

Evaluating Intracellular Redox Status in L02 Cells on Microchip

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Um novo dispositivo microfluídico integrado com introdução contínua de células individuais e detecção de espécies redutoras intracelulares por detector de fluorescência induzida por laser (LIF) é relatado. Um canal central de amostragem foi desenhado sendo flanqueado por dois outros canais auxiliares. As espécies intracelulares redutoras foram derivadas por uma sonda fluorescente baseada na recém-sintetizada 2,2,6,6-tetrametil-piperidina-1-oxilo (TEMPO). As células marcadas foram hidrodinamicamente focadas pelas correntes da bacia de fluxo e sequencialmente introduzidas no canal de amostragem sob pressão hidrostática gerada por ajuste dos níveis de líquido nos reservatórios. O nível de redução intracelular em células vivas intactas L02 foi detectada por LIF sem citólise. Após estimulação com 100 $\mu\text{mol L}^{-1}$ de peróxido de hidrogênio, durante 30 minutos, o nível de redução intracelular diminuiu em resposta ao estresse oxidativo. Foi obtida uma taxa de transferência de 41-44 células min^{-1} .

A novel microfluidic device integrated with continuous introduction of individual cells and detection of intracellular reducing species by laser induced fluorescence (LIF) detector is reported. A single channel with one sheath-flow channel located on each side of the sampling channel was designed. The intracellular reducing species were derivatized by a newly synthesized 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO)-based fluorescent probe. The labeled cells were hydrodynamically focused by sheath-flow streams and sequentially introduced into the sampling channel under hydrostatic pressure generated by adjusting liquid levels in the reservoirs. Intracellular reducing level in intact living L02 cells was detected by LIF without cytolysis. Upon stimulation with 100 $\mu\text{mol L}^{-1}$ hydrogen peroxide for 30 min, the intracellular reducing level decreased in response to oxidative stress. A throughput of 41-44 cells min^{-1} was obtained.

Keywords: single-cell analysis, microfluidics, high-throughput

Introduction

Redox system has a pivotal role in maintaining the reducing environment in cells. There are many redox couples existing in living cells, and they work together with antioxidant enzyme systems to maintain the balance of the intracellular redox status.¹ Intracellular reducing species levels change dramatically in the response to oxidative stress. Thus, the quantitative detection of intracellular reducing species is of great importance for investigating cell functions.² L02 cells are normal hepatocytes; study of variation of intracellular reducing species levels in normal hepatocytes in the response to oxidative stress plays an important role in oncology. During the past two decades, much attention has been given to the analysis of chemical components inside single cells. A variety of methods for

single-cell analysis have been developed.³⁻⁹ Microfluidic chips with laser induced fluorescence (LIF) detection have also been developed for separation and detection of fluorescent dyes permeated into cells.^{3, 10-12}

However, in the majority of the reported whole-cell injection techniques that rely on chip-based CE, the analysis throughput is rather low and limits their practical application.¹³ Therefore, high throughput analysis of intracellular components in single cells are highly desirable.¹⁴⁻¹⁶ Recently, we reported several novel high-throughput methods for analysis of reactive oxygen species (ROS) and glutathione (GSH) in single cells.^{17,18} In these methods, however, cells must be lysed before detection, causing the release of cellular debris and intracellular biological macromolecules be adsorbed onto the wall of the microchannel, thus interfering with the next determination. One way to perform high-throughput single-cell analysis is not to lyse cells during determination.

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In our current work, we developed a high-throughput method for single-cell analysis without cytolysis. A newly synthesized 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO)-based redox-sensitive fluorescent probe was used to label the intracellular reducing species. The labeled cells were hydrodynamically focused by sheath-flow streams followed by detection on a home-built LIF detector. Owing to the introduction of sheath-flow streams, continuous sampling of the aligned individual cells in sampling channel with high throughput was realized by using hydrostatic pressure created by adjusting liquid levels in the reservoirs. The intracellular reducing levels of intact living cells were analyzed by using the proposed system.

