High-throughput single-cell manipulation system for a large number of target cells

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A sequential and high-throughput single-cell manipulation system for a large volume of cells was developed and the successive manipulation for single cell involving single-cell isolation, individual labeling, and individual rupture was realized in a microhydrodynamic flow channel fabricated by using two-dimensional simple flow channels. This microfluidic system consisted of the successive single-cell handlings of single-cell isolation from a large number of cells in cell suspension, labeling each isolated single cell and the lysate extraction from each labeled single cell. This microfluidic system was composed of main channels, cell-trapping pockets, drain channels, and single-cell content collection channels which were fabricated by polydimethylsiloxane. We demonstrated two kinds of prototypes for sequential single-cell manipulations, one was equipped with 16 single-cell isolation pockets in microchannel and the other was constructed of 512 single-cell isolation pockets. In this study, we demonstrated high-throughput and high-volume single-cell isolation with 512 pocket type device. The total number of isolated single cells in each isolation pocket from the cell suspension at a time was 426 for the cell line of African green monkey kidney, COS-1, and 360 for the rat primary brown preadipocytes, BAT. All isolated cells were stained with fluorescence dye injected into the same microchannel successfully. In addition, the extraction and collection of the cell contents was demonstrated using isolated stained COS-1 cells. The cell contents extracted from each captured cell were individually collected within each collection channel by local hydrodynamic flow. The sequential trapping, labeling, and content extraction with 512 pocket type devices realized high-throughput single-cell manipulations for innovative single-cell handling, feasible staining, and accurate cell rupture. © 2011 American Institute of Physics.

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I. INTRODUCTION

A sequential handling and manipulation of cells and cell suspensions are essential for scientific research and clinical diagnostic applications for cellular function analysis as postgenome researches. Cells generally exist with contacting or neighboring with other cells using conven-

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tional methods with culture dish or microwell. Cells are cultured in large-scale environments such as the dish and analyzed for specific proteins or nucleic acid for the basis of metabolic models. Therefore, it is difficult to analyze individual cells without an influence of other cells. Recently, a difference between individual cells sometimes at the condition of temperature distribution or irregularity was reported.¹

Microfluidic devices, which are mainly the microchannel devices, have been widely applied for the single-cell diagnosis because of the advantages on the cost of measurement, minimal consumption of reagents, and accurate cell handlings by simple microfluidic control.^{2–7} Microfluidic devices also isolate cells from large number of cells in cell suspension by simple fluidic control inside the microchannel for the real-time monitoring. Thus, the microchannel platform offers the systematic understandings of cellular function at the single-cell level because a single cell is ideally isolated from cell suspension, labeled and examined its characteristics or morphology, successively. Actually, researches have been reported such as cell trap,^{8–12} electric cell lysis,^{13–16} chemical cell lysis,¹⁷ dielectrophoretic cell lysis,¹⁸ gene transfer of single cell,¹⁹ cell-by-cell patch clamping,^{20,21} and sequential cell sorting.^{22–24} Toriello *et al.* employed an electric field for single-cell capture inside a channel and successfully isolated single cells and observed their division.²⁵

Since electric stimulation and dielectrophoretic force sometimes influence the behavior of biomolecules, for example, destroying the weak interaction of protein and organelle in the cell,²⁶ hydrodynamic single-cell trapping has been preferred as the application of microfluidic devices in the cell biological researches. In particular, a cell membrane was deformable and generally fragile and delicate, noninvasive and damage-free isolation of single cells was strongly required with hydrodynamic pressure. Several researches of single-cell array using hydrodynamic cell trapping,^{27,28} single-cell assays in a compact disc (CD)-like lab chip²⁹ have been reported. These hydrodynamic methods of isolation cell enabled to capture noninvasively, automatically, and sequentially large number of cells. Single-cell array was expected to obtain enormous quantity of statistical data from single-cell assays such as the drug stimulation and to distinguish the diversity of the individual cell activities.

