Selective cell capture and analysis using shallow antibody-coated microchannels

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Demand for analysis of rare cells such as circulating tumor cells in blood at the single molecule level has recently grown. For this purpose, several cell separation methods based on antibody-coated micropillars have been developed (e.g., Nagrath *et al.*, *Nature* **450**, 1235–1239 (2007)). However, it is difficult to ensure capture of targeted cells by these methods because capture depends on the probability of cell-micropillar collisions. We developed a new structure that actively exploits cellular flexibility for more efficient capture of a small number of cells in a target area. The depth of the sandwiching channel was slightly smaller than the diameter of the cells to ensure contact with the channel wall. For cell selection, we used anti-epithelial cell adhesion molecule antibodies, which specifically bind epithelial cells. First, we demonstrated cell capture with human promyelocytic leukemia (HL-60) cells, which are relatively homogeneous in size; *in situ* single molecule analysis was verified by our rolling circle amplification (RCA) method. Then, we used breast cancer cells (SK-BR-3) in blood, and demonstrated selective capture and cancer marker (HER2) detection by RCA. Cell capture by antibody-coated microchannels was greater than with negative control cells (RPMI-1788 lymphocytes) and non-coated microchannels. This system can be used to analyze small numbers of target cells in large quantities of mixed samples. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4771968>]

INTRODUCTION

Single-cell gene analysis is becoming important to genomics, cell biology, and molecular biology. It is also efficient for personalized medicine or diagnosis of cancer and infectious disease. On the other hand, rolling circle amplification (RCA)¹ is a versatile DNA amplification method that employs a single DNA primer and circularized padlock probes. These probes are oligonucleotides designed to circularize via ligation in the presence of DNA target sequence in a highly specific reaction. The amplified DNA molecules are detected by hybridization with fluorescent oligonucleotide probes and visualization of amplification products as 1- μ m fluorescent spheres that are then counted. The technique can distinguish a single nucleotide difference and provides clear DNA amplification and fluorescent detection signals.^{2–5} Thus, single molecule detection can be achieved with minimal use of nonspecifically bound reagents or media. This method can also be used for *in situ* experiments, allowing single molecule counting in cells.⁶

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However, the method requires large numbers of cells on glass slides. Typically, $\sim 10^4$ cells are used for one analysis. It is difficult to analyze targeted cells in such a large background.

A microchip is a powerful tool that can be used to overcome this problem. Generally, microchips enable the creation of extremely efficient analytical devices with the advantages of micron dimensions, short diffusion distances, low Reynolds numbers, high interface-to-volume ratios, and small heat capacities.^{7–11} Thus, single molecules can be measured in small sample aliquots. We developed a microchip-based RCA system in which primer DNA molecules were patterned on the surface of a microchannel for the analysis of small samples ($\sim$ nl).¹² However, since this system can be used in homogeneous conditions, *In-situ* RCA in a microchip system has not yet been developed.

Using a microchip for a small number of cells presents substantial difficulties in practical applications such as circulating tumor cell (CTC) or cancer stem cell analysis, as these cells are present at 10^{-9} in whole blood. Cell selection is necessary to analyze these extremely rare cells. Fluorescence-activated cell sorting (FACS) is a widely used method that has recently been integrated on microchips.^{13,14} There have also been several reports on cell-selective structures based on antibody-coated micropillars,^{15–18} herringbone structures,¹⁹ or aptamer-coated flat microchannels²⁰ or micropilars.²¹ Although these methods achieve high selective capture rates, it is difficult to securely capture the desired cells in the target area because capture depends on the likelihood of collisions between cells and capturing molecules. This is inconvenient for single molecule analysis such as RCA, which requires high precision and analysis in a narrow area. Here, we propose a new structure that actively exploits cellular flexibility for certain capture of desired cells within a targeted area.

The objective of this study was to develop a new selective cell-capture structure for *in situ* RCA analysis. First, we designed and demonstrated cell capture on the new structure and integrated *in situ* RCA analysis on a glass microchip. Then, we used cancer cells to demonstrate selective capture and cancer marker detection by RCA.

