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Continuous cell introduction and rapid dynamic lysis for high-throughput single-cell analysis on microfludic chips with hydrodynamic focusing

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ABSTRACT

A chip-based microfluidic system for high-throughput single-cell analysis is described. The system was integrated with continuous introduction of individual cells, rapid dynamic lysis, capillary electrophoretic (CE) separation and laser induced fluorescence (LIF) detection. A cross microfluidic chip with one sheath-flow channel located on each side of the sampling channel was designed. The labeled cells were hydrodynamically focused by sheath-flow streams and sequentially introduced into the cross section of the microchip under hydrostatic pressure generated by adjusting liquid levels in the reservoirs. Combined with the electric field applied on the separation channel, the aligned cells were driven into the separation channel and rapidly lysed within 33 ms at the entry of the separation channel by Triton X-100 added in the sheath-flow solution. The maximum rate for introducing individual cells into the separation channel was about 150 cells/min. The introduction of sheath-flow streams also significantly reduced the concentration of phosphate-buffered saline (PBS) injected into the separation channel along with single cells, thus reducing Joule heating during electrophoretic separation. The performance of this microfluidic system was evaluated by analysis of reduced glutathione (GSH) and reactive oxygen species (ROS) in single erythrocytes. A throughput of 38 cells/min was obtained. The proposed method is simple and robust for high-throughput single-cell analysis, allowing for analysis of cell population with considerable size to generate results with statistical significance.

1. Introduction

Cell is the fundamental unit of life, and individual cells often differ significantly from their neighbors. Determination of the chemical composition in individual cells would greatly improve the probability of discriminating infected cells from healthy ones and provide a solid foundation on study and development in various fields including biochemistry, medicine, and pathology clinic [1,2]. However, the small amount of analytes present in a single cell makes the analysis challenging [1].

Microchannel with dimensions at micrometer level is ideally suitable for introduction, manipulation, lysis, separation and detection of single cells [3–9]. Therefore, chip-based systems for single-cell analysis are now attracting broad interests. Wu et al. [10] and Hong et al. [11] demonstrated high integration of single-cell analysis on microfluidic chips, but the manipulation and chip design were relatively complicated. Alternatively, cross [12–17] and double T [18,19] microfluidic chips are commonly used for single-cell analysis. The cells were positioned near the entry of the separation channel by electroosmotic flow (EOF) [18,19], hydrostatic pressure combined with EOF [12–14] or optical tweezers [15,20] and then lysed under static condition. The intracellular constituents were electrophoretically separated and determined by various high sensitivity detectors, such as LIF detector [12–15], electrochemical detector [18] and chemiluminescence detector [17]. However, cell immobilization and lysis under static condition greatly reduced the throughput to 25 cells/h or less [21].

Up to now, to the best of our knowledge, only a few microfludic devices have been reported for single-cell analysis with high-throughput [9,16,22]. In these studies, the analysis throughput was greatly improved based on continual lysis and analysis of cells distributed in a flowing stream. However, PBS in the cell suspension also injected into the separation channel along with single cells could induce significant Joule heating and undermine the separation efficiency. Furthermore, expensive accessories, such as special power supply and syringe pump were required to manipulate the cells, which inevitably increased the cost and complexity of the devices and ultimately hindered their applicability.

In this study, we proposed for the first time a chip-based microfluidic system with 2 sheath-flow channels to meet different requirements of multiple on-chip processes in single-cell analysis. Owing to the introduction of sheath-flow streams, continuous

