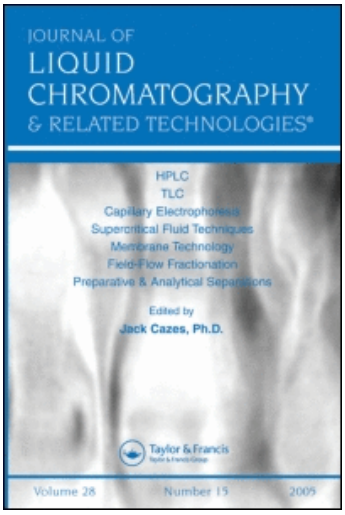

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Intracellular Labeling Methods for Chip-Based Capillary Electrophoresis

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Intracellular Labeling Methods for Chip-Based Capillary Electrophoresis

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Abstract: Recently, the development of chip-based electrophoresis demonstrated the feasibility of integrating the whole process of analysis for intracellular constituents in individual cells. Laser induced fluorescence detection is one of the dominant detection techniques used for chip-based capillary electrophoresis. However, a large number of compounds found in single cells are nonfluorescent and the cell membrane acts as a barrier to the free diffusion of derivatization reagents into cells. Considerable efforts on derivatization of intracellular contents have been made to sensitively and selectively detect intracellular species. This review aims to highlight the recent development of intracellular labeling methods. The approaches on integrating the whole process for single cell analysis on one microfluidic chip are also addressed.

Keywords: Chip-based capillary electrophoresis, Intracellular labeling, Microchip, Single-cell analysis

INTRODUCTION

Cells play a critical role in life activities such as metabolism, signal transduction and heredity. Determination of the chemical composition of individual cells and monitoring how the composition changes in response to external stimuli are essential to a better understanding of

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basic cellular functions and intra- and intercellular communication.[1] Moreover, characterization of heterogeneous cell populations is of considerable importance. Analysis results of pooled cell lysates often conceal the important information vital for diagnosis of certain disease. Determination of the intracellular components in single cells would greatly increase the probability of discriminating infected cells from healthy ones and provide a solid foundation on study and development in fields including biochemistry, medicine, and pathology clinic. The development of single-cell analysis has already become the focus of the frontiers in analytical chemistry.

However, the technical requirements for the determination of the chemical composition of individual cells are strict due to the complex composition and minute amount of analyte present in a single cell. The diameter of single cells is only on the order of tens of microns with a volume of about 1 pL.[2,3] Intracellular constituents include macromolecules, such as DNA/RNA and protein, inorganic ions and small organic molecules. Their amounts are typically present at femtomole to zeptomole levels ($10^{-15} - 10^{-21}$ mol). It is estimated that each cell may contain more than 100,000 different proteins having a wide disparity in abundance with <100 molecules of many receptors, 1,000-10,000 molecules of various signaling enzymes, and 10^8 molecules of some structural proteins.[3] This state of affairs requires employment of analytical methods with high sensitivity, high selectivity, high resolution and minimal dilution during sample preparation and analysis.

Capillary electrophoresis (CE) has been demonstrated to be valuable in the determination of intracellular contents of single cells[4] for the following reasons: (1) its ability to perform rapid, high-efficiency separations in biocompatible buffers; (2) its ability to sample extremely small volumes; (3) its ability to be coupled with ultrasensitive detection methods, such as laser induced fluorescence (LIF) and electrochemical detection; (4) its low cost. However, although these characteristics of CE are impressive, it is necessary to use invert microscope and precise micromanipulator for manual injection of individual, intact cells into the capillary.[5] The operation for manipulation, injection and lysis of single cells is labor intensive and throughput low. Typically only 8–10 cells can be analyzed in one day. To improve the throughput, Dovichi's group reported the construction of a capillary holder to better facilitate cell injection and buffer washes.[6] Lillard has also reported a continuous injection method for red blood cells.[7] While these developments have the potential to enhance the throughput, they do not completely address the issue of automating the entire cell analysis process owing to the one-dimensional structure of CE.[1]

Compared to conventional CE, the ability of integrated microfluidics to accurately manipulate, handle and analyze very small volumes has

opened up new opportunities for analysis of intracellular constituents in individual cells.[8] Recently, the development of chip based electrophoresis demonstrated the feasibility of integrating the whole process for single cell analysis on one microfluidic chip including single cell injection, position, on chip derivatization, lysis, separation and detection of cellular constituents by CE.[1,9–12] The automation and integration of all these cell handling and processing steps along with rapid separation will enable high-throughput analysis of individual cells. While a large number of strategies have been reviewed to perform a variety of investigations concerning on-chip cell cultivation, cell sampling, cell manipulation, trapping and sorting, cell treatment, and cell analysis on chip,[3,8,13–19] few have been addressed the issue of integration of the entire cell analysis process on microchip.

Due to its high sensitivity and easy incorporation into microfluidic devices, LIF detection is one of the dominant detection techniques used for chip-based CE. The recent development in LIF detection for microchip separations has been reviewed by Yang[19] and Karst.[20] However, a large number of compounds found in single cells are non-fluorescent in their natural state. Fluorescent labeling of biomolecules has been demonstrated as an indispensable tool in many biological studies. It not only improves the sensitivity, but also increases the selectivity, if the derivatization reagent reacts specifically with the target analyte. Although a number of derivatization reagents have been developed for labeling the compounds found in cells, such as proteins, amino acids and peptides,[21,22] it should be noted that cell membrane acts as a barrier to the free diffusion of the derivatization reagents into cells. In order to label intracellular analytes without evident dilution, considerable efforts have been made to sensitively and selectively detect certain intracellular species with CE and chip-based CE.