EXPERIMENTAL SECTION

Design and principle

Fig. 1(a) illustrates the principles of microchannel cell capture. A shallow channel with a depth slightly smaller than the cell diameter was prepared. The microchannel wall was coated with cell-capturing antibodies. Cells are certain to touch the shallow channel wall, and so cells are captured in this area. The microchannel is linear with a shallow area in the center. Liquid flow rates are controlled by syringes (Hamilton or Terumo) and microsyringe pumps (KD Scientific). All liquids (protein solution, cell suspension, reagents, and medium) were introduced into the microchannel by this method.

A glass microchip with a shallow channel was fabricated using a 2-step photolithographic wet-etching method.²² The fabrication scheme is briefly summarized, here. A borosilicate glass (Tempax) substrate was coated with Cr, Au, and photoresist layers. The deep microchannel pattern was transferred by irradiating UV light through a photomask. The deep microchannel pattern was etched by HF solution after the development of the resist, Au, and Cr layers. The shallow microchannel pattern was transferred by UV irradiation through a photomask. The deep and shallow microchannel patterns were etched with HF solution. After stripping the resist, Au, and Cr layers, the fabricated substrate was thermally bonded with a cover glass plate. The length, width, and depth (deep area) of the microchannel were 6 cm, 500 μ m, and 100 μ m, respectively. The microchannel depth of the shallow area was fabricated as described in the following experiments. Figs. 1(b) and 1(c) show a fabricated microchip and the microchannel around the shallow channel.

Surface modification

To capture target cells in a shallow channel, the microchannel was modified with cell-capture antibodies. Electrostatic interactions were used to immobilize the antibodies.

FIG. 1. Design and fabrication of a microchip. (a) Principle of selective cell capture using antibodies. Shallow channel depth is designed to be slightly smaller than the cells. Cells are captured in this part. (b) The fabricated microchip (70 mm $\times$ 30 mm). (c) The microchannel around the shallow channel (4 $\times$ magnification).

Surface chemical modification was performed as described previously.^{23,24} Briefly, the microchannel was cleaned with 0.1 M NaOH aqueous solution at room temperature (RT) for 30 min at 5.0 μ l/min, followed by a rinse with deionized water at 5.0 μ l/min for 60 min. After drying the channel, the inner surface was modified. First, 3% (v/v) 3-aminopropyl-triethoxysilane (APTS) (Sigma-Aldrich) in chloroform was introduced by syringe at RT for 2 h, followed by washes with chloroform, ethanol, deionized water, and N,N-dimethylformamide (DMF) with a microsyringe pump at 20.0 μ l/min for 10 min each. Then, vapor-phase APTS (0.5 ml) was used in an oil base at 70 $^{\circ}$ C for 1 h under vacuum, followed by washes with ethanol, deionized water, and DMF with a micro-syringe pump at 5.0 μ l/min for 30 min each. These microchannel surfaces were observed under a microscope to confirm the condition of the surfaces, which were positively charged.

Then, negatively charged antibodies were immobilized on the surface. We used FITC-labeled anti-epithelial cell adhesion molecule (EpCAM, mouse anti-human monoclonal (FITC) antibody, Lifespan Bioscience). EpCAM is an emergent antigen in cancer epithelial cells such as CTCs. Antibody solution was introduced by capillary force into a microchannel at 100 μ g/ml and maintained at RT overnight. Finally, the microchannel was washed with pure water.

RCA protocol

The RCA protocol is illustrated in Fig. 2. *In-situ* RCA methods for HL-60 and SK-BR-3 in a microchannel are described.