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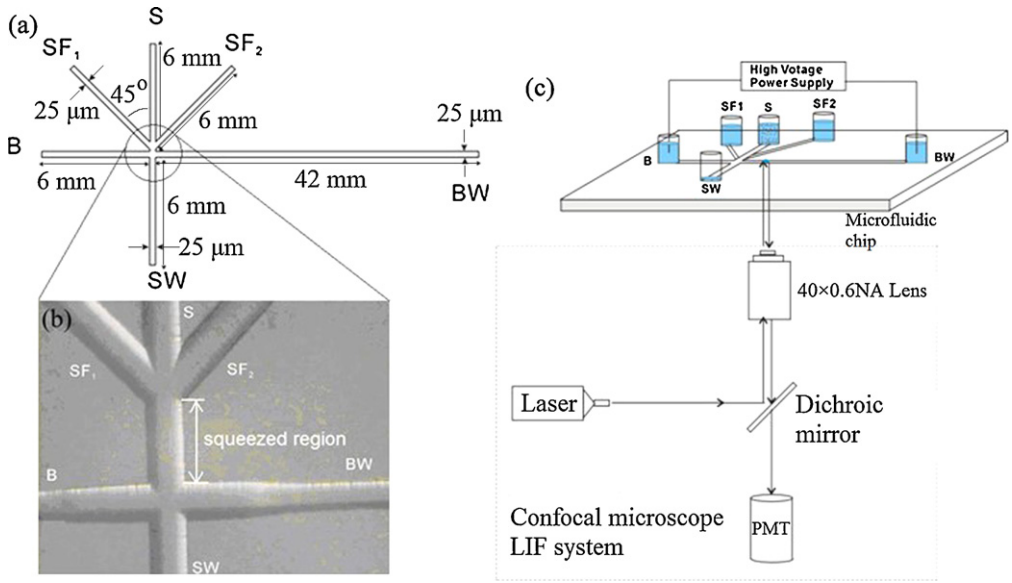

Fig. 1. (a) Schematic diagram of the channel design of the microfluidic chip. (b) Photograph of the partly enlarged cross section of the microfluidic chip. (c) Schematic diagram of the experimental setup.

injection of the aligned individual cells into the separation channel with high throughput was realized by using only a single DC power supply combined with hydrostatic pressure created by adjusting liquid levels in the reservoirs. In addition, the sheath-flow solution containing electrophoretic buffer and lysing reagent not only decreased the PBS concentration in the separation channel and thus Joule heating during electrophoresis, but also lysed the moving cells quickly at the entry of the separation channel.

2. Experimental

2.1. Microchip fabrication

The schematic diagram of the chip design is shown in Fig. 1a, in which the channel between sample reservoir (S) and sample waste reservoir (SW) was used for sampling and the channel between buffer reservoir (B) and buffer waste reservoir (BW) was used for separation. One sheath-flow channel was located on each side of the sampling channel. The line widths of the channels on the photomask were all 25 μm. After the microchip design on photomask was transferred onto the photoresist layer on the glass substrate, a two-step etching was used to fabricate the multi-depth microchip [13]. In the first step, with the separation channel covered with an adhesive tape, the other unprotected channels were etched with 1 mol/L HF + 1 mol/L NH_4F for 15 min with an eching rate of 1 μm/min. In the second step, the adhesive tape was removed and all the channels were etched for another 20 min with the same solution. The separation channel was etched to a depth of 20 μm, while the sampling channel and two sheath-flow channels a depth of 35 μm. Owing to the isotropic character of glass in wet etching, the average widths of sampling channel, sheath-flow channels and separation channel were about 95, 95 and 65 μm each. Access holes were drilled into the top etched plate with 1.5 mm diameter diamond-tipped drill bit at the terminals of the channels. After permanent bonding by a thermal bonding procedure, the access hole used for cell sampling was milled into the bottom plate for a further 200 μm with the same diamond-tipped drill bit to avoid its blockage by accumulated cells [23]. Five micropipette tips with an inner diameter of 5.5 mm and a height of 9.0 mm were epoxyed onto the chip surrounding the access holes of S, SF_1, SF_2, B and BW with epoxy glue (EPO-TEK 301, Epoxy Technology, Billerica, MA, USA). The epoxy glue does not contaminate the sample. A micropipette tip with an inner diameter of 7.0 mm and a height of 25.0 mm was used for the sample waste reservoir (SW). A photograph of the partly enlarged cross section of assembled microfluidic chip is shown in Fig. 1b.

2.2. Instrumentation

The schematic diagram of the experimental setup used for continuous single-cell analysis is shown in Fig. 1c. It consists of a home-built confocal microscope LIF system and a single high voltage power supply.

Fluorescence images were obtained from a CCD camera (YH-9682, Yonghui, Shenzhen, China) mounted on an inverted microscope (Jiangnan Optics & Electronics Co., Nanjing, China) equipped with an air-cooled solid-state laser source. The laser beam was expanded on the chip at an angle of about 45°. A 510 nm cut-off filter was directly positioned next to the window of the CCD camera.