Derivatizations can be performed pre-, on- and post-column with respect to the electrophoretic separation. This review summarizes the methods used for labeling intracellular contents of single cells. The developments and approaches for single cell analysis with chip-based CE are also addressed, with examples of integrating the whole process for single cell analysis on one microfluidic chip, including single cell injection, position, on chip derivatization, cell lysis, analytical separation and detection.

INTRACELLULAR LABELING METHOD

Pre-Column Derivatization

Pre-column derivatization in single cell analysis was first reported by Jorgenson and co-workers in 1989.[23] Single cells have been physically

isolated and placed in 200-nL microvials for derivatization.[23,24] One giant neuron with ca. 125 μm diameter and 1.0 nL volume from land snail was derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) to determine amino acids in individual cells. The final volume present after derivatization is ca. 25 nL. A large number of peaks have been observed using CE with LIF detection.[23] In this case, the contents of a single cell in 1.0 nL volume are diluted by a factor of ca. 100.[25] If this method is used to derivatize the contents in normally sized mammalian cells with a diameter of ca. 20 μm and a volume of ca. 4.2 pL, the dilution factor would be more than 10^4. Moreover, the microchemical operation concerning micropipet manipulation of nanoliter volume solutions is labor intensive.

It is known that microinjection[26–29] can be used to introduce chemical materials into cells. The main drawback of microinjection is that the needle-tips are very fragile and difficult to positioning. Moreover, microinjection techniques are considered to be relatively invasive due to the large mechanical force applied, which can induce permanent membrane damage and even cellular membrane rupture.[30]

In order to avoid diluting the intracellular analytes during pre-column derivatization, approaches have been made to deliver derivatizing reagent across cell membrane into cells as detailed below, classified into microderivatization of living cells, electroporation and intracellular derivatization mediated by liposome and Polyethylene glycol (PEG).

Microderivatization of Living Cells

Hogan and Yeung[5] described an intracellular derivatization approach for microderivatization of living cells. They derivatized thiols in single erythrocytes by incubating living cells with a cell-permeable derivatizing reagent, monobromobimane. In the derivatization process, the cell itself acts as a subpicoliter reaction chamber. Small nonpolar derivatization reagents surrounding the cell can penetrate through the lipophilic cell membrane and react with the analyte to be labeled. The derivatized product is much larger than the original reagent and will not migrate back out through the cell membrane. Attomole levels of glutathione have been determined in erythrocytes with CE and LIF detection. During the derivatization, almost no dilution of the cellular contents occurred. 100% derivatization of the analyte is also possible for slow labeling reaction, as long as the incubation time is long enough. The exterior phase with its excess reagents and contaminants can be completely removed via washing and centrifugation. Some reagents used in fluorescence microscopy and flow cytometry[21,31] are also cell-permeable and may be useful. Owing to the advantages described above, this pre-column derivatization scheme has been widely used in single cell analysis. Some examples of cell-permeable fluorescent reagents used for single cell analysis are listed in Table 1.

Table 1. Cell-permeable reagents used for microderivatization of living cells

Reagents	Excitation wavelength	Target analytes	References
Monobromobimane	Ar ion (350–360 nm)	Thiols	5
NDA	Ar ion (488 nm)	Reduced glutathione (GSH)	10,11,32,33,34
	Solid laser (473 nm)		
Hydroethidine (HE)	488 nm	Superoxide anion	35
	488 nm		36
Dihydrorhodamine 123 (DHR-123)	Solid laser (473 nm)	Reactive oxygen species (ROS)	34
	Ar ion (488 nm)		11,32,37
HPF	488 nm	Hydroxyl radical	38
APF			
Fluo-3	Ar ion (488 nm)	Ca^{2+}	39,40,41
	490 nm		42
Fluo-4	Ar ion (488 nm)		40,43,44,45,46
	a xenon arc lamp (480 nm)		47
BCECF-AM	mercury lamp	Cell viability	48
6-Carboxyfluorescein diacetate,	Ar ion (488 nm)	6-Carboxyfluorescein	49
5-carboxyfluorescein diacetate,		5-carboxyfluorescein	
Oregon Green diacetate		Oregon Green	
Zinpyr-1	507 nm	Zn^{2+}	50
ZnAF-R1	329 nm		51
ZnAF-R2	335 nm		51
PF1	488 nm	H_2O_2	52
PR1	543 nm		
PX1	704 nm		
anti-mouse IgG-FITC	Ar-ion 488 nm	P-glycoprotein	53

Electroporation

One of the approaches to introduce polar cell-impermeable fluorescent reagents into single cells is electroporation. This technique is based on the electric-field-induced permeabilization in biological membranes with pulse generator and electrodes. Electroporation was used to deliver DNA to a population of mammalian cells in the early 1980s.[54] Upon the brief application of an electric field, an extra transmembrane potential is developed across the poorly conducting cellular membrane. The transmembrane potential is linearly proportional to the electric field

strength and the cell radius. Under the stress from the transmembrane potential, hydrophobic pores appear randomly in the membrane.[55]

The modified electroporation method was used to introduce polar cell-impermeable fluorescent reagents into single cells. When the polar fluorescent reagent is added to the exterior solution of the cells, and an electric field is then applied, polar derivatizing reagents can be induced into cells.[30,56,57] It is important to ensure the viability of living cells after electroporation, because some fast enzymatic cellular reactions could vary analyte concentration by an order of magnitude within a second.[1] Therefore, stringent optimization of conditions, such as electric pulse strength, pulse duration, and pulse number, for achieving reversible electroporation (i.e., self-healing of the pores following electroporation) is required, which is a difficult procedure for bulk electroporation.[58]