Experimental

Microchip fabrication

The schematic diagram of the channel design is shown in Figure 1a, in which the channel between sample reservoir (S) and sample waste reservoir (SW) was used for sampling. 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 wet chemical etching procedure was performed to fabricate soda-lime glass microchip described elsewhere.¹⁹ The channels were etched to a depth of 35 μm and a width of 95 μm . 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.²⁰ Four micropipette tips with an inner diameter of 5.5 mm and a height of 9.0 mm were epoxied onto the chip surrounding the access holes of S, SF₁, SF₂ and SW with epoxy glue. The epoxy glue does not contaminate the sample. Figure 1b and 1c are photographs of the junction of the assembled microfluidic chip and the microchip, respectively.

Instrumentation

The schematic diagram of the experimental setup used for single-cell analysis is shown in Figure 1d. It consists of a microfluidic chip and a home-built confocal LIF system. As described previously,¹² an air-cooled solid-state laser (473 nm, 10 mW, Optoelectronic Technologies, Changchun, China) was coupled to an inverted microscope

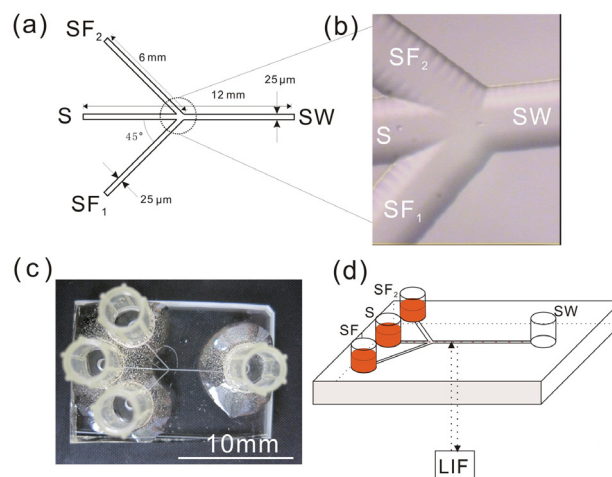


Figure 1. (a) Schematic diagram of the channel design of the microfluidic chip. (b) Photograph of the partly enlarged junction of the microfluidic chip. (c) Photograph of the microfluidic chip. (d) Schematic diagram of the experimental setup.

(Jiangnan Optics & Electronics, Nanjing, China) with necessary optical components. The laser beam was reflected by a dichroic mirror and focused into a 20 μm spot on the sampling channel from the bottom of the chip. The emitted light was collected and focused by the same focusing system, and directed to a pinhole by the optics of the inverted microscope. A photomultiplier tube (PMT, CR114, Hamamatsu, Beijing; bias: 600V) was mounted on the top of the microscope tube with a cutoff filter to reduce the background noise caused by any scatter and stray light below 520 nm and connected to an amplifier (GD-1, Reike Electronic Equipment, Xi'an, China). Data acquisition and processing were carried out using a N2010 A/D converter (Zheda Instruments, Hangzhou, China) and a computer.

Reagents and cell culture

All chemicals used were of analytical grade unless mentioned otherwise and Millipore purified water was used throughout. Phosphate buffered saline solution (PBS), which consisted of 8.00 g L⁻¹ NaCl, 0.20 g L⁻¹ KCl, 1.56 g L⁻¹ Na₂HPO₄·H₂O and 0.20 g L⁻¹ KH₂PO₄ (pH 7.4), was used for washing and preserving L02 cells. Hydroxypropyl methylcellulose (HPMC) was purchased from Sigma and a PBS-HPMC solution (40 mg HPMC dissolved in 10 mL PBS) was used to prepare the cell suspension.

TEMPO-based redox-sensitive fluorescent probe was kindly provided by Yanguang Wang group (Department of Chemistry, Zhejiang University, China). Detailed synthesis methods were described elsewhere.²¹ The stock solution of TEMPO-based probe was prepared

at a concentration of 10^{-3} mol L $^{-1}$ in dimethylsulfoxide (DMSO) for derivatizing reduced species in cells and kept in the dark at -20°C .