2.3. Reagents

All chemicals used were of analytical grade unless otherwise mentioned and Millipore purified water was used throughout. PBS, which consisted of 0.9% NaCl and 0.02 mol/L NaH_2PO_4–NaOH (pH 7.4), was used for washing and preserving human erythrocytes. Triton X-100 was purchased from Sigma. The sheath-flow solution was prepared by dissolving 1% Triton X-100 in 20 mmol/L borate buffer (pH 9.2), which also acted as the medium for cell lysis as well as electrophoretic separation. Dihydrorhodamine 123 (DHR 123), rhodamine 123 (Rh123) and fluorescein diacetate (FDA) came from Molecular Probes (Eugene, OR, USA). L-Glutathione (GSH, reduced form) was purchased from Sigma, and 2,3-naphthalenedicarboxaldehyde (NDA) from Aldrich (St. Louis, MO, USA). Two milligrams of NDA was dissolved in 1 mL acetonitrile for derivatizing GSH in cells. The solution of DHR 123 for derivatizing ROS in cells was prepared at a concentration of 0.1 mg/mL in acetonitrile and kept in the dark at −20 °C before use.

2.4. Sample treatment

Human blood from a healthy adult was obtained from the Hospital of Zhejiang University. 10 μL of blood sample was cen-

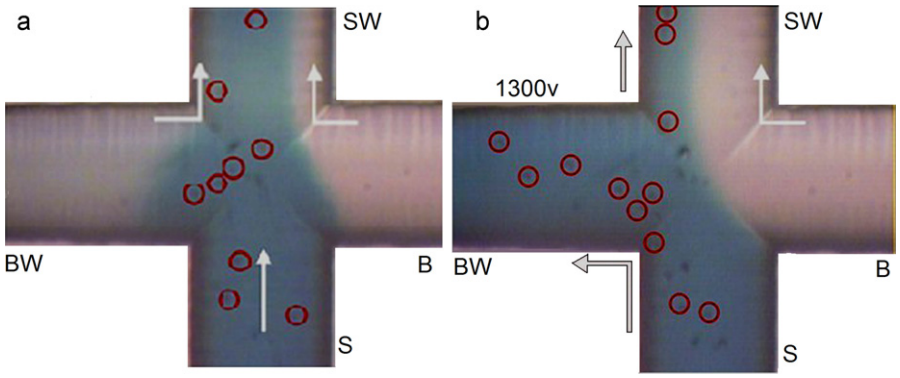

Fig. 2. Images of cell sampling in cross microchip. (a) A hydrostatic pressure of −0.5 mbar applied on SW. (b) −0.5 mbar on SW with 270 V/cm between B and BW. The cell suspension stream is visualized by dissolving 2% brilliant blue in PBS. Arrows indicate the direction of liquid flow.

trifuged at 2000 rpm for 5 min to separate out the erythrocytes. The supernatant was discarded and the erythrocytes were washed with PBS 3–5 times by centrifuging until a clear supernatant was obtained. After the supernatant was discarded, the erythrocytes were resuspended in 1 mL PBS, and 10 μL DHR 123 stock solution was added to the suspension and reacted with ROS in the dark at room temperature for 30 min. The cells were washed with PBS again for 3 times and resuspended in 1 mL PBS. Then, 10 μL NDA acetonitrile solution was added to the suspension to label GSH at room temperature for 10 min, according to a procedure described in previous reports [13,23]. The cells were washed with PBS twice to remove untreated NDA and resuspended in PBS solution to obtain a cell population of 1.2×10^5 cells/mL.

2.5. Continuous single-cell analysis on microchip

100 μL of 20 mmol/L borate buffer was added to the reservoirs B and BW. 100 μL of sheath-flow solution was added to both reservoirs SF_1 and SF_2. 100 μL of the labeled cell suspension was added to reservoir S with reservoir SW kept empty. Under hydrostatic pressure created by liquid-level difference between the reservoirs, the cell suspension flowed from S to SW and was focused by two sheath-flow streams. EOF produced by the potential applied along the separation channel would drive the moving cells continuously from the sampling channel into the separation channel. At the same time, the cells would be lysed at the entry of the separation channel by Triton X-100 added in the sheath-flow solution combined with electrical lysis. The liberated intracellular ROS and GSH were sep-