The significant understanding of single-cell functions was required analyzing the content of individual single cell isolated from cell suspension. The procedures for the on-chip cell content analysis were (1) single-cell isolation from cell suspension for the single-cell array, (2) labeling particular cell content, and (3) single-cell content extraction by cell lysis. In our previous work, all of the sequential single-cell handlings for COS-1 cells were confirmed in the fabricated device which consists of 16 single cell-trapping pockets.³⁰

In this study, we proposed a large-scale of microfluidic device which enables sequential single-cell manipulations of trapping, labeling, and content extraction for large number of cells with both actions taking place in the same pocket. We demonstrated a noninvasive isolation from cell suspension using two types of COS-1 cell in diameter of 20 μm and BAT cells in diameter of 30 μm . The individual cells were isolated, directly labeled by fluorescence dye, ruptured by surfactant, and corrected the cell lysate through the individual lysate correction channel in the same microfluidic device. This microfluidic channel provided various biological analyses, noninvasive bioanalysis, and the morphology of single cells.

II. EXPERIMENTAL METHOD

A. Microchip geometry and fabrication

The structure of microchannel and the principle for sequential single-cell manipulations are illustrated in Fig. 1. The microchannel was constructed to perform four successive biochemical processes which were cell trapping, isolation, labeling, and rupture in the same channel. Cell suspension, fluorescence reagent, and the surfactant were injected through the main channel. The main channel (width: 50 μm) was repeatedly a serpentine channel to trap the cell from the cell suspension and mounted a half circular trapping pocket (20 μm in diameter) to isolate each cell at the top of each winding shape. The trapping pocket prevented the captured cells from being

1. Cell suspension injection

2. Fluorescence dye injection

3. Surfactant injection

FIG. 1. Diagrammatic sketch of the structures of the serpentine microchannel and the sequential process of the biochemical manipulations of trapping, labeling, and content extraction of single cells. (a) A single cell was individually trapped in each trapping pocket by the trapping flow into the drain channel. (b) After cell trapping, a particular cell content was stained with a fluorescence dye injected into the main channel. (c) Finally, the stained cell contents were extracted beside the cell membrane by cell lysis with a surfactant injected into the main channel. The cell contents extracted from each captured cell were individually collected into each collection channel.

washed away by the injection of the reagent into the main channel. And at the top of each winding shape the drain channel (the channel width: $3\ \mu\text{m}$) was connected to correct the lysate of the individual single cell trapped on the pocket. The drain channel was connected to the collection channel (the channel width: $50\ \mu\text{m}$) to collect individually the extracted cell contents of each single cell. All of the microchannels were uniformly $20\ \mu\text{m}$ in depth.

The microchannel device was fabricated from polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning & Toray, Japan) using single-layer soft lithography technique. The mold was formed from SU-8 3025 negative photoresist (Kayaku MicroChem Co., Ltd., Japan). A layer of SU-8 3025 negative photoresist was spin-coated at 3800 rpm on a silicon substrate for $20\ \mu\text{m}$ thick and prebaked at $95\ ^\circ\text{C}$. The coated silicon substrate was exposed to ultraviolet through the mask in hard-contact mode using a mask aligner (MA-6, SUSS Microtec, AG, Germany). After exposure,

the coated silicon substrate was post-exposure-baked at 65 °C for 1 min and 95 °C for 3 min, and developed with SU-8 developer (Kayaku MicroChem Co., Ltd., Japan) and 2-propanol. Finally, the developed silicon substrate was hard-baked at 130 °C for 15 min. Following fabrication of the SU-8 mold, the PDMS microchannel was fabricated using soft lithography with the SU-8 mold. PDMS was prepared according to instructions. PDMS was degassed in a vacuum desiccator for 30 min, poured onto the SU-8 mold surrounded by an acrylic plate, and cured in an oven at 75 °C for 1 h. After curing, the cured PDMS was carefully peeled from the SU-8 mold. The fabricated PDMS structure was placed on a glass slide (Matsunami Glass Ind., Ltd., Japan) to enclose the channels. Both the surfaces of the PDMS structure and the glass slide were treated with oxygen plasma (Aiplasma, Matsushita Electric Works, Ltd., Japan) to form a strong adhesion and bonded.