L02 cells were cultured in a 160 mL glass culture bottle at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), penicillin ($100\text{ }\mu\text{g mL}^{-1}$, Sigma), and streptomycin ($100\text{ }\mu\text{g mL}^{-1}$, Sigma). Cells were diluted at a ratio of 1:3 every 3 days to maintain in the exponential growth phase.

Sample treatment

In the exponential growth phase, the L02 cells were harvested by adding Trypsin-EDTA (Sigma) to the culture and centrifuged at 1000 rpm for 5 min. After the supernatant was removed, the cells were resuspended in PBS solution. Then the cells were divided into two groups, one group of cells were pretreated with $100\text{ }\mu\text{mol L}^{-1}$ hydrogen peroxide for 30 min, while another group acted as control. The cells were washed with PBS twice to remove extracellular hydrogen peroxide and resuspended in 1 mL PBS. $20\text{ }\mu\text{L}$ TEMPO-based probe DMSO solution was then added to label the intracellular reducing species at room temperature for 30 min. The cells were washed with PBS twice to remove untreated probe and resuspended in PBS-HPMC (0.4%) solution to obtain a cell population of 10^5 cells mL $^{-1}$.

Single-cell analysis on microchip

$100\text{ }\mu\text{L}$ of PBS was added to both reservoirs SF_1 and SF_2 , $100\text{ }\mu\text{L}$ 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 focused by two sheath-flow streams. When the cell flowed through the detection point one by one, the fluorescence emitted from the cell was detected by LIF. Simultaneously, the data acquisition and processing system was activated to record the fluorescence signal.

Results and Discussion

General considerations in microchip design

In our preliminary design, a single-channel microchip with two reservoirs S and SW was used to explore the possibility of single-cell sampling. To simplify the system, hydrostatic pressure generated by adjusting the liquid level in SW lower than that of S was used to drive the cells from reservoirs S to SW as shown in Figure 2a. However,

using such a simple microfluidic chip, the throughput is rather low. By adjusting the liquid level in SW reservoir 3 mm lower than that in S reservoir, individual cells in the cell suspension were separated with an average of 2 mm apart at a flow rate of 0.2 mm s^{-1} from S to SW. Once the liquid-level difference between SW and S was increased, the flow rate of single cells was also increased. However, it has been observed that multiple cells occupied the channel at any given point as shown in Figure 2a. To continuously introduce individual cells into the sampling channel with a high throughput, 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 and sheath-flow buffer solution were drawn simultaneously from S, SF_1 , and SF_2 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 to SW with a high speed as shown in Figure 2b.

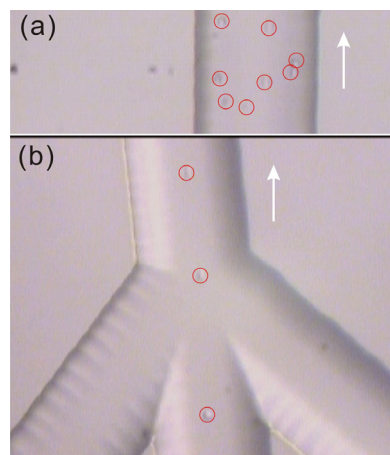


Figure 2. (a) Image of cell sampling in single-channel microchip with a hydrostatic pressure of -0.4 mbar applied on SW. (b) CCD image showing sample flow hydrodynamically focused by sheath flow streams with a hydrostatic pressure of -0.4 mbar applied on SW. Arrows indicate the direction of cell flow.

Hydrostatic pressure and cell concentration for high-throughput single-cell analysis

Hydrostatic pressure is a key factor for high throughput analysis. Greater the hydrostatic pressure applied, faster the cells migrate and shorter the distance between the cells, which may lead to an overlapping signal peak profile. Lower the hydrostatic pressure applied, slower the cells migrate and greater the distance between the cells, which lead to a low throughput, we found that a hydrostatic pressure of -0.4 mbar is appropriate for acquiring high throughput analysis.