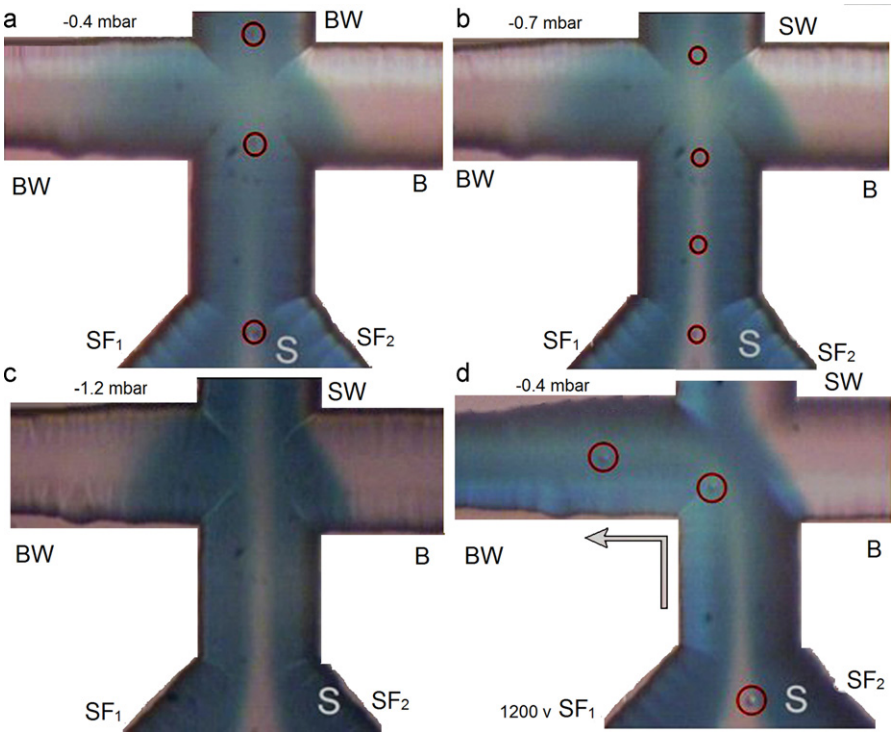

Fig. 3. CCD images showing sample flow hydrodynamically focused by sheath flow streams under different conditions. (a) A hydrostatic pressure of −0.4 mbar applied on SW, (b) −0.7 mbar on SW, (c) −1.2 mbar on SW, (d) −0.4 mbar on SW combined with an electric field of 250 V/cm applied between B and BW. Sheath flow stream is visualized by dissolving 2% brilliant blue in a borate buffer solution.

arated by electrophoresis in the separation channel, and detected by LIF. Simultaneously, the data acquisition and processing system was activated to record electropherograms.

3. Results and discussion

3.1. Continuous introduction of individual cells

In preliminary experiments, a simple cross microfluidic chip with four reservoirs S, SW, B and BW was used to explore the possibility of continuous single-cell analysis with integrated functions of continuous single-cell sampling, lysing, CE separation and LIF detection. To simplify the system, hydrostatic pressure generated by adjusting the liquid level in SW lower than those in the other 3 reservoirs was used to drive the cells from reservoir S to SW as shown in Fig. 2a. When an electric field was applied on the separation channel, the EOF in the separation channel counteracted the Poiseuille flow induced by the liquid-level difference between SW and BW. When the EOF is greater than the Poiseuille flow, the net flow will be directed towards the BW reservoir. The cells were negotiated by the corner of the intersection along with the cell suspension into the separation channel and moved along the separation channel to BW as shown in Fig. 2b. The result indicates that cells could be continuously introduced into the separation channel by hydrostatic pressure combined with EOF. However, with such a simple cross microfluidic chip, a number of difficulties remain for continuous analysis of single cells. Firstly, the throughput is rather low. By adjusting the liquid level in SW reservoir 3 mm lower than the other 3 reservoirs, individual cells in the cell suspension were separated with an average of 2 mm apart at a flow rate of 0.2 mm/s via the crosspoint to SW reservoir. About 30 s was required to transport a cell from the sample reservoir S to the channel crossing. Once the liquid-level difference between SW and other 3 reservoirs was increased, the flow rate of single cells was also increased. However, it has been observed that multiple cells occupied the channel crossing at any given time resulting in multi-cell loading as shown in Fig. 2b. Secondly, the erythrocytes should be washed with PBS and resuspended in PBS to provide the cells with an isotonic medium, thereby minimizing cell stress and avoiding rupture of cell membrane. However, the conductivity of PBS solution was higher than that of electrophoretic buffer solution. From Fig. 2b, it could be seen that PBS in cell suspension entered into the separation channel along with the dispensed cells and replaced the electrophoretic buffer solution added in the separation channel, which could induce significant Joule heating and undermine the separation efficiency. Thirdly, it was difficult to rapidly lyse a moving cell in a microchannel, since the cell was surrounded by PBS solution [16].