The use of microfluidic devices for cell electroporation offers apparent advantages compared to common bulk electroporation set-ups. Lee et al. developed a polymeric chip that can selectively immobilize and locally electroporate single cells. This easy-to-use chip allows parallel and reversible single cell electroporation using low applied voltages.[58] Lu developed a simple technique to electroporate mammalian cells with high throughput on a PDMS microfluidic platform.[59] Weber et al. studied the effect of cell size and shape on single-cell electroporation. Cells are more readily permeabilized and are more likely to survive if they are large and hemispherical as opposed to small and ellipsoidal ones with a high aspect ratio. The dependence of the maximum transmembrane potential across the cell membrane on cell size is much weaker for single-cell electroporation than it is for bulk electroporation.[60]

Recently, a new micro electroporation cell chip with three-dimensional electrodes has been fabricated through microelectromechanical systems (MEMS) technology, and tested on cervical cancer (HeLa) cells. Different sizes of molecules under fluorescence mode, including Rhodamine B-dextran with a molecular weight of 10 kDa, Rhodamine B isothiocyanate (RITC)-dextran with a molecular weight of 20 kDa, fluorescein isothiocyanate (FITC)-dextran with a molecular weight of 40 kDa and 70 kDa, and a gene-coding green fluorescent protein, were introduced into HeLa cells.[61]

Electroporation has also been used for the determination of amino acids in individual cells.[25,62] Cell-impermeable fluorescent reagent FITC was introduced into living cells by electroporation for derivatization of amino acids. After completion of the derivatization reaction, a single cell was injected into the separation capillary tip and lysed there with 0.1 mol/L NaOH. The derivatized amino acids were separated by CE and detected by LIF. Nine amino acids were quantitatively determined, with amounts of the amino acids ranging from 3.8 to 32 amol in single erythrocytes[25] and 2.68×10^{-5} mol/L to 18.18×10^{-5} mol/L in single protoplasts.[62]

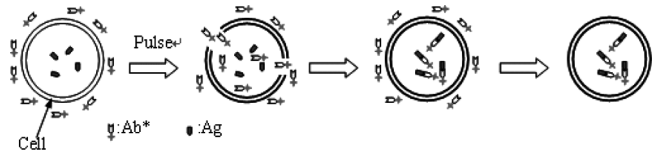

Figure 1. Schematic diagram showing the process of intracellular immunoreaction. (a) Before electroporation; (b) During electroporation; (c) Intracellular immunoreaction; (d) After removing residual Ab*. Adapted from Ref. [63].

Recently, Jin demonstrated a highly selective method for the determination of human interferon-gamma (IFN-γ) isoantigens in single natural killer (NK) cells by using intracellular immunoreaction and CE with LIF detection.[63] Anti-IFN-γ monoclonal antibody labeled with fluorescein isothiocyanate (Ab*), which is cell membrane impermeable, was introduced into NK cells by electroporation. IFN-γ isoantigens (Ag) were selectively labeled in cell interior after completion of the intracellular immunoreaction between Ag and Ab*, as illustrated in Fig. 1. When a proper pulse-shaped electric field was applied across cell membrane, pores on the cell membrane were formed. During the effective pore-open time, Ab* in the extracellular medium diffused into the cell (Fig. 1 b). Once the electric field is removed, the cell membrane reseals automatically (Fig. 1 c). After completion of the intracellular immunoreaction, the exterior phase with excess Ab* can be completely removed via washing (Fig. 1 d). Then, one NK cell containing the complexes of Ag with Ab* was electrokinetically injected into the capillary. The cell adsorbed on the tip of the capillary was lysed by ultrasonication. Finally, the complexes of the two forms of IFN-γ in the cell were separated and detected by CE-LIF with a detection limit of zeptomole. Since the antigens or antibodies labeled with fluorescent reagents are commercially available, the combination of the intracellular immunoreaction and CE/chip based CE-LIF detection will be a useful tool for single cell analysis.

Derivatization Mediated by Liposome and Polyethylene Glycol (PEG)

Liposomes are artificial phospholipid vesicles that are formed by self-assembly of lipids into spherical or quasispherical structures, containing an aqueous central cavity.[64,65] Because of its similarity to cell membrane structures, liposome has been utilized for drug delivery and targeting,[66] for the transfer of genetic materials[67] and fluorescent dyes[68] into cells. Recently, an intracellular derivatization method mediated by liposome has been developed for chip-based CE.[69] Liposomes with an average

diameter of 100 nm were produced from phosphatidylcholine to encapsulate fluorescent dyes by an ultrasonic method. Cell membrane impermeable fluorescent reagent FITC was delivered into living cells without cell viability decrease and dilution from extracellular media, even after a long incubating time of 24 h to complete the labeling reaction. A large number of peaks have been observed using chip-based electrophoretic separation with LIF detection, which revealed that liposome-membrane fusion occurred after entrance of liposomes into the cells, and intracellular amino acids and some proteins were labeled by FITC released from the liposomes.