Cell concentration should match with hydrostatic pressure of -0.4 mbar. Lower the cell concentration, greater the distance between the cells (see Figure 3), and lower the analysis throughput. However, higher cell concentrations lead to multi-cell sampling. When a hydrostatic pressure of -0.4 mbar is used, a cell concentration of 1.225×10^5 cells mL^{-1} is found to be appropriate for high throughput analysis of single cells.

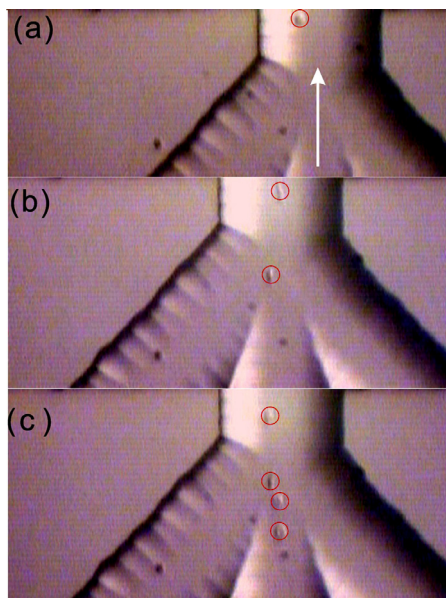
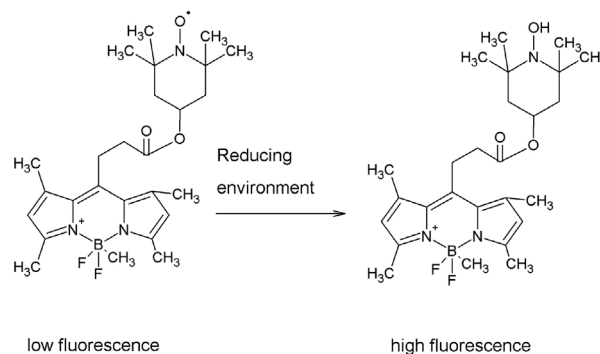


Figure 3. CCD images of cell sampling at different concentration: (a) 1.225×10^5 cells mL^{-1} . (b) 2.227×10^5 cells mL^{-1} . (c) 3.06×10^5 cells mL^{-1} . Arrow indicates the direction of cell flow.

Considerations in redox-sensitive fluorescent probe for living cells

Fluorescent probes are promising tools for the exploration of the world inside cells. So far, many fluorescent probes have been designed to detect various reactive oxygen species (ROS),²² but few probes are effective for evaluating the reducing intracellular environment. Therefore, it is necessary to develop new probes as a supplement in order to achieve reliable results. In our experiment, we applied a TEMPO-based redox-sensitive fluorescent probes which was newly synthesized to evaluate the intracellular redox status in L02 cells. In living cells, the nitroxide moiety of the probe is reduced to hydroxylamine quickly and becomes highly fluorescent.²¹ The reaction principle of the TEMPO-based fluorescent probe was detailed in Scheme 1.²³ The newly synthesized probe was used to evaluate the changes of redox status of L02 cells which was pretreated by H_2O_2 . Since the cell membrane is permeable to the new dye, the incubation time of 30 min was used to guarantee complete derivatization of intracellular reducing species.



Scheme 1. Structure of the TEMPO-based fluorescent probe.

Analysis of intracellular redox status in single L02 cells

A reducing Intracellular environment is necessary for living cells. The reducing level will change with cell apoptosis and oxidative stress.²⁴ One group of L02 cells was treated with H_2O_2 while another group was not. Then, the intracellular reducing levels of the two groups of L02 cells were analyzed on the microfluidic chip. Figure 4a and Figure 4b show the analytical results of reducing levels of 44 L02 cells without treatment and 41 L02 cells with treatment in a 1-min segment, respectively. Since one peak was defined as an individual cell, Figure 4 indicated the average analysis throughput of intracellular reducing levels was 41-44 cells min^{-1} . Although the analysis throughput could be increased by increasing the cell concentration or hydrostatic pressure, overlapping signals produced by two or more cells may be easily obtained at higher concentrations or speeds, which pose difficulty in acquiring the reducing levels of individual cell. Compared with the results shown in Figure 4c, a decrease of reducing level was observed for the H_2O_2 stimulated L02 cells. The results clearly demonstrated that normal hepatocytes conserve high levels of reducing species, while liver injury caused by oxidative stress can induce depletion of reducing species.