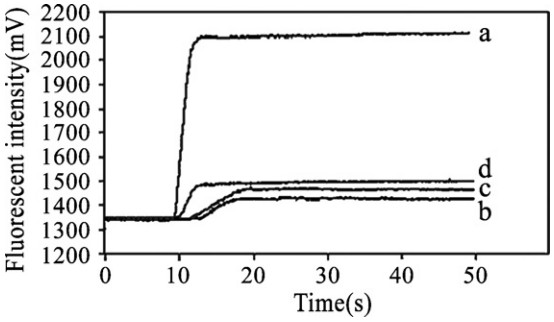

Fig. 4. Fluorescent signal for 10^{-6} mol/L Rh123 without dilution (a), and diluted by sheath-flow solution with different hydrostatic pressure applied on SW (b) −0.4 mbar, (c) −0.7 mbar, (d) −1.2 mbar. The electric field of 416 V/cm was applied between B and BW.

To continuously introduce individual cells into the separation channel with a high throughput, a multi-depth chip with 2 sheath-flow channels as shown in Fig. 1a was designed and fabricated. The sampling channel and two sheath-flow channels were 35 μm deep and 95 μm wide, while the separation channel had a depth of 20 μm and a width of 65 μm. The sampling channel with larger dimension is beneficial for high-throughput cell sampling even with low hydrostatic pressure. With such a design, sheath-flow streams were introduced on both sides of the sample fluid stream. Under hydrostatic pressure generated by the differences in liquid levels in the reservoirs, cell suspension, buffer solution and sheath-flow buffer solution were drawn simultaneously from S, SF_1, SF_2, B and BW across the intersection to SW. The sheath-flow streams not only constrained the sample stream to the central region of the sampling channel, but also compressed its width. Individual cells in the sample stream migrated sequentially through the intersection