Mitochondrial DNA in HL-60 cells was detected as shown in Fig. 2(a). The RCA protocol was established based on previous reports.^{6,25} Cells were suspended in 4% paraformaldehyde in 0.1 M PBS (phosphate-buffered saline) and introduced by syringe pump to capture cells in the

FIG. 2. Scheme of *in situ* RCA on captured cells. (a) Detection of mitochondrial DNA in HL-60 cells. (b) Detection of mRNA (HER2) in SK-BR-3 cells.

shallow channel at the defined flow rate. After 30 min at RT, the microchannel was washed with 0.1 M PBS at 1.0 $\mu\text{l}/\text{min}$ for 10 min. Then, 0.1 M HCl was introduced for 2 min at RT to dissolve the cell membrane, and the microchannel was washed with 0.1 M PBS at 1.0 $\mu\text{l}/\text{min}$ for 10 min. Next, 0.5 U/ μl restriction enzyme *MscI* (New England Biolabs Inc.), 0.5 U/ μl exonuclease T7 (New England Biolabs Inc.), in 1 $\times$ NEB4 buffer (New England Biolabs Inc.) supplemented with 0.2 $\mu\text{g}/\mu\text{l}$ bovine serum albumin (BSA, Sigma Aldrich) was introduced at 1.0 $\mu\text{l}/\text{min}$ for 10 min, then allowed to react for 40 min at 37 $^{\circ}\text{C}$ after the flow was stopped. The microchannel was washed with washing buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20, the same hereinafter) at 1.0 $\mu\text{l}/\text{min}$ for 10 min. Then, padlock probes were hybridized and ligated. T4 DNA ligase (0.1 U/ μl ; New England Biolabs Inc.), 1 $\times$ ligase buffer, 0.2 $\mu\text{g}/\mu\text{l}$ BSA, 0.1 μM padlock probe (5'-P-CTGCCATCTTAACAATTCCTTTTACGACCTCAATGCTGCTGCTGTACTACTCTTCTGCGATTACCGGGCT-3', Sigma Genosys), 1 mM ATP, and 0.25 M NaCl were introduced at 1.0 $\mu\text{l}/\text{min}$ for 10 min, then allowed to react for 40 min at 37 $^{\circ}\text{C}$ after the flow was stopped. The microchannel was washed with washing buffer at 1.0 $\mu\text{l}/\text{min}$ for 10 min. Then, the RCA reaction was performed with a solution of 10 U/ μl Φ 29 polymerase (New England Biolabs, Inc.), 1 $\times$ Φ 29 buffer, 250 μM dNTPs (Takara Bio Inc.), 0.2 $\mu\text{g}/\mu\text{l}$ BSA, and 5 wt. % glycerin at 1.0 $\mu\text{l}/\text{min}$ for 10 min, incubated for 90 min at 30 $^{\circ}\text{C}$ after the flow was stopped. The microchannel was washed with washing buffer at 1.0 $\mu\text{l}/\text{min}$ for 10 min. Then, fluorescent probes were hybridized. A solution of 2 $\times$ SSC, 20% formaldehyde,

and 0.25 μM fluorescent probe (5'-Cy3-CCTCAATGCTGCTGCTGTACTAC-3', Sigma Genosys) was introduced at 1.0 $\mu\text{l}/\text{min}$ for 10 min, then allowed to react for 20 min at 37 °C after the flow was stopped. The microchannel was washed with washing buffer at 1.0 $\mu\text{l}/\text{min}$ for 10 min. Finally, nuclei were stained with 2 $\mu\text{g}/\text{ml}$ DAPI (4,6-diamidino-2-phenylindole) introduced at 1.0 $\mu\text{l}/\text{min}$ for 10 min at 37 °C. The microchannel was washed with 1 $\times$ PBS and ethanol at 1.0 $\mu\text{l}/\text{min}$ for 10 min each.

In SK-BR-3, mRNA for the HER2 cancer marker was detected as shown in Fig. 2(b). Methods on glass slides were previously described in detail²⁶ and are briefly summarized here. First, 4% paraformaldehyde (Sigma) in PBS was used to fix the cells in the microchannel at 1.0 $\mu\text{l}/\text{min}$ for 15 min, then allowed to react for 30 min at RT after the flow was stopped. The microchannel was washed with diethylpyrocarbonate (DEPC) in PBS at 1.0 $\mu\text{l}/\text{min}$ for 10 min. The cells were dehydrated with 70%, 85%, and 99% ethanol at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT. The cells were permeabilized by 0.1 M HCl at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT. After 10 min, the cells were washed with DEPC-PBS at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT.

