipulation and detection functions, is a breakthrough providing several unique advantages for single-cell analysis.

The size of a mammalian cell is close to the dimension of the microchannel, combining with nonmechanical fluidic micropumps or microvalves, the precise and automation manipulation of a singe cell and chemical reagent handling could be easily realized; the microfluidic device allows the integration of various tasks such as reagent delivery, cell culture, sorting, manipulation, lysis and separation, which enable very rapid, highly efficient single-cell analysis to be performed. Furthermore, many different detection schemes could also be integrated on a microdevice and multiple information from a single cell could be obtained simultaneously. In addition, microfluidic devices provide the capability of mimicing the natural physiological environment cells subjected to bio-functionization of the surfaces and control of extracellular matrix with microfluidic channels, which therefore facilitate them to be the multifunctional platforms for single-cell analysis.

Owing to its unique advantages, microfluidic technique has been become the most potential platform for single-cell analysis, and rapid progress has been made in this burgeoning field. Numerous excellent reviews have been also published in the past few years, which covered the advances that have been made in the critical areas [1,4–9]. In this section, we will focus on the recent crucial advances in cell culture, cell manipulation, chemical cytometric analysis and cellular physiological studies.

3.1. Cell culture

Compared to traditional static cell culture in homogeneous conditions across the substrate, microfluidic perfusion culture can effect a defined artificial microenvironment by continuously controlling the supply and removal of soluble factors [40], which is more accurately mimicing the *in vivo* situations. Moreover, microfabrication techniques offer the advantages of increased fluid control, ability to address the cellular length scale, approximating the physiological culture environment, improved culture efficiency, and batch fabrication of high-throughput arrays [41]. Furthermore, owing to microtechnologies and biocompatible materials (e.g., PDMS, glass, silicon) as well as surface treatment techniques, cells can be deposited in designed situation thus further analysis about physiological and biomedical response can be easily executed.

Cells are particularly sensitive to the microenvironments, and an accurate assay will depend upon the success of the culture technique. Microfluidic perfusion culture can create the background of soluble factors as well as constantly change the nutrients and then become the most popular mode of chip-based cell culture. Gu et al. designed a device which can precisely control fluid flow inside elastomeric capillary networks by a refreshable Braille displays and cultured each cell subpopulation under perfusion for up to 3 weeks [42]. Braille display could power integrated pumps and valves through localized deformations of PDMS channel networks aligned above. This design was capable of doing reconfiguration by the compartmentalization between a cell seeding state and a cell culture state. Seeding procedure assigned precise locations for cells to be seeded and required only one loading event to seed multiple sites with cells. While, seeded cells were broken into different compartments by valves during culture (Fig. 5A). Tourovskaia et al. presented a microfluidic perfusion system suitable for a long-term (>2 weeks) culture of muscle cells spanning the whole process of dif-

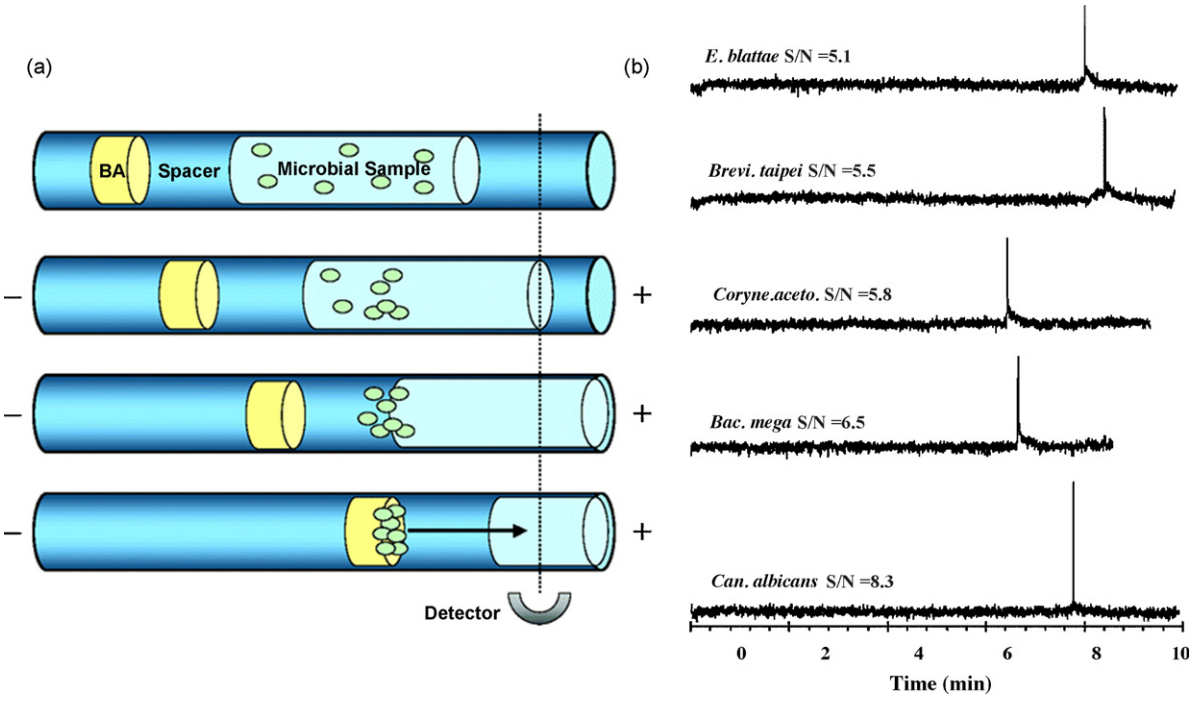

Fig. 4. (a) Schematic of CE/microfluidic-based test for microbial contamination. The entire capillary is initially filled with running buffer containing CTAB surfactant. Three injections are made prior to the run: (1) a large plug of sample containing microorganisms; (2) a spacer plug of running buffer and CTAB; and (3) a short plug of blocking agent (BA). Cells present in the sample are represented by ovals. (b) Electropherograms of single cells of various bacteria and fungi using the revised CE-based sterility test. Reproduced, with permission, from Ref. [38].