B. Measurement setup

The measurement was performed with inverted fluorescence microscope (IX71, Olympus, Japan). The microchannel device was placed on the microscope stage and the real-time imaging of consecutive cell behaviors, such as cell trapping, labeling, and content extraction, was taken with a high-sensitive charge-coupled device camera (Olympus DP70, Olympus, Japan) connected to an on-line controller (DP-BSW, Olympus, Japan) on the port in microscope. The injections of cell suspension, medium, fluorescent dye, and surfactant were performed with a disposable plastic microsyringe (Top Surgical Taiwan Co., Taiwan) placed on a syringe pump (KD Scientific, Inc., USA).

C. Cell preparation

COS-1 cells, whose cell line was derived from African green monkey kidney, were purchased from Health Science Research Resources Bank (Health Science Foundation, Osaka, Japan). The COS-1 cells were grown to 80% confluence in Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Biowest, Miami, Florida, USA), 100 units/mL potassium penicillin G, and 0.1 mg/mL streptomycin sulfate at 37 °C in a 5% CO₂ atmosphere. Roughly 4×10^6 COS-1 cells were washed twice with Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) to remove the residual substrate from the culture medium, and then collected via trypsin processing with a 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution.

The rat primary brown preadipocytes (BAT cells) were purchased from Takara Bio, Inc. (Shiga, Japan). The adipocytes were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air in DMEM supplemented with fetal bovine serum, penicillin, streptomycin, pantothenic acid, biotin, ascorbic acid, octanoic acid, and triiodothyronine (Takara Bio, Inc., Otsu, Japan). After incubation for 24 h, the cells were washed with PBS (Life Technologies Japan, Ltd., Tokyo, Japan) and incubated in the same medium. The cells grown to 95% confluence were washed twice with PBS to remove the residual substrate from the culture medium, and then collected via trypsin processing with a 0.25% trypsin-0.02% EDTA solution (CosmoBio Co., Ltd., Tokyo, Japan). Mitotracker Green (Invitrogen, USA) and Hoechst 33342 (Invitrogen, USA) were used for labeling of captured cells. Mitotracker Green selectively stains intracellular mitochondria. 10% sodium dodecyl sulfate (Sigma-Aldrich, USA) was used for cell lysis.

III. RESULTS AND DISCUSSION

A. Optimization by finite element fluidic analysis

In order to realize single-cell mass-trapping, the structure of the main channel and the drain channel dimensions were optimized with simulation by finite element fluidic analysis (COVENTORWARE). Figure 2(a) shows the flow velocity distribution in the microchannel when the water was injected at 1 μ L/min into the left hand side of the inlet. In the serpentine channel, higher flow velocity around the inner arc was generated, which caused nonuniform flow velocity distribution in the main channel, as shown in Fig. 2(a). In contrast, the straight main channel generated the uniform flow velocity distribution in which the fastest flow velocity area was located at the center

FIG. 2. Finite element fluidic analysis for optimizing the structure of the main channel and the drain channel dimensions. (a) Flow velocity distribution in the microchannel when the water of 1 $\mu\text{L/min}$ at flow velocity is injected into the left hand side of the inlet. (b) Microchannel structure for the analysis. (c) Flow velocity distribution of C-C' cross-section in the drain channels of 30 and 100 μm in length. (d) Graph of the flow velocity in D-D' segment.

of the main channel. In the serpentine channel, the nonuniform flows worked to press cells against the outer channel wall, which included the trapping pockets. Therefore, the serpentine channel yielded higher trapping efficiency as compared to the straight channel shape.

We also investigated the optimum cell-trapping structure focused on the length of the drain channel using finite element fluidic analysis [Fig. 2(b)]. Figure 2(c) shows the flow velocity distribution of C-C' cross-section shown in Fig. 2(b) in the drain channels of 30 and 100 μm in length, when the water was injected at 1 $\mu\text{L/min}$ into the left hand side of the inlet. Details of the flow velocity in D-D' segment was shown in Fig. 2(c) and 2(d). Optimal drain channel length for single-cell trapping was required to choose in consideration of the flow velocity in the drain channel. According to Figs. 2(c) and 2(d), when the drain channel length decreases, the flow velocity in the drain channel increases. Therefore, the length of the drain channel can control the flow velocity in the drain channel. Consequently, we investigated the optimal drain channel length by the cell-trapping experiment with drain channel length of 30 and 100 μm .