Chen used PEG to transfer FITC into plant cells based on the fact that PEG can increase the permeability of cell membrane and has been used successfully in gene transformation in plants. After intracellular FITC-derivatization, the amino acids in plant cells were determined by CE.[70]

On-Column Derivatization

In 1995, Gilman and Ewing[71] developed the on-column derivatization scheme for single cell analysis with CE. The process is illustrated in Fig. 2. In step 1, a separation capillary was filled with a running buffer containing both a lysing reagent for cells and a derivatization reagent for the analyte. Then, a single intact cell was drawn into the front end of the separation capillary by electroosmotic flow (EOF) with the aid of an invert microscope and a precise micro-manipulator. The sampled cell adhered to the capillary wall after removal of the EOF. In step 2, the cell was staticly lysed with the lysing reagent in the running buffer. Sodium dodecyl sulfate (SDS) and digitonin have often been used as cell lysing reagents in single cell analysis. In step 3, free analytes to be analyzed were liberated from the cell and labeled with the derivatizing reagent in the running buffer. In steps 4 and 5, after completion of the derivatization reaction, separation voltage was applied to the separation channel and the derivatized analytes were electrophoreticly separated and detected.

With this method, femtomole to attomole levels of dopamine, amino acids and endogenous histamine after on-column derivatization have been quantitatively determined in individual cells, such as PC12 cells (*ca.* 15–25 μm in size),[71] single neuron from the snail *P. corneus* (*ca.* 75 μm in size),[72] mouse peritoneal macrophages (*ca.* 15–25 μm in size)[73] and rat peritoneal mast cells.[74] Recently, proteins and other primary amines in single MCF-7 cells were characterized using 2-D CE after on-column derivatization using the fluorogenic dye 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ).[75]

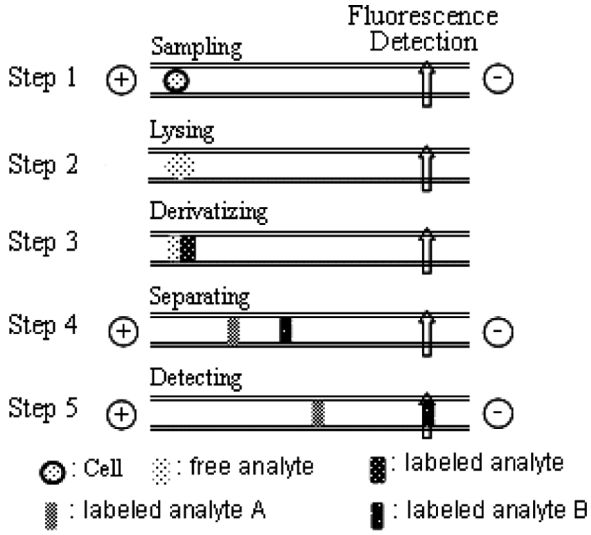

Figure 2. Schematic representation of the on-column derivatization method for single cell analysis.

During on-column derivatization, although the walls of the capillary can keep the cell contents from dilution by restricting diffusion to the longitudinal axis of the capillary, dilution effect due to latitudinal diffusion in the capillary is not avoidable. Therefore, the reaction rate for on-column derivatization is very important. According to calculation,[71] the contents of a 20 μm diameter cell with a 4.2 pL volume are diluted by a factor of *ca.* 100 for 10 min reaction time during the on-column derivatization of dopamine, amino acids with NDA in present of cyanide. Obviously, the dilution factor increases with prolonged reaction time. Use of a reagent that reacts very slowly with the analytes of interest in a cell will result in unacceptable dilution due to diffusion during derivatization and will result in decreased resolution and sensitivity for the electrophoretic separation by the same process.[71]

On-column derivatization has also been used for chip-based single cell analysis. Ocvirk et. al reported the use of microfluidic glass chips for continuous single-cell lysis, on-chip derivatization and assay of internal β-Galactosidase (β-Gal) content in single HL-60 cells.[76] Scheme of on-chip lysing and on-chip reaction of intracellular β-Gal with fluorogenic substrate fluorescein-di-β-D-galactopyranoside (FDG) is shown in Fig. 3. Substrate and lysing agents were placed in ports A-C. Cells were placed in port D and suction was applied at port E. Single cell streams were generated and transported toward the Y-shaped mixing

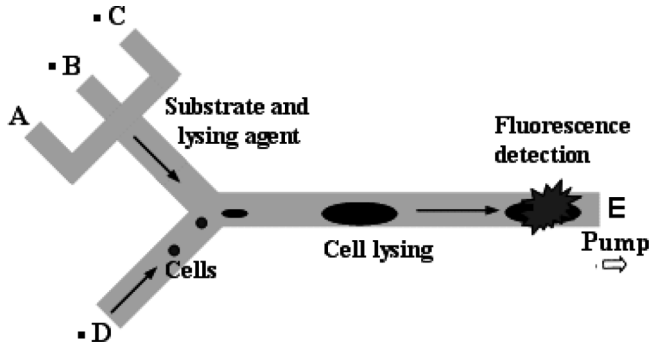

Figure 3. Scheme of on-chip lysing and on-chip incubation of HL-60 cell with substrate. Adapted from Ref. [76].

junction. A fluorescence detector was located downstream of the mixing point. Fluorescence peaks, due to the enzymatic product fluorescein mono-β-D-galactopyranoside (FMG), were detected downstream of the cell lysis point.

A microchip electrophoresis method has been developed in our laboratory for simultaneous determination of ROS and GSH in individual erythrocyte cells. ROS was pre-column labeled with DHR 123 in the intact cell, while GSH was on-chip labeled with NDA.[32] After electric lysing, the reaction between the released GSH and NDA contained in electrophoresis buffer can be carried out in the microchip separation channel during electrophoresis and was completed after traveling 5 mm downstream in 22 s. The average separation efficiency for GSH was 2.4×10^6 plates/m, which was not significantly different from that of pre-column derivatization. The GSH migration times on the on-column mode and off-column mode were 116 s and 110 s, respectively.[77] The method can maintain favorable kinetics for the labeling reaction, simplify the cell treatment and reduce the sample and reagent amount used in the analysis.