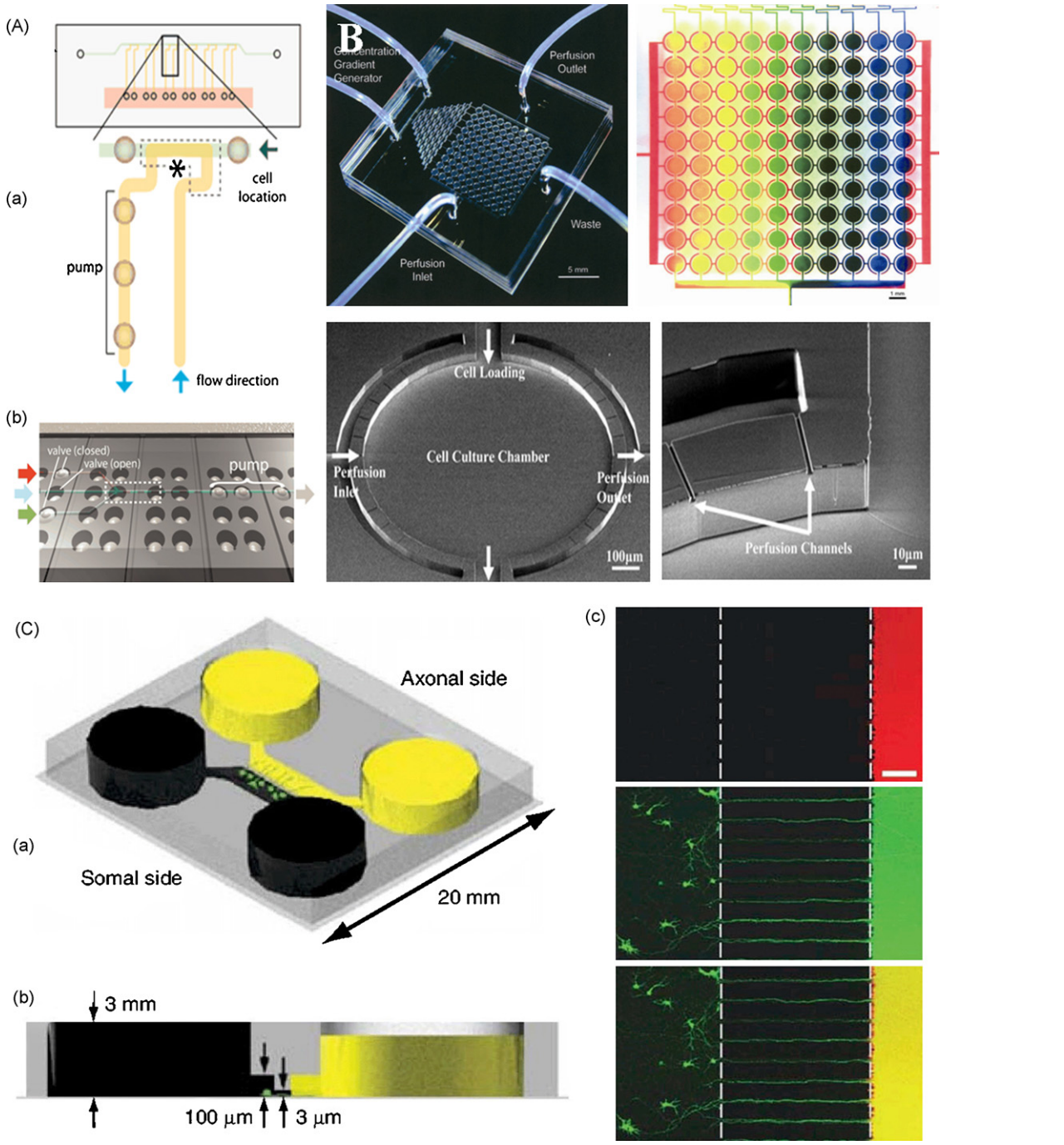

Fig. 5. (A) A typical PDMS-based perfusion cell culture device and its working scheme. (a) A scheme of a close-up of a single flow loop. Cells are only seeded in the green-colored channel. (b) Overview of the typical experimental setup. The channels are aligned above a grid of Braille pins which can conveniently and flexibly control over the fluidic flow. Reproduced, with permission, from Ref. [42]. (B) Microfluidic cell culture array (10 × 10) for high-throughput cell-based assays and a concentration gradient generator was connected to the 10 columns at the top of the device. And the multiple perfusion channels of single culture units (the two SEM pictures) provide refreshable and uniform nutrient access throughout each microchamber for long-time cell culture. Reproduced, with permission, from Ref. [41]. (C) The microfluidic-based culture platform directs axonal growth of CNS neurons and fluidically isolates axons (a) The culture chamber consists of a PDMS mold containing a relief pattern of somal and axonal compartments. (b) A volume difference between the somal side and axonal side (∼50 μl) allows chemical microenvironments to be isolated in two sides.(c) Fluidic isolation of Texas red dextran (top panel) to the axonal compartment demonstrates that axonal or somatic microenvironments can be independently manipulated using this culture platform. Reproduced, with permission, from Ref. [47]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ferentiation from myoblasts to myotubes by a gravitational flow, combining with cell-adhesive and cell-repellent micropatterns, the tracks of C2C12 cells transformed into precisely aligned myotubes were traced [43]. Lee's group have developed many innovations on microfluidic chip-based cell culture [41,44–46]. They demonstrated a microfluidic-based hydrodynamic trapping method for creating arrays of single adherent cells with dynamic control of perfusion possible. Additionally, cell division, adhe-

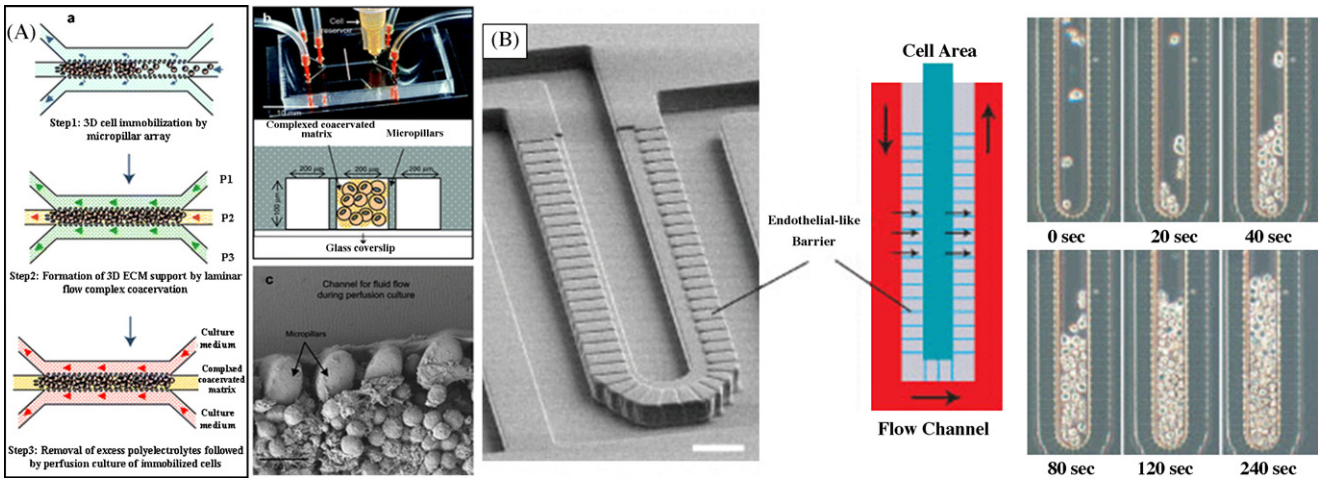

Fig. 6. (A) Implementation and characterization of the 3D microfluidic channel-based cell culture system (3D-μFCCS). (a) Cells were three-dimensionally immobilized in a microfluidic channel by dynamic seeding through a micropillar array and stabilized and supported by 3D matrices (b) Prototype of 3D-μFCCS with a cross sectional illustration (indicated by white line). (c) SEM micrograph of hepatocytes three-dimensionally immobilized in a microfluidic channel with an array of 30 μm × 50 μm elliptical micropillars. Reproduced, with permission, from Ref. [53]. (B) Microfluidic endothelial-like barrier properties. (a) SEM micrograph depicting the microfluidic sinusoid unit and mass transport properties of the microfluidic liver sinusoid. The right part shows pictures of hepatocyte loading into the artificial sinusoid. Reproduced, with permission, from Ref. [46].

sion, and apoptotic behavior were comparable to static culture on the same substrate, indicating cells were not stressed above normal culture conditions [44]. They [41,45] pioneered a high aspect ratio (∼20) microchambers array (10 × 10), single unit of the array consisted of a circular microfluidic chamber 40 μm in height surrounded by multiple narrow perfusion channels 2 μm in height, for high-throughput cell culture with continuous perfusion of medium for a long time, which could also assay 100 different cell-based experiments in parallel. Meanwhile, a series of manipulations such as reagent handling and real-time optical analysis could be achieved in this microdevice (Fig. 5B). They also created a biologically inspired liver sinusoid with a high fluidic resistance-based microfluidic endothelial-like barrier that has mass transport properties similar to the liver acinus such as extensive cell–cell contact, defined tissue and fluid transport regions and continuous nutrient exchange. It could efficiently load hepatocytes into the culture area at a high volumetric density and maintain cell survival for over 2 weeks (Fig. 6B) [46].

Micropatterning cell culture plays an important role in orientation and guidance to the growth of nerve cells. For example, Taylor et al. described a microfluidic culture platform that polarized the growth of central nervous system (CNS) axons into a fluidically isolated environment without the use of targeting neurotrophins. The culture chamber consisted of a PDMS mold containing a relief pattern of somal and axonal compartments connected by microgrooves (10 μm wide, 3 μm high). Rat CNS neurons were added to the somal-side reservoir and were drawn into the somal channel by capillary action. Within 3–4 days, axonal growth was guided into the axonal side through the microgrooves (Fig. 5C) [47]. Vogt et al. grew rat embryonic cortical neurons on a grid pattern of proteins created by microcontact printing. And experiments showed that cells attached and differentiated normally on the pattern and form functional, mature synapses following the predefined geometry [48].

In animals, cells typically reside in environments with very specific three-dimensional (3D) features. Cells are sensitive to the presence of neighboring cells of similar or different types and often make long-lasting mechanical and biochemical connections to them [7]. It is reported that cells mirror their *in vivo* counterparts more closely when cultured in 3D microenvironments through properly regulated cell–cell and cell–matrix interactions [5]. Some researches about cell–cell (cell coculture) or cell–matrix (cell culture in 3D cellular scaffold) have been reported.