B. Process sequence for cell manipulation with 16 pocket type

We fabricated and demonstrated two kinds of devices with different main channel shapes (serpentine or straight) and different drain channel lengths (30 or 100 μm) with 16 trapping pockets to investigate the actual functionalities of the serpentine channel shape as well as the optimal drain channel length. Cells used for the trapping experiment were COS-1 cells whose diameter was approximately 20 μm . A concentration of cell suspension was 20×10^4 cells/mL in all devices. Loading cell suspension into the main channel was performed using flow rate of 0.1–1.0 $\mu\text{L/min}$ with the syringe pump.

When cell suspension was injected into the main channel, cells in cell suspension were isolated and immobilized one by one in each trapping pocket. Once the single cell was trapped in the trapping pocket, the trapping flow was dammed by the captured cell, and other cells flowing in the main channel passed over the trapping pocket which has already captured the single cell. The cell-trapping pocket functioned to prevent the trapped cell to slip away into the main channel. The

TABLE I. Experimental results of 16 pocket type devices with different main channel shapes and different drain channel lengths.

Channel structure	Straight channel		Serpentine channel	
	30	100	30	100
Drain channel length (μm)	30	100	30	100
Capture ratio (%)	0	0	12.5	87.5
Operation time (min)	40	40	33	2

FIG. 3. Distribution of flow velocity in a cell-trapping pocket. (Red color denotes faster velocity than the blue one.)

FIG. 4. SEM images of the SU-8 mold of 512 pocket type device. (a) Structure of 512 pocket type device. (b) Magnification of the single cell-trapping microchannel.

capture rate of 100 μm drain channel in straight channel, 30 and 100 μm drain channel in serpentine channel was shown in Table I. The velocity of the vicinity of a trapping pocket was fast at the straight channel which resulted to captured cells that were easy to be released (Fig. 3). Flow velocity on the edge of trapping pocket at the straight channel was 1.5 times faster than that of serpentine channel based on CFD simulation.

The serpentine microchannel with 100 μm drain channel yielded higher capture rate as compared to the straight microchannel with 100 μm drain channel. The capture rate with 16 pockets was defined as the following equation: (total of captured cells)/(total of the trapping pockets) $\times 100$. Thus, the serpentine shape was more appropriate to the single-cell trapping than the straight channel shape. In terms of the length of the drain channel, the serpentine shaped microchannel with the 30 μm drain channel yielded higher capture rate of 87.5% as compared to the serpentine shaped microchannel with 100 μm drain channel. Additionally, this single-cell isolation was completed within only 2 min. From both simulation and experimental results, high efficiency cell trapping was preferred in the serpentine channel shape and the 30 μm drain channel. The optimized microchannel structure can be applied to large-scale flow systems for single-cell mass-trapping.

C. Trapping experiment of single cell with 512 pocket type device

We applied the optimized microchannel structure, which consisted of the serpentine channel and the 30 μm drain channel, to 512 pocket type device. Figure 4 shows the SEM image of the SU-8 mold of the 512 pocket type device. The single cell-trapping experiment with the 512 pocket type device was performed with COS-1 cells. Cell suspension of 20×10^4 cells/mL was injected into the main channel at flow rate of 2.0 $\mu\text{L}/\text{min}$ and the cells were trapping individually with the 512 pockets under the conditions shown in Table II. The capture rate was 83.2%, in which 426 cells were individually trapped in each trapping pocket. The result was larger than that obtained in other researches on individual cell capture. Additionally, we applied the 512 pocket type device for larger size cells, BAT cells, whose diameter was approximately 30 μm . A concentration of cell suspension for injection was 10×10^4 cells/mL, a flow rate of the cell suspension was controlled in the range of 2.0 $\mu\text{L}/\text{min}$. The capture rate of 70.3% was achieved, as shown in Table II.

Figure 5 shows the picture of COS-1 cells and BAT cells captured in the trapping pockets. Both COS-1 cells and BAT cells were trapped individually in each trapping pocket without cell

TABLE II. Results of the single cell-trapping experiments with 512 pocket type device.