M-MuLV reaction buffer was introduced at 1.0 $\mu\text{l}/\text{min}$ at RT for 15 min. Then, 1 μM of cDNA primer (P-HER2: 5'-G+AG+CT+GG+GT+GC+CT+CGCACAATCCGCAGCCT-3', + symbol denotes LNA bases, Integrated DNA Technologies) with 20 U/ml Revert Aid H minus M-MuLV reverse transcriptase (Fermentas), 500 μM dNTP (Fermentas), 0.2 $\mu\text{g}/\mu\text{l}$ BSA (Sigma), and 1 U/ μl RiboLock RNase Inhibitor (Fermentas) in M-MuLV reaction buffer was introduced at 1.0 $\mu\text{l}/\text{min}$, then allowed to react for 3 h at 37 °C after the flow was stopped. Then, the cells were rinsed with PBS-T (DEPC-PBS with 0.05% Tween 20, Sigma) at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT and 4% paraformaldehyde in PBS was introduced at 1.0 $\mu\text{l}/\text{min}$ for 15 min, then allowed to react for 30 min at RT for post fixation after the flow was stopped. The microchannel was washed with PBS-T at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT.

Ampligase buffer (20 mM Tris-HCl, pH 8.3, 25 mM KCl, 10 mM MgCl_2 , 0.5 mM NAD, and 0.01% Triton X-100) was introduced at 1.0 $\mu\text{l}/\text{min}$ for 10 min at RT. Ligation was then performed with 100 nM padlock probe (PLP-HER2: 5'-TGCCAGCCTGTCCTTCCTGCATCG TCTTAATCACTAGTCGGAAGTACTACTCTCTTACGCTTACAAGTACTGCTCACCTACCTGC CCACCAA-3', Biomers) solution in a mix of 0.5 U/ μl Ampligase (Epicentre), 0.4 U/ML RNase H (Fermentas), 1 U/ μl RiboLock RNase Inhibitor, Ampligase buffer, 50 mM KCl, and 20% formamide was introduced at 1.0 $\mu\text{l}/\text{min}$, then allowed to react for 30 min at 37 °C after the flow was stopped, followed by 45 min at 45 °C with no flow. The microchannel was washed with DEPC-treated 2 $\times$ vSSC with 0.05% Tween 20 at 1.0 $\mu\text{l}/\text{min}$ for 15 min at 37 °C and rinsed with PBS-T at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT.

The cells were preincubated in 1 $\times$ Φ 29 DNA polymerase buffer (Ferments) at 1.0 $\mu\text{l}/\text{min}$ for 15 min at RT. Then, RCA was performed with 1 U/ μl 1 $\times$ Φ 29 DNA polymerase (Ferments) in the supplied reaction buffer, 1 U/ μl RiboLock RNase Inhibitor, 250 μM dNTPs, 0.2 $\mu\text{g}/\mu\text{l}$ BSA, and 5% glycerin at 1.0 $\mu\text{l}/\text{min}$ for 15 min, then incubated for 60 min at 30 °C after the flow was stopped. The microchannel was washed with PBS-T at 1.0 $\mu\text{l}/\text{min}$ for 15 min. Fluorescent probe hybridization was performed with 100 nM fluorescent probe (DP-5: 5'-Cy5-AGTCG-GAAGTACTACTCTCT-3', Eurogentec) in 2 $\times$ SSC, 20% formamide at 1.0 $\mu\text{l}/\text{min}$ for 15 min, then incubated for 20 min at 37 °C with no flow, followed by a PBS-T wash at 1.0 $\mu\text{l}/\text{min}$ for 15 min. Finally, nuclear staining was performed with 2 $\mu\text{g}/\text{ml}$ DAPI at 1.0 $\mu\text{l}/\text{min}$ for 15 min. The microchannel was washed with 1 $\times$ PBS and ethanol at 1.0 $\mu\text{l}/\text{min}$ for 10 min each.