Conclusions

In summary, we have demonstrated a simple microfluidic system for high-throughput analysis of intracellular reducing levels without cytolysis. One sheath-flow channel located on each side of the sampling channel was used to generate a compressed sample stream, and the driving force was provided by hydrostatic pressure created by adjusting liquid levels in the reservoirs. The introduction of sheath-flow streams aligns individual cells in sampling channel. The changes of intracellular reducing level in L02 cells were observed by the proposed system before and after treatment. The simple and robust method shows great potential in high-throughput single-cell analysis. Combined

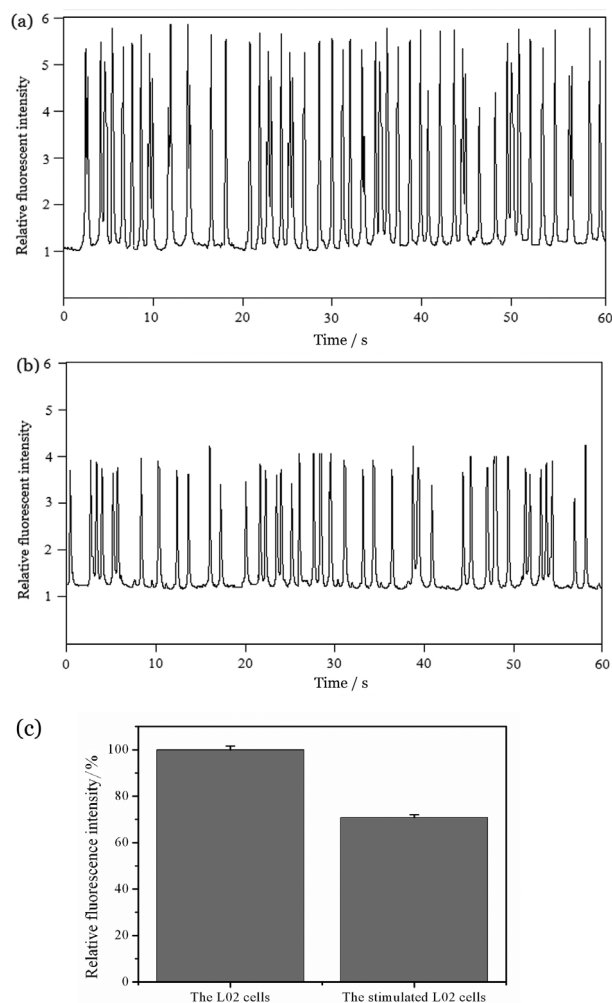


Figure 4. Typical recordings of the fluorescence signal peak profiles of (a) L02 cells without treatment (b) L02 cells with treatment by $100 \mu\text{mol L}^{-1} \text{H}_2\text{O}_2$ for 30 min. Hydrostatic pressure applied on SW is -0.4 mbar . (c) Comparison of the reducing levels of the L02 cells and the stimulated cells. The L02 cells without treatment were used as control. The fluorescent intensity value of the control sample was set as 100%.

with high selective antibody-based stains, it may also be useful for discriminating infected cells from healthy ones to generate a result with statistical significance for clinical diagnosis.

Acknowledgement

This work was supported by the Guangdong Provincial Natural Science Foundation of China (Grant S2011040002246 and S2012040007274) and research start-up fund of Hanshan Normal University (Grants QD20110616 and QD20120521).

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Submitted: September 26, 2012

Published online: March 1, 2013