Cell type	COS-1	BAT
Average diameter (μm)	20	30
Capture ratio (%)	83.2	70.3

FIG. 5. Optical photomicrographs of COS-1 cells and BAT cells captured in the trapping pockets. (a) COS-1 cells captured in the trapping pockets. (b) Magnification of captured COS-1 cells and BAT cells. Both COS-1 cells and BAT cells were individually trapped in each trapping pocket without cell deformation and intrusion into the drain channel.

deformation and intrusion into the drain channel and nuclei and many mitochondria were observed inside of those cells. Noninvasive single-cell trapping for the large-scale cell array was realized using the fabricated device.

D. Labeling of cell in the trapping pockets

After trapping individual cells, we consecutively preceded the labeling process of the isolated cells with Mitotracker Green for mitochondria and Hoechst 33342 for nuclei in the 512 pocket type microfluidic devices. Mitotracker Green was injected into the main channel at the flow rate of 0.3 $\mu\text{L}/\text{min}$ for 10 min to stain the mitochondria in cell completely. The diameter of the trapping pockets was 20 μm in consideration of the target cell size in order to prevent the unexpected release of the captured cells by sudden pressure change in the main channel. Fluorescence image of the Mitotracker Green stained BAT cells in the trapping pockets was shown in Fig. 6. Also, labeling nuclei in COS-1 cells with Hoechst 33342 was demonstrated. From these results, the

FIG. 6. Fluorescence image of the mitochondrial-stained BAT cells in the trapping pockets. Mitotracker Green was injected into the main channel at the flow rate of $0.3 \mu\text{L}/\text{min}$. Labeling mitochondria in BAT cells without releasing captured cells was performed.

device enables the treatment with the drug injections without releasing the captured cells. This result indicated that the microfluidic device was applicable for the real-time monitoring of the single-cell level response by the drug stimulation.

E. Extraction of cell contents

After the labeling, we performed the extraction and the collection of the stained cell contents. Stained cells were lysed by surfactant injected into the main channel. Phospholipid bilayer of cell membrane was disrupted by the surfactant action, and cell contents are extracted. After labeling, 10% sodium dodecyl sulfate for cell lysis was injected into the main channel at the flow rate of $0.4 \mu\text{L}/\text{min}$. Simultaneously, the solution in the main channel was sucked to each collection channel by a syringe pump in order to collect the extracted cell contents efficiently. Figure 7 shows the time-lapse fluorescence micrographs at cell lysis of the captured COS-1 cell, which was stained nuclei using Hoechst 33342. The stained nuclei inside the cell membrane were extracted inside the trapping pocket and collected into the collection channel by cell lysis. As a result, sequential manipulations of trapping, labeling, and content extraction of single cells were confirmed.

IV. CONCLUSION

Sixteen pocket type device with the serpentine channel and $30 \mu\text{m}$ drain channel length which fabricated based on the finite element fluidic analysis yielded high capture rate of 87.5%. In

FIG. 7. Time-lapse fluorescence micrographs at cell lysis of the captured COS-1 cell, which was stained nuclei with Hoechst. After labeling, 10% sodium dodecyl sulfate was injected into the main channel at the flow rate of $0.4 \mu\text{L}/\text{min}$. Simultaneously, the solution in the main channel was sucked to each collection channel by a microsyringe pump. The stained nuclei inside the cell membrane were extracted inside the trapping pocket by cell lysis, and then the extracted nuclei were perfectly collected into the collection channel.

addition, 512 pocket type device also yielded high capture rate of 83.2% for COS-1 cells. Non-invasive single-cell trapping for the large-scale uniform cell array was achieved with the fabricated device. Captured cells were stained by Mitotracker Green and Hoechst 33342 without releasing the captured cells. The possibility of application to the real-time monitoring of response to the drug stimulation in single-cell array was confirmed. In addition, the extraction and the collection of the stained cell contents after labeling process were performed for COS-1 cells. The sequential single-cell manipulations of trapping, labeling, and content extraction were achieved with 512 pocket type device. In the future, this device will be directly applied for the electrophoresis to quantify the variability of the molecular weight in organelles like mitochondria.

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