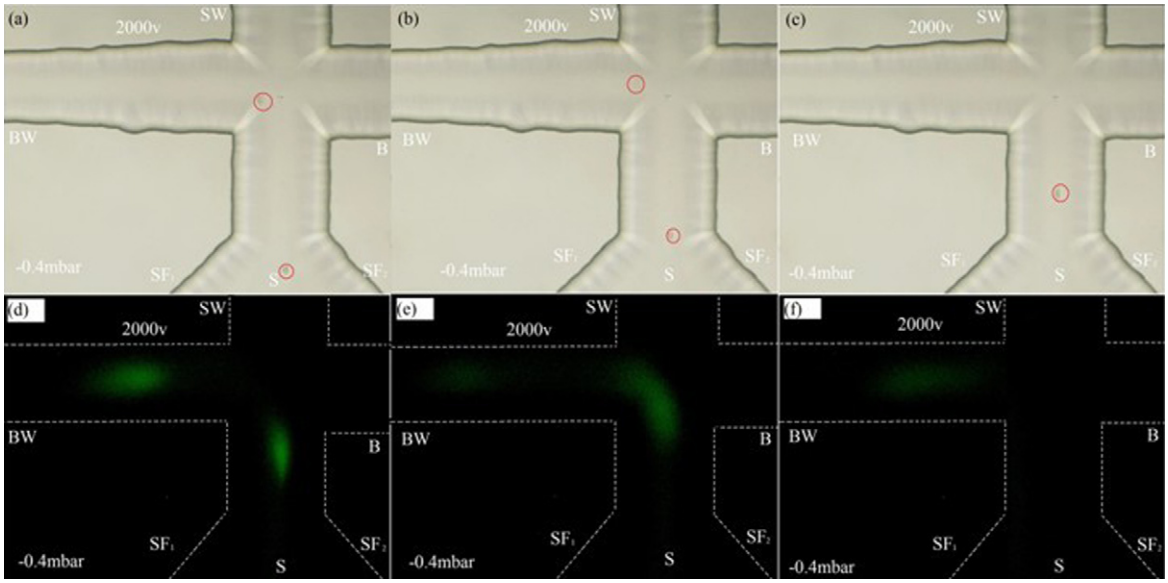

Fig. 5. CCD images of cell lysing in the microchip with the squeezed region length of 0.15 mm. (a–c) Light field images of cell lysing process; (d–f) fluorescent images of lysing process for cells labeled with fluorescein diacetate (FDA). The cell appears distorted in the image because the exposure time of the CCD camera was long compared to the cell velocity. Separate images were taken at intervals of 16.7 ms.