Cell–cell interactions are critical to the function of many organ systems. Further understanding of cell–cell interactions play an important role in the investigation of their physiological traits and functions so as to gain fundamental biological insight as well as suggest approaches that will allow the manipulation of tissue function *in vitro* for clinical applications. Microfluidic system provides the capability of coculturing different type of cells by microstructure and microfluidic design, precise reagent control and surface treatments. Wei et al. presented a novel microfluidic coculture system to improve the accuracy of evaluating the interaction between coculture cell types and then determined macrophage–osteoblast interaction induced by mimetic implant degradation produces PMMA debris [49]. Li et al. employed self-assembled monolayer (SAM) modification to activate the "inert surface", resulting in a pattern of multiple types of cells on the surface. By electrochemical desorption of SAMs, patterned cells could spread out and interact with other cells [50].

Extracellular matrix (ECM) is a central aspect of the cellular environment, which has been shown to affect growth, adhesion, differentiation, morphology and cell signaling in multiple cell lines [51]. In addition, due to the structural properties of the ECM, it encourages physiological cell growth into a 3D configuration. The composition and proportion of matrix components can be adjusted to influence cell behavior

and better mimic the cellular microenvironment *in vitro* [52]. Toh et al. developed a microfluidic system with an array of micropillars that allows cells to be perfusion cultures in 3D by supporting them with adequate 3D cell–cell and cell–matrix interactions (Fig. 6A) [53]. Rosenthal et al. established the Bio Flip Chip (BFC), a microfabricated polymer chip containing thousands of microwells, each sized to trap down to a single-stem cell. Then they used it to pattern small groups of cells, with and without cell–cell contact, allowing incremental and independent control of contact-mediated signaling [54]. Some researchers used proteins (e.g. gelatin, collagen, laminin, fibronectin, or poly-L-lysine [55]) and biopolymers (such as collagen, collagen–chitosan, matrigel and fibrin, etc.) to construct 3D matrix for cell culture. Gottwald et al. described a multipurpose platform for the three-dimensional cultivation of tissues, which was constructed either as a grid of microcontainers or as an array of round recesses. By the suitable modification, the cellular microenvironment could be tailored to cell type-specific applications. And experiments showed that the chip systems were able to support and maintain mesodermally differentiated cells, both from embryonic carcinoma and embryonic stem cells [56]. Tan and Desai [57] built a 3D microscale hierarchical tissue-like structure inside a microchannel through surface modification and layer-by-layer deposition. In standard human embryonic stem cells (hESCs) culture, completely separated systems are needed for the cell renewal and the differentiation respectively. Gerecht et al. [58] created a single, controllable 3D culture system containing hydrogel scaffolds to mimic the developmental milieu in which hESCs can be maintained as undifferentiated cells and differentiate in response to specific cues within the same culture setting.

There are also some reports about cell coculture in 3D matrix. Co et al. demonstrated a new method based on polyelectrolyte assembly for controlling the multiple cell types with subcellular resolution on biomaterials and they observed coculture of 3T3 fibroblasts with line patterns of endothelial cells on chitosan [59]. Tan and Desai [60] constructed a layer-by-layer microfluidic platform for 3D coculture which exerted spatial control over how multiple cell types were placed in relation to one another in 3D and with control over the ECM environment. Influences on the nature and degree of homotypic and heterotypic cell–cell interactions by configuration were studied.

3.2. Cell manipulation

The first critical step in single-cell analysis is the development of methods that can reproducibly transport cells to precise locations for further analysis [6]. Usually it contains the separation of single cells and trapping them in wanted positions. Several methods for single-cell manipulation have been developed and the mechanisms include electroosmosis, electrophoresis, dielectrophoresis (DEP), optical interference, acoustic standing waves, splitting laminar flows, mechanical obstacles and restrictions, or magnetic forces, etc. [61].

The most popular method for cell manipulation is hydrodynamic flow for its easy operation. By special microstructure fabrication and precise control over fluidic flow, people usually design some barrier-structures to constrain the pathways of the flow to sieve objective cells or transport them to determined locations. Since some different physical or chemical specialty of desired cells, selection and trapping of cells within the microchannels can be achieved through fabricating constriction structures such as microchambers [44], microgripper [62], microfilters [63], microwells [64], etc.

Lee's group have made many efforts on developing microfluidic techniques for cell manipulation. They [44] designed a device which consisted of arrays of physical U-shaped hydrodynamic trapping structures with geometries without surface modification that were biased to trap only single cells and cell loading could be done in less than 30 s (Fig. 7A). Microgrippers [62] created by them could manipulate single cells in physiological ionic solutions and held a cell without employing any forces in the closed position (Fig. 7C). They also developed a microfluidic patch-clamp array with cell trapping sites which fixed single cells by negative pressure (Fig. 7D), which could realize parallel experiments effectively on patch-clamp [65]. Irimia and Toner [63] fabricated a microstructured membrane design for the purpose of cell handling on the chip which was multifunctional by one actuation step. Rettig and Folch [64] made highly effective single cells trapping in large arrays of microwells and examined occupancy of microwells by adherent cells and nonadherent cells (Fig. 7B). VanDelinder and Groisman [66] conducted and tested a microfluidic device that employs an array of microchannels in cross-flow to separate white blood cells from whole human blood and be enriched according to their obvious difference in sizes. Murthy et al. [67] created a size-based microfluidic device and separated myocytes and non-myocytes from the neonatal rat myocardium (Fig. 7E).

A key need for dynamic single-cell measurements is the ability to gently position cells for repeated measurements without perturbing their behaviors. Lutz et al. described a new method that used a gentle secondary flow to trap and suspend single cells, including motile cells, at predictable locations in 3D. Trapped cells could be more dense or less dense than the surrounding medium [68]. A novel biocompatible method for cell entrapment and release on a microchip is presented by Braschler et al. [69]. The growth of a gel bar was used to enclose and immobilize yeast cells and the trapped cells were released during shrinkage of the gel. Their experiments showed that the technique they presented not only allowed the reversible immobilization of cells under gentle conditions but also offered the potential of long-term cell culture.

Recently, there is an increasing interest in optical manipulation of biological species on microfluidic device due to its non-contact and contamination-free manipulation process. Optical manipulation using optical tweezers offer high resolution in single-cell trapping. Chiu's group used optical trapping to manipulate and translate the desired particle to the interface of the two immiscible fluids so that the particle would be enclosed within the droplet during the droplet formation process and then transported it to designed position [70]. Hellmich et al. employed optical tweezers to trap, inject, steer and deposit single biological cells in a PDMS chip [71]. Optical tweezers offer high resolution for trapping single particles, but have a limited manipulation area

owing to tight focusing requirements. Chiou et al. developed an optoelectronic tweezer (OET) which utilized direct optical images to create high-resolution DEP electrodes for the parallel manipulation of single particles (Fig. 8A) [72]. It has been suggested that confining the optical field to only the critical junctions of a microfluidic network is a more efficient use of optical power. Wang et al. fabricated a fast, active, all-optical control switch for live cells in a high-throughput, fluorescence-activated microfluidic cell sorter, the cells were firstly aligned to the center of the channel by flow focusing, and analyzed at the analysis region followed by optical switching based on their detected fluorescence [73]. Additionally, to meet the requirements of small size and high throughput as well as low costs for microchips, vertical cavity surface emitting lasers (VCSELs) and array of VCSELs took place in optical tweezers designs. Shao et al. developed a microscope-integrated VCSELs array trapping system capable of independent control, rotation, and batch processing of biological cells, which enabled parallel processing of multiple objects [74].

Besides optical manipulation, the other contact-free methods including magnetic, DEP and acoustic techniques have also been developed for cell handling. Local magnetic field can be used to trap and move cells targeted by magnetic beads. Furdui and Harrison [75] implemented a one-step immunomagnetic separation technique for the isolation and the purification of T cells from blood samples (Fig. 8B). Furlani [76] employed an array of integrated soft-magnetic elements embedded adjacent to a microfluidic channel to directly and continuously separate red and white blood cells in plasma. Ramadan et al. demonstrated magnetic beads trapping, concentration, transportation and sensing in a continuous sample flow by employing high magnetic field gradients generated by novel multifunctional magnetic microdevices [77].