RESULTS AND DISCUSSION

Capturing test

Before using target cells for rare cell analysis, we used leukemia cell line HL-60 to investigate shallow channel depth versus cell diameter and to demonstrate integration of RCA analysis. We chose HL-60 because it is nearly spherical and its size is relatively homogeneous ($12.4 \pm 1.2 \mu\text{m}$).²⁷ We also demonstrated RCA in this new cell capture structure using HL-60. We selected mitochondrial DNA targets because they are present in high quantities and are easily detected.

HL-60 cells (lymphoblast-like, provided by Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan) were cultured in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (FBS). We investigated the capture rate of various channel depths and flow rates.

Capture rate was calculated by dividing the captured cell number in the shallow channel by the total number of cells introduced (calculated by adding number of pass-through cells to the number of captured cells). The microchannel was not coated in this experiment.

Shallow channel depths were 6, 8, and 10 μm , slightly smaller than the cell diameter. Fig. 3(a) shows a shallow channel and captured cells at an 8- μm channel depth. Cells were captured without breaking. Fig. 3(b) shows the capture of HL-60 cells versus channel depth. At 6 μm , cells were completely collapsed due to stress in the shallow channel. On the other hand, at 10 μm , most of cells were not captured. Although this is a very rough measurement, we found the structure should be smaller than the cell diameter but larger than the half-diameter.

RCA of HL-60

After capturing the HL-60 cells, mitochondrial DNA was detected by RCA. The image was acquired by a fluorescence microscope (IX71, Olympus) through a cooled CCD camera (Hamamatsu Photonics) using a 20 $\times$ objective lens. To observe nuclei, the excitation and emission

FIG. 3. HL-60 cell capture and RCA. (a) Captured HL-60 cells in a shallow channel. (b) Capture rate versus flow velocity in a shallow channel at channel depths of 8 μm and 10 μm .

wavelengths were 350 nm and 465 nm, respectively (using a DAPI filter set). For RCA product observation, the excitation and emission wavelengths were 606 nm and 626 nm (using a Texas red filter set). The acquired images were analyzed by AQUACOSMOS software (Hamamatsu Photonics).

In this experiment, it is difficult to know the exact number of targets (mitochondrial DNA). Therefore, we reduced the padlock probe concentration below the target concentration and varied it across a range to confirm the RCA process. The results are shown in Fig. 4. We clearly observed red dots in HL-60 cells. Increasing the concentration of padlock probes increased the number of dots per cell. Thus, we conclude RCA can be performed in captured cells on a microchip.

Surface modification result

We performed fluorescence microscopy to confirm cell-capturing antibody anti-EpCAM was immobilized on the surface of the microchannel. For observation, the excitation and emission wavelengths were 492 nm and 535 nm, respectively (using a FITC filter set). Fig. 5 shows the fluorescence images of the antibody-immobilized surface and non-immobilized surface (negative control) after washing the channel. The difference was apparent. Thus, immobilization of anti-EpCAM was verified.

Selective cell capture

We investigated the target cell capture rate with SK-BR-3 (breast cancer cells) (DS Pharma Biomedical) as a model for CTCs. To model non-target cells, we used RPMI-1788 (lymphocytes) (DS Pharma Biomedical). We prepared 3 conditions (A–C) to demonstrate selective

FIG. 4. RCA detection of mitochondrial DNA in captured HL-60 cells. (a) Bright field image. (b) Fluorescence image. (c) Dots per cell versus padlock probe concentration. Values are represented as mean $\pm$ SD; $n = 50$.