Cheng's group developed a fluorescence detection system with a Hg-lamp as the excitation source and a photon counter as the detector used for the determination of neurotransmitters in single PC 12 cells by chip-based electrophoresis with on-column derivatization.[78] O-Phthaldialdehyde (OPA) was employed to label the catecholamine neurotransmitters such as dopamine (DA), norepinephrine (NE), and amino acids including alanine (Ala), taurine (Tau), glycine (Gly), glutamic acid (Glu), and aspartic acid (Asp). After a single cell was electrokinetically loaded at the double-T intersection area on the microfluidic chip, the docked cell was lysed by 0.6 mM SDS prepared in 20 mM borate buffer (pH 9.0) working electrolyte. The neurotransmitters and amino acids liberated from the lysed cell and labeled by OPA added in cell

Table 2. On-column derivatization used for single cell analysis

Analytes	Reagents	Excitation wavelength	References
Glutamate	Glutamate dehydrogenase, Glutamic pyruvic transaminase	Ar ion laser (305 nm)	79
Protein	3-(2-furoyl)quinoline-2-carbox-aldehyde (FQ)	Ar ion laser (488 nm)	80, 81
IFN-γ	Anti-IFN-γ labelled with FITC	Ar ion laser (488 nm)	82
Lactate Dehydrogenase	Lactate and NAD^+	340 nm	83
DNA	SYTO 9	Mercury arc source	84
	Ethidium bromide	Solid phase laser (532 nm)	85
Amino acid	OPA, 2-ME	340 nm	86

suspension for 2 min. Then separation voltage was applied to the separation channel to activate electrophoretic separation.

Some other applications of on-column derivatization used in single cell analysis are summarized in Table 2.

Wu et al. fabricated an integrated PDMS microfluidic chip for analysis of the amino acids in a single Jurkat cells with functional integration of single-cell introduction, on-chip lysis, derivatization, separation and detection.[12] Fluorescent derivatization was accomplished in a reaction chamber with a volume of 70 pL by using two valves formed by multilayer soft lithography to minimize dilution due to latitudinal diffusion during on-column derivatization. Schematic illustration of such a microfluidic chip is shown in Fig. 4. This chip consists of four sections: cell manipulation channels (channels 5–7), reagent introduction channels

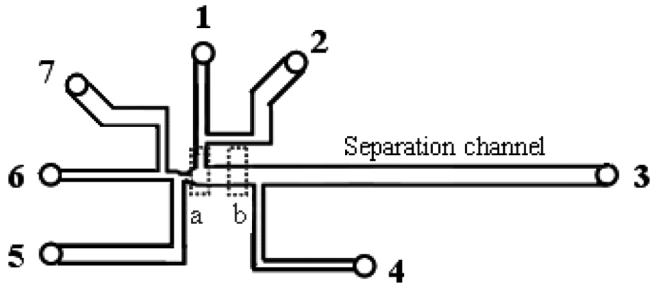

Figure 4. Schematic illustration of a microfluidic chip used for single-cell analysis. Adapted from Ref. [12].

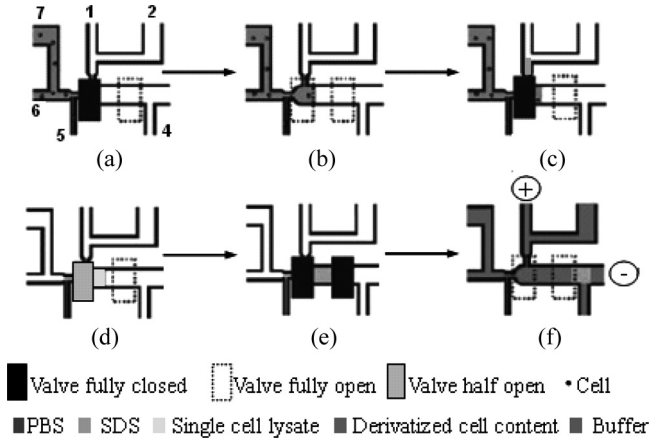

Figure 5. Schematic of the process of single-cell lysis and derivatization on an integrated microchip. Adapted from Ref. [12].

(channels 1 and 2), a reaction chamber between two valves and a separation channel (channel 3). A three-state valve (a) regulates flow of cells and reagents, and a two-state valve (b) connects the reaction chamber to the separation channel. By controlling the pressure on the valve, the three-state valve has three states as follows: (i) when no pressure is applied, the fluidic channel is open to flow in all directions; (ii) when a little pressure is applied, the left horizontal channel is blocked, but the side channel remains open to the right horizontal channel; and (iii) when higher pressure is applied, the side channel is also blocked and no flow takes place through this valve in any direction.

The process of manipulation and analysis of single cells with the mentioned chip is shown in Fig. 5. First, the three-state valve is fully closed. After a suspension of cells are introduced into inlet 7, cells flow from channel 7 to channels 5 and 6 (Fig. 5a); Once a cell is observed to be near the three-state valve (the region joining channels 5 and 6), valve a is opened to introduce the cell into the chamber (Fig. 5b) and then the valve is closed (Fig. 5c). By controlling the valve half open to deliver a plug of 20 mM borate buffer (pH $\sim$9.2) with 1% SDS and 30 mM sodium cyanide to the reaction chamber, SDS lyses the cell immediately (Fig. 5d). Picoliter amounts of NDA and cyanide are delivered to the reaction chamber. Both valves are closed and the cell contents are derivatized with NDA in the reaction chamber (Fig. 5e). Finally, both valves are open, filled with running buffer and the contents in the reaction chamber are separated by CE (Fig. 5f). This chip-based device can be generally used for on column derivatization with limited dilution effect, even though the reaction time is very slow.