As a polarizable particle, when subjected to an inhomogeneous electric field, a cell is either attracted toward the higher field (pDEP) or pushed away from it (nDEP) [61]. Taff and Voldman presented a passive, scalable architecture for trapping, imaging and sorting individual microparticles including

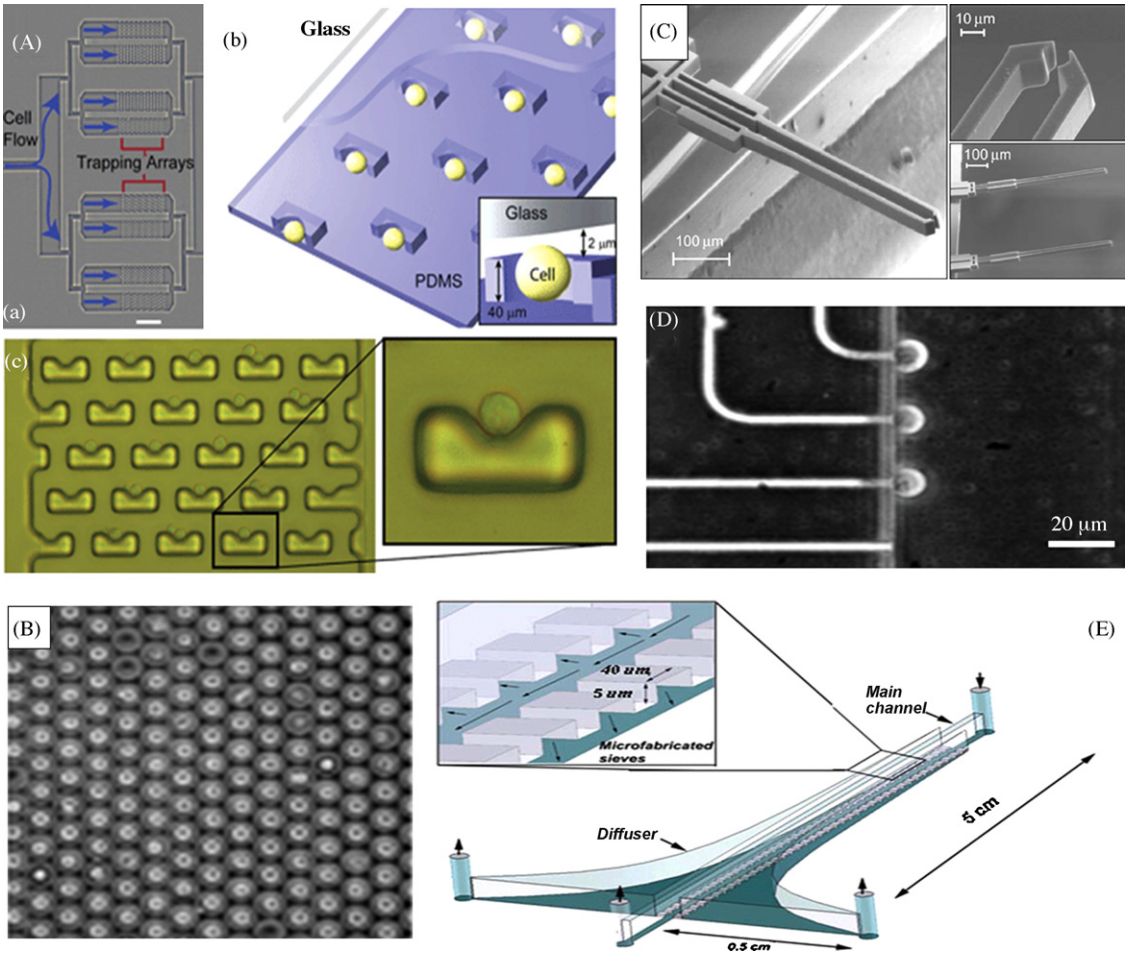

Fig. 7. (A) Single-cell trapping arrays. (a) A photograph of the cell trapping device is shown demonstrating the branching architecture and trapping chambers with arrays of traps. The scale bar is 500 μm. (b) A diagram of the device and mechanism of trapping is presented. (c) A high resolution brightfield micrograph of the trapping array with trapped cells is shown. Reproduced, with permission, from Ref. [44]. (B) Seeding of RBL-1 cells in PDMS microwells (diameter: 20 μm; depth: 27 μm). Reproduced, with permission, from Ref. [64]. (C) SEM pictures and close-ups of the overhanging fabricated SU-8 microgripper. Reproduced, with permission, from Ref. [62]. (D) Darkfield optical microscope image of cells trapped by applying negative pressure at three capillary orifices. Reproduced, with permission, from Ref. [65]. (E) Schematics of a size-based cell separation device which can be used as a diffusive filter for cell isolation and enrichment. Reproduced, with permission, from Ref. [67].

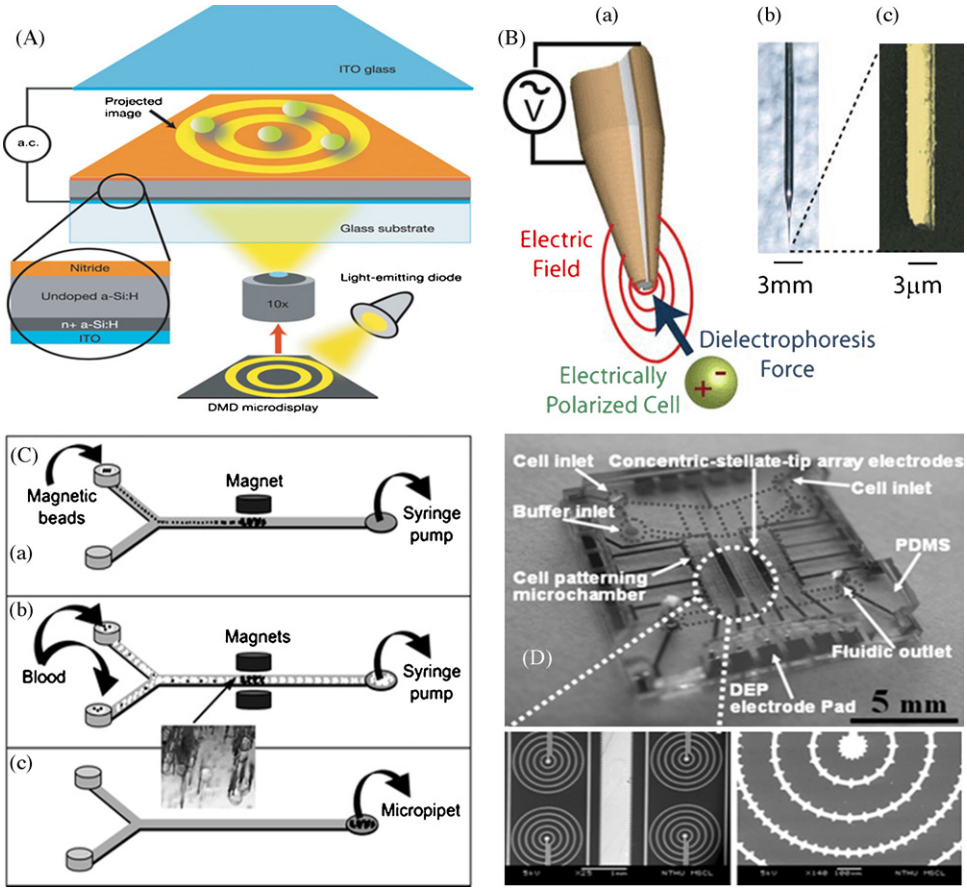

Fig. 8. (A) Device structure used in optoelectronic tweezers. The liquid containing the cells or particles of interest is sandwiched between an upper ITO-coated conductive surface and a lower photoconductive surface. Reproduced, with permission, from Ref. [72]. (B) Immunomagnetic separation of T cells using Y-intersection device; (a) syringe pump draws magnetic beads into the channel to capture with a magnet, (b) T cells are captured from blood sample, (c) magnets were removed and captured cells and magnetic beads were transferred from the outlet with a micropipet. Reproduced, with permission, from Ref. [75]. (C) DEP tweezers for cell manipulation. (a) Schematic of DEP tweezers in operation, (b) photograph of DEP tweezers, (c) SEM image of the tweezer tip. Reproduced, with permission, from Ref. [79]. (D) The chip for rapid heterogeneous liver-cell on-chip patterning via the enhanced field-induced DEP trap and the SEM image of the detail electrode geometry as well as close-view of the concentric ring electrodes with stellate-tips. Reproduced, with permission, from Ref. [80].

cells, using a pDEP trapping array for the first time [78]. Hunt and Westervelt reported dielectrophoretic tweezers which could position a single cell in three dimensions, holding the cell against with fluid flow of hundreds of microns per second with more than 10 pN of force (Fig. 8C) [79]. Ho et al. designed a chip with concentric-stellate-tip electric array to generate radical-pattern electric fields for the DEP manipulation of the live liver cells. The specially arranged DEP electrodes enhance the desired spatial electric field gradients to guide and snare individual cells to form the desired biomimetic pattern (Fig. 8D) [80].