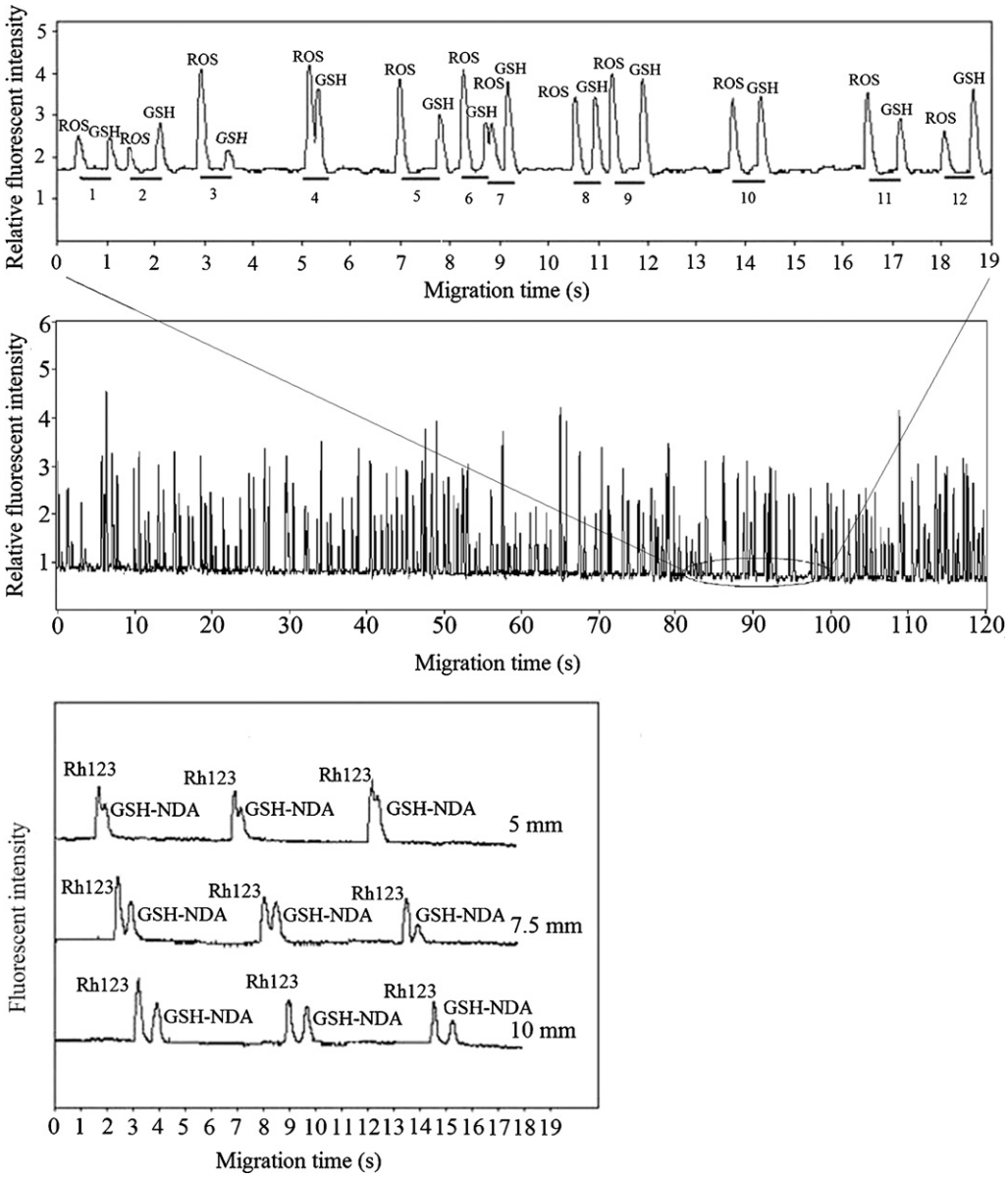

Fig. 6. (a) Electrophoretic separation of ROS and GSH released from individual cells. The electric field strength is 416 V/cm; the effective separation length is 1.0 cm; hydrostatic pressure on SW is −0.4 mbar; (b) electrophoretic separation of Rh123 plus GSH-NDA with different effective separation length. 5×10^{-6} mol/L Rh123 plus 1×10^{-4} mol/L GSH-NDA solution was used as the sample.

region with a high speed. The flow rate of single cells increased with increasing of the hydrostatic pressure applied on the SW, but the width of the sample stream became larger as shown in Fig. 3a–c. When an electric field was applied between B and BW, the hydrodynamically focused cells were driven sequentially into the separation channel by the electrokinetic force as shown in Fig. 3d. In our experiment, the maximum rate of 150 cells/min could be achieved by applying a hydrostatic pressure of −0.7 mbar on SW combined with electrokinetic force (Supporting Information available).

As shown in Fig. 3d, the electrophoresis medium in the separation channel was composed of a mixture of sheath-flow solution and PBS in cell suspension. This means that the PBS concentration in the electrophoresis medium could be diluted by sheath-flow solution. To evaluate the dilution effect of sheath-flow solution on the concentration of PBS in the electrophoresis medium, an experiment was performed as the following. With 10^{-6} mol/L Rh123 added in SF_1, SF_2, S reservoirs and buffer solution in B and BW reservoirs, and a hydrostatic pressure of −0.4 mbar applied on SW and a voltage of 2000 V between B and BW, Rh123 solution in SF_1, SF_2, S reservoirs was continuously introduced into the separation channel. Before Rh123 solution reached the detection point, a steady background signal of 1.36×10^3 mV was detected from the buffer solution. After the 10^{-6} mol/L Rh123 reached the detection point, a steady fluorescent signal (Fig. 4a) of 2.12×10^3 mV appeared in the electrophorogram. When Rh123 solution in SF_1 and SF_2 was replaced with sheath flow solution while kept in S alone, a steady fluorescent signal of 1.44×10^3 mV (Fig. 4b) was obtained owing to the dilution effect of the sheath-flow solution. With the hydrostatic pressure applied on the SW increasing, the width of the sample stream became larger, which resulted in the increase of the concentration of Rh123 in the separation channel, thus increased the signal intensity as shown in Fig. 4c and d.

Since concentration of Rh123 at the detection point is proportional to fluorescent intensity, the dilution ratio (DR), which is termed as concentration ratio of diluted Rh123 to the original

Rh123, could be calculated from the following equation:

$$DR = (I - I_0)(I_a - I_0)^{-1}$$

where I_0 is the base-line florescent intensity; I_a is the florescent intensity of 10^{-6} mol/L Rh123; I is the florescent intensity of 10^{-6} mol/L Rh123 diluted by sheath flow solution. The calculated DRs with hydrostatic pressure of −0.4, −0.7 and −1.2 mbar are 0.105, 0.145 and 0.184 each. The results indicated that hydrodynamic focusing produced by two sheath-flow steams significantly decreased PBS concentration in the electrophoretic buffer and thus reducing Joule heating during electrophoresis. In our experiment, the electric current was only 18 μA owing to the dilution effect from the sheath-flow streams, even though a high separation voltage of 2000 V was used.