Post Column Derivatization

Post column techniques allow improved detection of analytes after separation in their native state, while pre- and on- column derivatizations change both the detection and the electrophoretic properties. For example, it was reported that labeling of proteins can lead to significant band broadening, which is interpreted as a result of multiple labeling of proteins[2,87], wherein one or more fluorescent molecules are bound to the protein. The heterogeneous reaction products with different mobilities, generate a broad peak during electrophoresis. Post-column derivatization overcomes this limitation.

Daunert et al. used the post-column derivatization to examine biotinylated species in a crude mammalian cell lysate. After separation by CE, the effluent stream of the separation capillary is mixed with streptavidin-FITC. The vitamin biotin is strongly bound with the streptavidin-FITC. Covalent labeling of streptavidin with fluorescein yields a conjugate that can detect biotin compounds via fluorescence enhancement upon binding.[88] Owing to the limited microchip dimensions, post-column derivatization has not been widely used for single-cell analysis with chip-based CE.

DEVELOPMENT OF CHIP-BASED CAPILLARY ELECTROPHORESIS FOR SINGLE CELL ANALYSIS

The critical development for single-cell analysis is to integrate the cell treatment steps on one chip. While a large number of strategies exist to manipulate cells on chips,[13,18] few have been successfully combined with electrophoretic separation. Since chip-based CE for single-cell analysis is based on the same separation principle as CE separation, we will limit our discussion mainly to recent developments of cell manipulation used in chip-based CE.

To integrate the elemental operations for single-cell analysis, such as single cell injection, position and cell lysis, on a chip, techniques developed for the manipulation of single-cell include electroosmotic pumping, hydrodynamic flow manipulation, hydrostatic pressure combined with EOF and optical tweezers manipulation.

Electroosmotic Manipulation

In 1997, Harrison et al. first demonstrated the possibility to manipulate cells within a double-T microfluidic channels by using electrokinetic approaches.[89] The cell suspension was added in the sample reservoir.

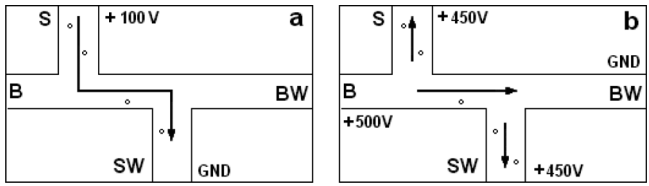

Figure 6. Schematic diagrams of cell transport in a double-T microfluidic device by EOF. Arrows indicate the direction of liquid flow. (a) Single cell loaded, (b) Single cell injected into separation channel (B-BW). Adapted from Ref. [89].

Potentials were applied to the various reservoirs as indicated in Fig. 6. Since electroosmotic flow follows the electric field lines, application of a potential between sample reservoir (S) and sample waste reservoir (SW) should cause the cells to negotiate the corners of the intersections in the double-T layout and form a plug of cells along the axis of the main channel (Fig. 6a). Once a plug of cells is formed under steady state condition at the intersection, it can be injected down the main channel by switching to another set of electric potentials to the four reservoirs, with the buffer reservoir (B) at 500 V, buffer waste reservoir (BW) grounded and both S and SW at 450 V (Fig. 6b). The directions of liquid flow produced by EOF were towards S, SW and BW as indicated by the arrows in Fig. 6b, and further cells were prevented from entering the separation channel. These preliminary experiments show that cell transport and manipulation are readily on-chip feasible by using valveless electroosmotic pumping. By optimization of the applied electric field and the density of cell suspension, a single cell could be loaded at the double-T injector with a similar electrokinetic procedure.[9,78] Once the cell appeared in the double-T, all reservoirs kept floating. The cell was docked on the channel walls around the double-T. Recently, Jin's group analyzed ascorbic acid in single wheat callus cells after electric lysis and electrophoretic separation.[9] Cheng's group reported a microchip electrophoresis system that integrated single cell injection, position, lysis, on-chip derivatization, separation and fluorescence detection.[78] The system was successfully applied for separation and determination of neurotransmitters in single rat pheochromocytoma (PC 12) cells. The average amounts of analyte per cell from a cell population were 2.5 fmol for DA, 3.3 fmol for Ala, 8.2 fmol for Tau, 4.0 fmol for Gly, and 1.9 fmol for Glu, each.

Hydrodynamic Flow Manipulation

Ramsey's group developed a hydrodynamic flow strategy for high-throughput continuous automatic analysis of single cells on a microfluidic

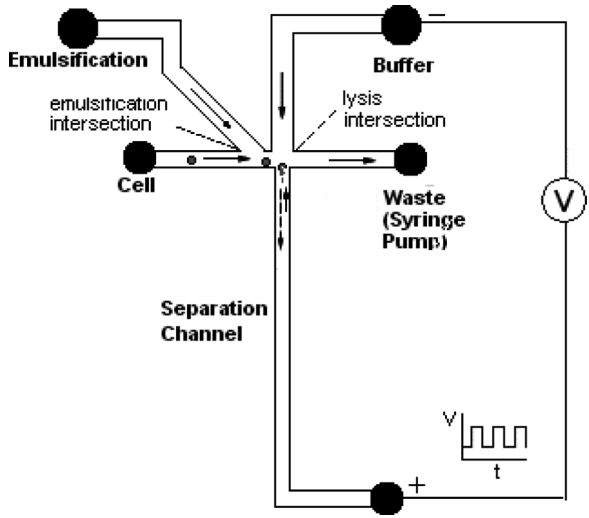

Figure 7. Schematic diagram of microchip device used for high-throughput analysis of single-cells. The solid arrows show the direction of bulk fluid flow and the dashed arrow shows the electrophoretic migration, direction of the labeled components in the cell lysate. Adapted from Ref. [1].