The ultrasonic techniques usually use an ultrasonic standing wave to create a pressure node that will attract/repel particles or cells, which can be used to trap and concentrate objects or separate different types of objects from each other. Laurell's group developed an acoustic tapping platform for perfusion-based cell handling and assaying [81]. It allowed long time of stable operation for direct microfabrication of microfluidic channels in the glass reflector, tests of three live cells showed satisfactory results. They also demonstrated a novel method of free flow acoustophoresis (FFA) for continuous separation of mixed particle suspensions into multiple outlet fractions, which was suitable for both biological and nonbiological suspended particles and could be used to perform complex separation tasks. Experiments further proved that acoustic manipulation causes no perceptible harm to red cells and other biological particles [82].

There are some works employing chemical trapping, which is usually performed by harnessing surface-modified microchannels with protein and antibodies. Murthy et al. effectively separated highly pure subpopulations of T and B lymphocytes from mixtures using microfluidic chambers coated with antibodies, even when the target cell concentration was low [83]. Chang et al. employed the natural, physiological mechanism of cell collection and separation to establish a biomimetic platform, which contained an array of square pillars and another with slender, offset pillars, and both were coated with E-selectin IgG chimera, accomplishing enrichment and partial fraction of HL-60 and U-937 cells from a continuously flowing sample [84].

Some new technique developed recently took different measures in separation and trapping of cells. Allbritton's group created a coated micropallet arrays for the separation of single, adherent cells. Single SU-8 pallets were released cells on

its top with a single focused pulse from a laser [85]. Xia et al. described a microfluidic device that could pull molecules and living cells bound to magnetic particles from one laminar flow path to another by applying local magnetic field gradient, and thus selectively removed them from flowing biological fluids without any wash steps [86]. Chiu's group [87] utilized a thermoelectric cooler (TEC) to control the temperature of select portions of a microfluidic chip as an "ice valve" to manage the fluid flow in a microchannel. Due to the vastly different freezing points for aqueous solutions and immiscible oils, in use of TEC, they enable to freeze streams of picoliter-volume droplets as they flow through the microchannel system as well as to freeze single biological cells contained within the droplets formed on-chip.

3.3. Chemical cytometry

Comparing with CE, chemical cytometry by using microfluidics allow the integration of several distinct functions including cell transport, lysis and separation of cellular contents, which make the whole process be performed rapidly on a massively parallel scale. Microfluidic chemical cytometry has been become an attractive hot point and achieved rapid development.

Gao et al. developed microfluidic system that functionally integrated cell sampling, single-cell loading, docking, lysing and CE separation with LIF detection by using a simplest glass micro-CE chip. The cell flow and single-cell loading into the separation channel were controlled by hydrostatic pressure and electrophoretic force respectively, and the single-cell docking was performed by repeatedly connecting and disconnecting a set of low potentials ("switching the HV on and off "). The docked cell was then directly lysed under an applied CE separation voltage of 1.4 kV (280 V/cm) with 40 ms without additional lysates, and the NDA-derivatized glutathione (GSH) in single human erythrocytes was determined with a average amount of 63 ± 29 amol [88]. Laser source is always used in LIF detection to obtain high detection sensitivity, but the fixed wavelength limits the choice of the labeling reagent, our group constructed a fluorescence detection system for microfluidic CE with an Hg-lamp as the excitation source and a photon counter as the detector. Various fluorescent probes with different excitation or emission wavelength could be adopted owing to the wide spectrum of Hg-lamp, the amino acid neurotransmitters in rat pheochromocytoma (PC 12) cells were separated and determined, and the intracellular Glu in individual PC 12 cells was quantified as 3.5 ± 3.1 fmol [89].

Carbon fiber nanoelectrodes have been used as the amperometric detector for microfluidic CE, the experimental results indicated that both the sensitivity and resolution of microchip CE with the carbon fiber nanoelectrode (CFNE) amperometric detection had been improved markedly comparing with the traditional microelectrodes. The detection limit of dopamine was one or two orders of magnitude lower than that reported so far. The nanoelectrode-based microchip CE system has been successfully applied to the determination of dopamine (DA) in cultured rat pheochromocytoma (PC12) cells, and the average content of DA in an individual PC12 cell is 0.54 ± 0.07 fmol [90].

Dilution of the intracellular contents of interest during the derivatization and the lysis processes inside the microchannel is a critical issue that restricts the determination of the intracellular compounds in low concentration. Intracellular derivatization can minimize the dilution during derivatization, but only derivatizing reagents that penetrate the cell membrane can be used with this approach. For those derivatizing reagents that cannot penetrate the cell membrane, Sun et al. developed a method by encapsulating the fluorescent dyes FITC into liposomes with an average diameter of 100 nm and intracellularly delivering the liposomes to label the intracellular species [91].

To obtain better reproducibility, precise by trapping single cells at a fixed position within the microchannel is essential, which can be achieved by structured microfluidic device. Ros's group fabricated a microfluidic device embodied the cell trap composed of microstructured obstacles at the channel crossing. Single biological cells were trapped, injected, steered, and deposited by means of optical tweezers in a PDMS microfluidic device and consecutively lysed at the microstructured obstacles defined position. The green fluorescent protein-construct (T31N-GFP) in single Sf9 insect cells (*Spodoptera frugiperda*) was separated and determined by native LIF [71,92]. Later they found that the incorporating carbon black particles into PDMS resulting in black microfluidic chip bodies could decrease the background fluorescence of the PDMS that was major source limiting the fluorescent detection sensitivity, the detection limit of tryptophan with 25 nM could be achieved, and the single-cell electrophoregram with native UV-LIF was demonstrated for the first time [93]. Sun and Yin fabricated a novel multi-depth microfluidic chip with a weir structure for cell docking and lysis. The sampling channel on the microchip was 37 μm deep, the separation channel was 12 μm deep, while a 1-mm long weir at which the channel was only 6 μm deep was constructed in the separation channel, the single cell was electrophoretically loaded into the separation channel where it was stopped by the weir and precisely positioned within the separation channel. The trapped cell was lysed and the intracellular-reduced glutathione (GSH) and reactive oxygen species (ROS) in single human carcinoma cells were separated and detected with an improved reproducibility [94].

Chemical cytometry by using valved microfluidic devices integrate different functions, and the each function can be isolated and performed independently by microvalve and micropumps, another advantage is that the chemical reaction such as derivatization reaction can accomplished without dilution caused by diffusion. Zare's group developed a microfluidic device which integrated the major steps in microfluidic chemical cytometry, including isolating an individual cell from bulk suspension, accurately metering and delivering chemical reagents, performing cell lysis and chemical derivatization reaction and separating derivatized cell contents by CE with LIF detection. A three-state (fully open, half open and fully closed) valve was described for the first time in their microfluidic device, by which a reaction chamber with a volume of 70 pl for the lysis and derivatization of the contents of a single cell was constructed, and the unwanted dilution of the intracellular chemical contents was reduced. The separation and detection of amino acids in a single