3.2. Rapid cell lysis

From Fig. 3, it could be seen that individual cells in the sample flow was squeezed into a narrow stream by the sheath-flow streams. In the squeezed region the sheath-flow solution replaced PBS surrounding the cell along the sampling channel. To lyse the moving cells quickly, initial attempts were made by adding 1% sodium dodecylsulfate (SDS) in the sheath-flow solution. A video clip revealed that human erythrocytes were quickly lysed in the squeezed region before entering the separation channel (Supporting Information available), which might result in an incomplete injection of intracellular components into the separation channel. As shown in Fig. 3d, when a separation electric field was applied on the separation channel, only a part of the sample stream was driven into the separation channel, while other part was introduced downstream of the intersection to SW. To achieve a complete injection of intracellular components into the separation channel, individual cells should be lysed in the stream to BW immediately after entering the separation channel. Therefore, a nonionic surfactant Triton X-100 (1%), in which the time required for lysing a cell is longer than that in SDS [24], was further used for optimizing the length of the squeezed region. The lysing process in three microchips with the squeezed region length of 0.1 mm, 0.15 mm and 0.25 mm were examined. The results indicated that individual cells could be lysed quickly in all three microchips, but an optimal position for cell-lysis was obtained on the microchip with the squeezed region length of 0.15 mm. CCD images of the lysing process in the microchip with the squeezed region length of 0.15 mm were shown in Fig. 5. Each image was captured at an interval of 16.7 ms. In the first image, the cell was intact (Fig. 5a). After 16.7 ms the cell was partly damaged (Fig. 5b) and completely lysed within 33 ms as shown in Fig. 5c. As shown in Fig. 5d–f, a complete injection of the cytosolic dyes into the separation channel was obtained. In our experiment, a microchip with squeezed region length of 0.15 mm was used (Supporting Information available).

3.3. Continuous analysis of intracellular ROS and GSH in single erythrocytes

ROS and GSH are two important species related to cell apoptosis and oxidative stress. After intracellular derivatization [13,23] by incubating living cells with derivatizing reagents DHR-123 and NDA in succession as described in the sample treatment section, GSH and ROS in single erythrocytes were analyzed on the microfluidic chip. A recursive two-peak pattern, with each peak pair corresponding to GSH and ROS from one cell was shown in Fig. 6a. The separation of ROS and GSH from 76 cells was completed in a 2-min run. The detection point was 10 mm from the cross intersection. An expanded view in Fig. 6a shows the separation of the GSH and ROS peaks from 12 cells in a 19-s segment. The difference between migration times of each pair ($\Delta t = 0.61 \pm 0.05$ s, $n = 12$) matched well with those for the lysate ($\Delta t = 0.62 \pm 0.02$ s, $n = 3$) shown in Fig. 6b. Nevertheless, individual discrepancy also led to different cell lysis point at the entry of the separation channel and different intervals between peak pairs, e.g. the interval of pair 4 in Fig. 6a is smaller than others. It can be seen from Fig. 6a that the peak area of ROS and GSH varied from cell to cell, which is expected to come from intracellular variation, as reported previously [23]. Although the maximum rate for introduction of individual cells into separation channel was about 150 cells/min, the actual rate for continuous analysis of single cell was limited by the difference between migration times of ROS and GSH. If the individual cells were introduced into the separation channel with higher sampling rate, the interval between the individual cells would be shorter, resulting in peak overlaps and a lower resolution between species from cells close to one another. By using shorter effective separation distance, the actual rate for continuous analysis of single cell could be enhanced, but the resolution between ROS and GSH in the same single cell was worse as shown in Fig. 6b. Therefore, to distinguish each cell's electrophorogram from those of nearby cells, the actual rate for continuous analysis of single cell will be depended on the component-number to be detected and difference in migrating time of these species. In our experiment, the effective separation distance of 10 mm was used and the average cell throughput was about 38 cells/min to realize separation of ROS and GSH in single cells.

4. Conclusion

We have demonstrated a microfluidic system for high-throughput single-cell analysis on a cross microfluidic chip with one sheath-flow channel located on each side of the sampling channel. The microfluidic system is rather simple, which is composed of a LIF detector, a single DC power supply combined with hydrostatic pressure created by adjusting liquid levels in the reservoirs. The introduction of sheath-flow streams not only align individual cells to sequentially enter the separation channel, but also ensure rapid dynamic lysis of the moving cells at the entry of the separation channel. In addition, the concentration of PBS injected into the separation channel along with single cells could be significantly diluted by sheath-flow solution, thus reducing Joule heating during electrophoresis. Moreover, single cell analysis is automatic and does not require a microscope for observing cell positioning. The simple and robust method shows great potential in high-throughput single-cell analysis. Combined with high selective antibody-based stains, it also might be useful for discriminating infected cells from healthy ones to generate a result with statistical significance for clinical diagnosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.11.049.

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