device.[1] The schematic diagram of the microchip is shown in Fig. 7. All of the channels and reservoirs were filled with working electrolyte buffer, except for the cell reservoir, which was filled with the cell suspension. Cells flow continuously through the channel manifold by applying a subambient pressure to the waste reservoir at the end of the waste channel using a syringe pump. To focus the cell to one side of the main channel and dissolve the membrane debris, an emulsification agent was added to the emulsification reservoir. When the cell entered the lysis intersection, cell lysis in less than 33 ms was achieved by pairing electrical and chemical lysis. The fluorescent analytes liberated from the ruptured cell were injected down the separation channel toward the anode and electrophoreticly separated by the applied voltage between the buffer and separation reservoirs. The average cell analysis rate was 7–12 cells/min, which was 100–1000 times faster than those reported using standard bench-scale CE.

Kelparnik and colleagues introduced cells into the separation crossing by a negative pressure-induced hydrodynamic flow on a compact disc (CD)-like microfludic device.[85] After on-chip cell lysis by sodium hydroxide present in the sieving medium, the released DNA fragments in a single apoptotic cardiomyocytes were labeled by ethidium bromide and detected by chip-based CE.

Hydrostatic Pressure Combined with EOF

We employed hydrostatic pressure combined with EOF to manipulate cells on a simple cross microfluidic chip.[10,32,90] After adding cell suspension in the sample reservoir (S) and the working electrolyte in the other reservoirs, individual cells were observed under microscope being transported in the direction from sample reservoir (S) to sample waste reservoir (SW) by hydrostatic pressure simply adjusted by the liquid levels in the four reservoirs (Fig. 8a). When a single cell moved within the crossed section of the channels, a set of electric potentials was applied to the four reservoirs, with buffer reservoir (B) at $+1200$ V, sample reservoir (S) and sample waste reservoir (SW) both at $+700$ V, and buffer waste reservoir (BW) grounded as shown in Fig. 8b. The sampled cell was transported toward the buffer waste reservoir (BW) by EOF (Fig. 8b). After 0.1 s, the set of potentials was switched off and the sampled cell settled within the channel adhering to its walls near the channel crossing. The docked cell was electrically lysed within 40 ms under the applied electric field in the working electrolyte without additional lysates. Then, the chip was shifted from the channel-crossing viewing position to the detection point, the set of electric potentials was resumed. The released intracellular constituents were separated by CE and detected by LIF. A throughput of 15 samples per hour, a retention time precision of 2.4% RSD was obtained for 14 consecutively injected cells. Further improvement was achieved by fabricating a multi-depth microchip with a weir as illustrated in Fig. 9.[11] The weir on the separation channel has two functions. First, it ensures the loaded cell to be precisely positioned within the separation channel. Unless the cell was lysed, the cell contents would not enter into the separation channel for separation and detection. Secondly, a phosphate-buffered saline medium (PBS) plug surrounding the trapped cell was rapidly replaced by the SDS containing buffer solution to speed up cell lysis. GSH and

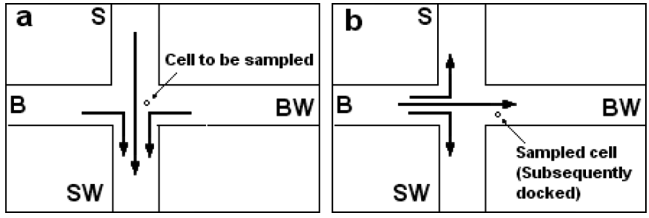

Figure 8. Schematic diagrams of cell transport in a crossing microfluidic device using hydrostatic pressure combined with EOF. Arrows indicate the direction of liquid flow. (a) Single cell loaded (b) Single cell injected into separation channel (B-BW). Adapted from Ref. [10].

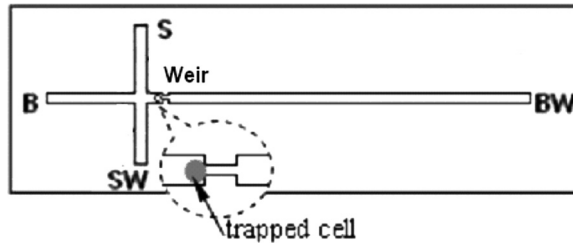

Figure 9. Schematic of multi-depth microchip with a weir on the separation channel.

ROS in single human carcinoma cells were separated by chip-based electrophoresis and detected by LIF, after the trapped cell was staticly lysed by SDS containing buffer solution.

Optical Tweezers Manipulation

Performing single-cell electrophoresis separations in multiple parallel microchannels offers the possibility of both increasing throughput and eliminating cross-contamination between different separations. Recently, Lilge's groups demonstrated a compact platform for single-cell CE in parallel microchannels that combines optical tweezers for cell selection and electromechanical lysis.[91] Calcein-labeled acute myloid leukemia (AML) cells were selected from an on-chip reservoir and transported by optical tweezers to one of the four parallel microfluidic channels. Each channel entrance was manufactured by laser ablation to form a 20- to 10-μm tapered lysis reservoir. Upon initiation of the electric field, cells would be transported along the 20 μm injection channel to the tapered opening of the 10 μm separation channel (Fig. 10). As the electric field pulled the cells into the opening with a diameter smaller than that of the cells, cells at the interface of the two channels were lysed by putting stress on the membrane. The cell contents were simultaneously injected into parallel channels and analyzed by microfludic electrophoresis. A potential drawback of using optical tweezers as a cell manipulation method is the slow selection time of each cell compared to systems described above. However, with optical tweezers the user gains the ability to examine, select, and analyze predetermined individual cells.