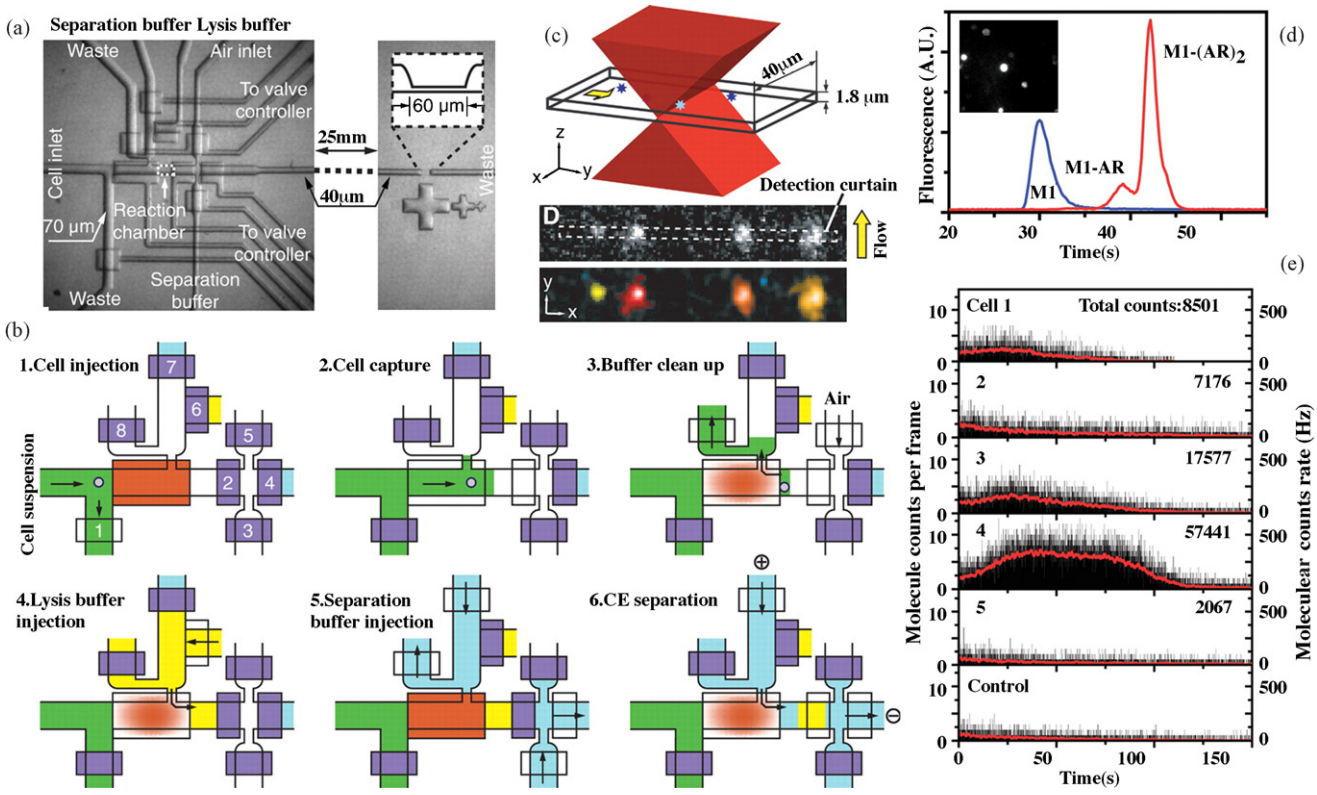

Fig. 9. The single-cell analysis chip and the analysis of β_2AR in SF9 cells. (a) Layout of the single-cell chip, showing the cell-manipulation section on the left and the molecule-counting section on the right. (b) Analysis procedure for a mammalian or insect cell. (c) Schematic illustration of the excitation laser focused by the microscope objective and the dimensions of the molecule-counting channel. (d) One frame from the CCD images of fluorescent molecules flowing across the molecule-counting section (upper panel) and the identification results (lower panel). (e) Electropherogram of Cy5-labeled M1 antibody against FLAG (M1); measurements are shown before and after adding an excess amount of purified β_2AR (AR) in a double-T chip. (f) Molecule-counting results of SF9 cells expressing β_2AR, showing the electropherogram of the M1-AR complexes. The red line represents the average count rate. Reproduced, with permission, from Ref. [96]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Jurkat T cell were demonstrated by using this novel integrated microfluidic device [95]. In their recent reports, the similar microfluidic device was used to count low-copy number proteins in a single cell, the cell was captured in the reaction chamber formed between the three-state valve and a conventional two-state valve, a lysing/labeling buffer was injected into the chamber to release the cell contents, and the generic labeling of proteins was achieved through fluorescent-antibody binding. They used a cylindrical optics to form a rectangular, curtain-shaped detection region across the channel, which enabled high efficiency counting of molecules in micrometer-sized channels. By using this microfluidic device combined with single-molecule fluorescence counting, they quantified β_2 adrenergic receptors expressed in SF9 cells (Fig. 9) and analyzed phycobiliprotein contents in individual cyanobacterial cells (*Synechococcus* sp. PCC 7942) and observed marked differences in the levels of specific complexes in cell populations that were grown under nitrogen-depleted conditions [96].

Single-cell gene expression analysis holds great promise for studying diverse biological systems, but it is challenging to process these precious samples in a reproducible, quantitative and parallel fashion by using conventional methods, while microfluidic device provides powerful and potential tool for the research. Quake's group developed microfluidic device with integrated micromechanical valves for single-cell RNA and DNA analysis. All steps including cell capture, cell lysis, mRNA purification, cDNA synthesis, cDNA purification were implemented in the device, single NIH/3T3 cell mRNA isolation and cDNA synthesis were demonstrated with quantitative calibration for each step in the process, and gene expression in individual cells was measured (Fig. 10) [97]. In their recent work, a higher throughput microfluidic digital PCR to amplify and analyze multiple, different genes obtained from single bacterial cells was demonstrated, many parallel reaction chambers (12 samples × 1176 chambers/sample) were formed by micromechanical valves to act as independent PCR reactors, and the multiplex microfluidic digital PCR of single cells was performed [98].

Information on how cells respond to changes in their external and internal environment is very important to understand cell functions, Eriksson et al. put a single cell under environmental gradient created by microfluidics, and observed the rapid cytological responses by moving a trapped cell repeatedly between the different environments using optical tweezers [99].

3.4. Cellular physiology

Studies of cellular physiology are typically performed with cells bathed in a physiological medium while adhered to culture plates or immobilized in a macroscale perfusion system, and chemical, electrical, optical, or mechanical measurements

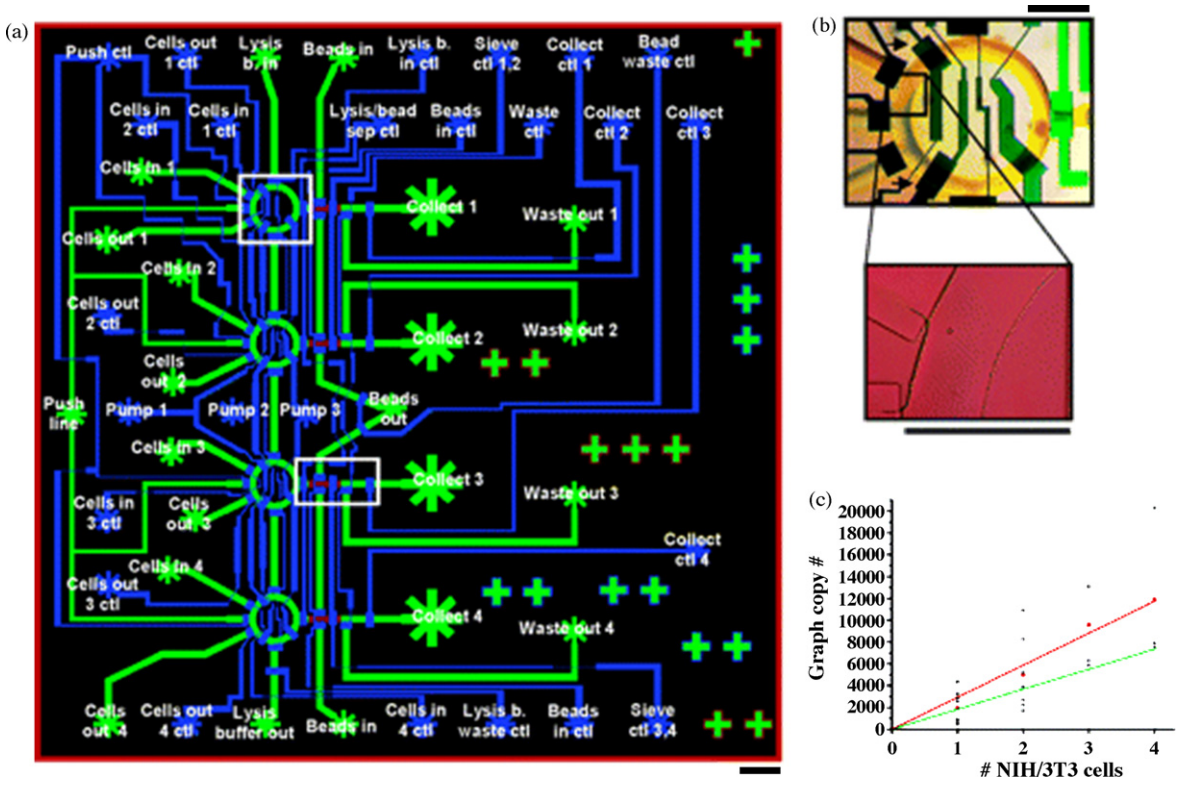

Fig. 10. 4plex mRNA isolation/first strand synthesis device. (a) AutoCAD drawing of a device with inputs and outputs labeled according to function. Rounded flow channels are depicted in green and control channels are shown in blue. Unrounded (rectangular profile) flow channels for affinity column construction are shown in red. Portions of drawing in white boxes are shown in (b). (b) Optical micrographs of the lysis ring and an NIH/3T3 cell captured in the ring. (c) The level of GAPDH gene expression was measured in different numbers (1–4) of NIH/3T3 cells (plotted as red circles fitted with red line) on the microchip shown in (a). Averaged copy number per NIH/3T3 cell obtained from a bulk experiment is represented by the green line. Reproduced, with permission, from Ref. [97]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

are made on the cells. Microfluidic device provides the capability of mimicing the sophisticated natural cell physiological experimental platforms that incorporate multi-parameter, highly parallel measurements on cells. Increased emphasis is now being placed on transferring traditional cellular physiological studies to the multiple functional and highly integrated microfluidic devices.