Recently, Ros's group have proposed a method for single cell analysis combining navigation and steering of single cells with optical tweezers, on-chip cell lysis and electrophoretic separation of proteins with subsequent detection by LIF.[92–94] As shown in Fig. 11, a poly(dimethyl-siloxane) (PDMS) microchip microstructured by vertical posts was used

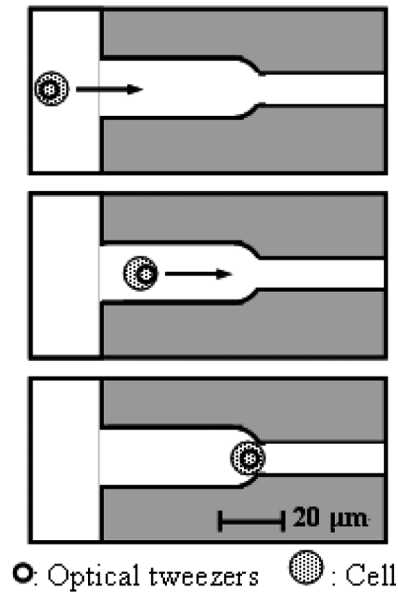

Figure 10. Top to bottom: transport of a cell from the cell chamber along the 20-μm injection channel to the tapered opening of the 10-μm channel. The circle indicates the position of the optical tweezers beam. Scale bar equals to 20 μm. Adapted from Ref. [91].

in their experiments. An individual cell can be selected from sample reservoir S and transported along the microchannel by optical tweezers to an intersection of two channels and trapped by the vertical posts. After the cell is dynamicly lysed at the intersection position, the protein components of the cell were electrophoreticly separated and detected by LIF.

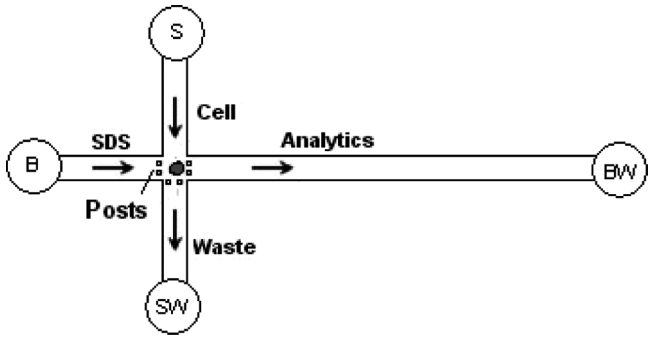

Figure 11. Schematic of PDMS microchip with vertical posts on the intersection of two channels. Adapted from Ref. [93].

The trapped Sf9 insect cells can be lysed by SDS containing buffers within seconds.[93] This lysing time can be further reduced to 50 ms by using high voltage pulses. Microfabricated electrodes were designed to create a maximum voltage drop across the cell's membrane at a minimum interelectrode voltage, achieving rapid cell lysis, which may broaden the application of chip-based electrophoresis to the study of cellular dynamics requiring fast lysing time.[49]

The lysis time determines the injected plug length and is critical in terms of band broadening for subsequent separation during the dynamic lysing.[94] The reason might be that intracellular components were continuously released during cell lysis and transported downstream through the separation channel by EOF.

Lysing the cell under static conditions can reduce band broadening, achieving a separation with higher resolution. This was accomplished by retaining the sampled cell in its docked position and switching off the separation potentials during the lysing period.[10,11]After cell lysis was completed, the separation potentials were resumed to activate the electrophoretic separation. The drawback of static lysing is the lower throughput compared with dynamic lysis.

CONCLUSIONS

Determination of intracellular components in single cells provides a powerful tool for researches and applications in the fields including biology, medicine, pathology clinic. The development of integrating the whole process for single-cell analysis on one microfluidic chip will promote the automation of single-cell analysis with high throughput.

The minute amount of cellular contents present in single cells requires the use of extremely sensitive detection methods. Laser induced fluorescence detection is one of the dominant detection techniques used for chip-based CE.

In most single-cell analysis, the sensitivity of detection is primarily dependent on the intracellular libeling method and the exogenous labeling agent. Considerable progress on derivatization method has been reported to label intracellular analytes without evident dilution, including microderivatization of living cells, electroporation, derivatization mediated by liposome and PEG, and on-chip single-cell derivatization.

There are thousands of compounds in one cell. Chip-based CE limits the column capacity due to the short separation channel. It is difficult to separate and identify all the species labeled by non-selective labeling agent. By using specific labeling agent, intracellular species could be easily identified and determined. Immunoassay has become a popular technique because of its ability to selectively target a compound at trace levels

in the presence of potential interferences at much higher concentrations. Derivatization of cellular contents for chip-based electrophoresis combines the selectivity of immunoassay and the high separation efficiency of CE, which makes targeting analysis possible on the single-cell scale.

It is also expected that more permeable fluorescent probes will be developed with high selectivity and sensitivity, favorable kinetics, long excitation and emission wavelengths and large florescence quantum efficiency.

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