Comparing with the bulk solutions, the volume and the microflow characteristics of the extracellular conditions around the cell within microfluidic systems more closely match the cells' environment *in vivo*, and the possibility therefore arises to precisely control the local environment and simulate near *in vivo* situation around the cell to perform studies of cellular physiology. Klauke et al. described the partition of the extracellular space around an electrically activated single cardiac myocyte constrained with a microfluidic device. They produced a hydrophobic gap-structure to divide the extracellular space into two distinct microfluidic pools, which allowed the two ends of a single cell to be independently superfused using the dual superfusion pipets. Using planar-integrated microelectrodes to electrically stimulate the cardiomyocyte and record the evoked action potential as well as simultaneous length and epifluorescence measurements. A variety of different biochemical manipulations including the microfluidic environment, the cellular membrane and the intracellular space were performed on this microfluidic system [100].

An essential physiological function is the secretion of chemicals from cells. A variety of chemicals including signal molecules such as hormones or neurotransmitters, trophic factors and metabolic products, are released from cells. Spatiotemporal monitoring of the chemical release from cells plays an important role in understanding the regulation of the secretory process. Amperometric measurements based on the microelectrodes are a powerful tool for spatiotemporal monitoring of the electroactive chemicals, but traditional monitoring experiment was performed in bulk solutions while the microelectrodes or nanoelectrodes and the stimulation pipets had to be manually positioned adjacent to the cells with micromanipulators [10,101], which made the measurements often technically elaborate. However, by using microfluidic system, all manipulations can be integrated in a microsystem and the monitoring can be performed in a simple and automated way. Our group made the initial efforts toward such systems to measure dopamine release from PC 12 cells, including the use of microfluidics to transport and trap a single cell while the stimulators was introduced from the microchannel and a carbon fiber microelectrode was positioned over the cell for amperometric measurement [102]. Amatore et al. integrated three band microelectrodes including Pt counter electrode, platinized electrode and Ag/AgCl electrode in the microfluidic device, murine macrophages were deposited and cultured into the microfluidic chamber and the ROS release from the cell was electrochemically measured by the integrated

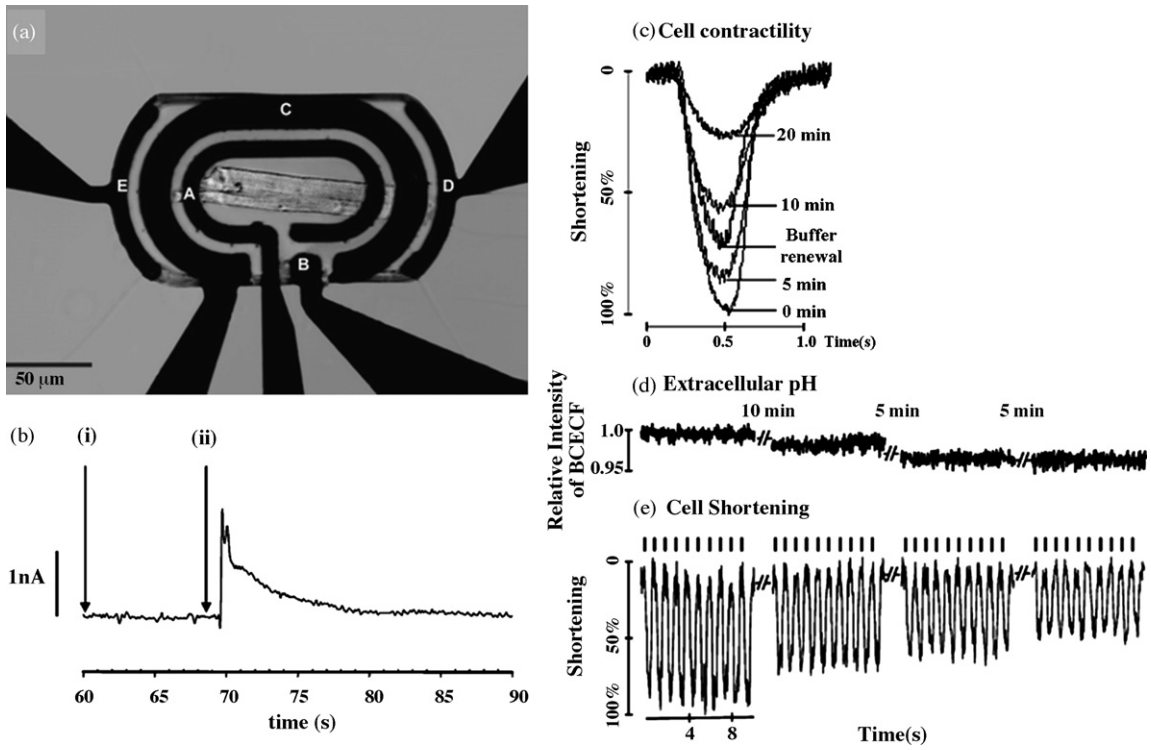

Fig. 11. (a) Micrograph of the microelectrodes and 20 μm deep microchamber. The different functional microelectrodes are indicated as A–E and a quiescent cardiomyocyte is shown in the microchamber. (b) Typical responses to lactate released from an eletropermeabilised cell, (i) in the absence of pacing, no lactate is present in the extracellular space, (ii) electrochemical response as lactate is released from the single cardiomyocyte after eletropermeabilisation. (c) Myocyte contractility measured as the change of the sarcomere length within the chamber when stimulated. (d) Recordings of extracellular pH during continuous contraction with stimulation. (e) Simultaneous recording of cell contraction with stimulation. The electrical stimulation was performed with a field strength of 75 V cm^{-1} and a frequency of 1.0 Hz (c), (d) and (e). Reproduced, with permission, from Ref. [104].

band microelectrodes [103]. Cheng et al. developed microfluidic system that integrated five functional microelectrodes to monitor real-time ionic and metabolic fluxes from electrically active, beating single heart cells. The five-electrode array comprised one pair of pacing microelectrodes for field-stimulation of the cell, and three other microelectrodes as the electrochemical lactate microbiosensor to measure the amounts of lactate produced by the heart cell. At the same time, the cell morphology, pH and Ca^{2+} were also measured, providing the details of the electrical and metabolic states of the heart cell (Fig. 11) [104].

Kennedy's group developed a microfluidic device that incorporated continuous perfusion with an on-line electrophoresis immunoassay to monitor insulin secretion from single islets of Langerhans. They improved their previous chip design [105] that enabled perfusion of the cells, the perfusion afforded more natural physiological environment for the cells and the truly dynamic measurements of insulin secretion was achieved, furthermore continually supplied fresh nutrients to the cells allowing longer-term cell survival, and the measurements could be performed up to 2 h. The cell chamber was perfused with cell culture media or a balanced salt solution at 0.6–1.5 mL min^{-1}. Perfusate was continuously sampled at 2 nL min^{-1} by electroosmosis and mixed on-line with fluorescein isothiocyanate-labeled insulin (FITC-insulin) and monoclonal anti-insulin antibody to react for 60 s. The reaction mixture was injected into a 1.5-cm separation channel as frequently as every 6 s, and the free FITC-insulin and the FITC-insulin-antibody complex were separated and the insulin secretion from islets was quantified [106]. Later they improved the assay throughput by performing the serial immunoassay in a parallel way, the improved microfluidic device contained four individual channel networks, each capable of performing electrophoresis-based immunoassays of the perfusate from islets. The device was used to complete over 1450 immunoassays of biological in less than 40 min, allowing the parallel monitoring of insulin release from four islets every 6.25 s [107].

Patch-clamp recording is a significant research tool for electrophysiology. Traditional patch-clamp recording is accomplished by using a micromanipulator to position the tip of a glass pipette against the membrane of a cell, and carefully applied negative pressure through the pipette tip causes the membrane to invaginate into the pipette and causes a gigaohm seal to form between the pipette and cell. Though big success of the traditional patch-clamp techniques has been achieved in electrophysiological research, it requires complex and expensive setups and remains highly laborious, which limits its wide application in the fields where high throughput and automated measurements are demanded. Chip-based patch clamp by using microchannel as the micropipette to suck a single cell provides the capability that the patch-clamp experiment to be performed in a high throughput automated way. Lee's group developed a 12-channel patch-clamp array based on PDMS microfluidic junctions between a main chamber and lateral recording capil-

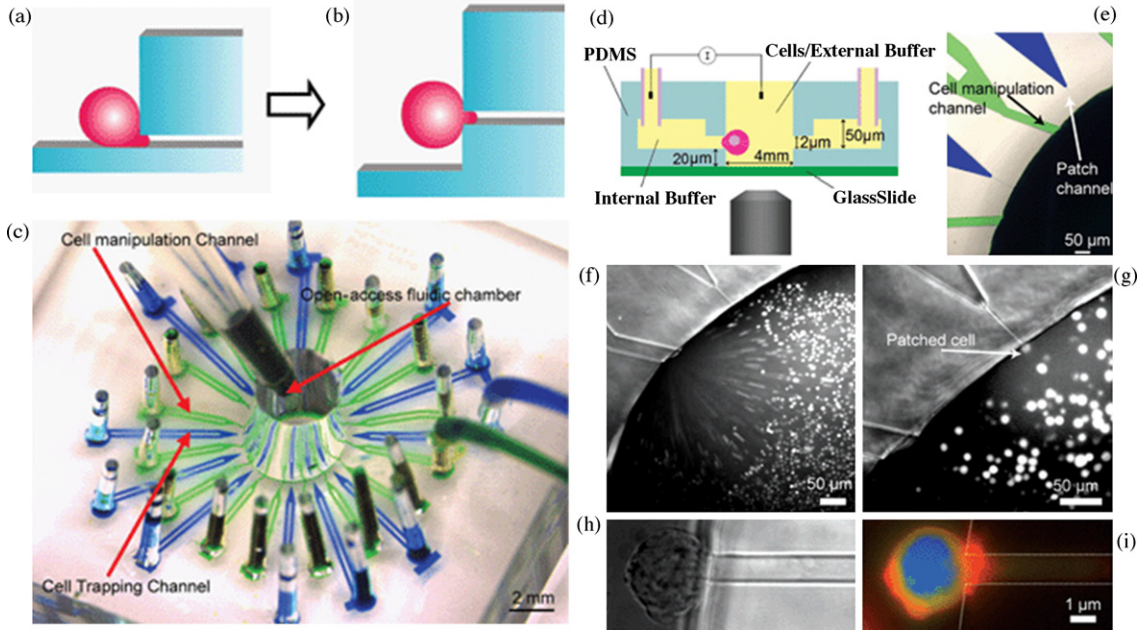

Fig. 12. (a) and (b) are the cross-sectional illustration showing how the cell is trapped in the previous lateral patch-clamp device and this device. (c) Angled view of the device. Patch channels and cell manipulation channels were filled with two different dyes. The open-access chamber is shown in the image, where cells or drug samples can easily be pipetted in. (d) Schematic of the set-up showing how the cell is being trapped onto the channel from a cross-sectional view. (e) Close-up image of the channels of the devices—small patch channel for trapping cells and larger channel for cell manipulation. (f) Long exposure image of cells being manipulated via the wide channel. The cells were dyed with calcein AM. (g) Image showing a cell being trapped and patched on the small channel. (h) Close-up of a trapped cell on the patch channel in bright field. (i) Fluorescent image of the same cell in (g). The cell is stained with calcein AM (cytoplasm, green), R18 (membrane, red), and Hoechst (nucleus, blue). Reproduced, with permission, from Ref. [108]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

laries. Microfluidic integration allowed recording capillaries to be arrayed 20 μm apart, for a total chamber volume of <0.5 nl and incorporated partial cure bonding, yielding robust seals on individually selected mammalian cells under optical observation. The device was used to record the activation of the voltage-gated potassium channel Kv2.1 in mammalian CHO cells. Seals were established without the need of vibration isolation equipment and Kv2.1 channel activation data corresponded well with data measured by the traditional pipette-based technique, using the same reagents and protocols [65]. However, the trapping sites in their initial chip were contacted at the bottom plane of the chip, which caused some deficiencies such as substandard seal quality formation with cells, uncommon deformation of the cells and inefficient fluidic exchange. Later they improved the microfluidics design by "raising" the cell trapping locations above the bottom plane of the chip to better resemble traditional glass micropipette openings and afforded better seal quality. The main fluidic chamber was open to the air, providing an easy-to-use platform for fluidic exchange. The seal resistance were characterized and correlated with the aperture dimensions. Whole cell patch-clamp measurements were carried out with CHO cells (Fig. 12). Their results showed that the microfluidic system was capable of performing whole cell measurements in a more efficient manner [108].

Direct cell–cell communication between adjacent cells is vital in multiple physiological functions including neurotransmission, immune system, transmitting action potential in cardiac myocytes and proper organ development. However, current biological techniques are difficult to scale up for high-throughput screening of cell–cell communication in an array format. Microfluidic devices provide the powerful platform tools for this study via the control of the physiological environment and the precise manipulation of the adjacent cells. Lee's group described an initial work by using microfluidic device to monitor cell–cell communication via gap junctions between individual cells. The microfluidic device contained main microfluidic channel with a height of 50 μm and a width of 20 μm while the 37 pairs of cell trapping channels with an opening of 2 μm by 2 μm located at the floor of the main channel. The cell pairs were trapped at the ports and the dye transfer between mouse fibroblasts (NIH3T3) placed in membrane contact [109]. Cooper's group described the regional microfluidic and electrical manipulation of two cardiac myocytes connected through the intercalated discs. The extracellular space was partitioned into three pools, the two aqueous reservoirs for the cell ends and the central sealing gap containing the region of the intercalated discs. A nanopipetting system was used to induce Ca^{2+} waves in a single cell and the Ca^{2+} propagation across the intercalated discs was monitored. The results demonstrated that Ca^{2+} waves travelled unimpaired along the longitudinal axis of the stimulated cell, however, under physiological conditions, stopped in front of the intercalated discs, while under non-physiological conditions, when the adjoining cell was either damaged or challenged by a drug, conditions which resulted in Ca^{2+} overload in this cell, the propagation of the Ca^{2+} wave across the cell junction was observed [110].

4. Conclusions

During the past two decades, CE has been successfully applied to single-cell studies in various research areas including neuroscience, proteome science, gene expression, and diagnosis, owing to its excellent ability to identify and quantify the single-cell contents. Though CE has gradually become a mature method for single-cell analysis, there are still many issues should be pursued since many biological processes and mechanisms in a singe cell are not well known. Subcellular sampling with higher spatial resolution would help us to obtain more precise information of the components of interest inside a single cell. More sensitive detectors should be developed and coupled with CE system for the determination of the crucial molecules in very low concentration. In addition, extension of its application in proteome science, metabolome science and disease diagnosis would also facilitate the developments of CE-single-cell analysis.

Microfluidic devices provide the capability of integrating of whole process of single-cell analysis and various detection techniques into a miniaturized microchip, and afford a versatile and automated platform and are emerging as the next revolution tools for single-cell analysis. Rather than offering the qualitative and quantitative information of cell contents, microfluidics devices make complex single-cell studies be performed, and many biological tasks that previously not available could be realized by using this multifunctional platform. As a developing method, although significant advances have been achieved over the past decade, tremendous challenges exist and numerous works should be done before it being an easy-to-use and robust tool. There is no doubt that highly integrated microdevices will find applications in many areas such as in basic biological research whereas cell functions must be investigated in a rapid and high throughput way; and in basic biomedical research whereas robust and portable point-of-care (POC) devices will be used.

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