FIG. 5. Fluorescence images of a microchannel with and without antibody coating. (a) An antibody-coated microchannel. (b) A non-coated microchannel.

capture of SK-BR-3 as shown in Fig. 6(a). In each condition, a cell suspension was introduced by a syringe pump. Captured and non-captured cells were counted under a microscope, and the flow was stopped when the total number of introduced cells reached around 100. Flow velocity in the shallow channel was varied at 0.2, 0.5, 1.0, and 2.0 mm/s.

Results are shown in Fig. 6(b), which illustrates a greater capture rate in condition C than in conditions A and B. Even at high flow velocity (2 mm/s), at which no cells were captured in condition B, 20% of cells were captured in condition C. This fact indicates that target cells can be selectively captured by immobilized antibodies. Fig. 6(c) shows cells captured in a 1 mm × 1 mm square shallow channel area. Therefore, the detection area could be limited to a relatively small area. This is significant for analysis of rare cells such as CTCs at the single molecule level, because it is very time-consuming and difficult to detect cells across larger areas.

However, the maximum capture rate was about 60% and the rate of non-targeted cell capture was about 30%. This rate is relatively lower than the capture rate of a micropillar array, which is near 90%.¹⁸ One of the possible reasons for the discrepancy is that SK-BR-3 cell size is not homogeneous; therefore, relatively small cells passed through the shallow channel. To prevent this, a combination of cell separation methods before capture may be a powerful way to enhance target cell capture in the shallow channel structure. There have been a number of pre-treatment to enrich target cells such as biotin-streptavidin interaction based enrichment,²⁸ antibody coated bead-based enrichment,²⁹ affinity based separation,³⁰ magnetic separation,³¹ or size based separation using ultra sound.^{32–34} In these options, ultra sound based separation will be the most suitable method for our analysis because that does not require any labels which may influence RCA analysis. After rough separation using this method, our dual size/affinity separation system will have much better capture rate.

RCA of SK-BR-3

In condition C, 2 mm/s, we demonstrated RCA detection of HER2 mRNA in SK-BR-3 cells. It is possible to perform direct amplification with a RNA primer,³⁵ but RNA is relatively unstable due to the hydroxyl group at the 2' position. Therefore, reverse transcription is better. After SK-BR-3 cell capture and RCA reaction in the shallow channel, we observed the RCA products by a fluorescence microscopy as described for HL-60.

Fig. 7 shows the captured images, based on which we verified the presence of several red spots representing HER2 signal. Signal number was lower than in previous experiments on glass slides,²⁶ possibly because the RCA protocols in microchannels are less than optimal. Four of the spots seem to be RCA products (arrowed ones), but it is difficult to draw conclusions regarding the very strong one. This can be an aggregate of signals or just an aggregate of fluorophores from the oligonucleotide stock. This result demonstrated RCA reaction and detection using captured epithelial cells.

FIG. 6. Cell capture in an antibody-coated shallow microchannel. (a) Cell selectivity under various conditions. Condition A included SK-BR-3 cells and a non-coated microchannel. Condition B included RPMI-1788 cells and an antibody-coated microchannel. Condition C included SK-BR-3 cells and an antibody-coated microchannel. In all conditions, channel depth was $16\text{ }\mu\text{m}$. The diameter of SK-BR-3 cells and RPMI-1788 cells is $12\text{--}24\text{ }\mu\text{m}$ and $16\text{ }\mu\text{m}$, respectively. (b) Capture rate versus flow velocity in each condition. (c) Captured SK-BR-3 cells in condition C.

FIG. 7. RCA detection of HER2 in captured SK-BR-3 cells. (a) Bright field. (b) A fluorescence image.

CONCLUSIONS

We have demonstrated a new selective cell-capture method using a sandwich structure on a microchannel coated with cell-capturing antibodies. First, we demonstrated cell capture and *in situ* single molecule analysis (RCA) with HL-60 cells, which are relatively homogeneous in size. Then, we used SK-BR-3 cells as a cancer cell model in blood, and demonstrated selective capture and cancer marker detection by RCA. Results showed apparent increases in captured cell number in comparison to negative control cells (RPMI-1788) and non-coated microchannels. Thus, target cells could be selectively captured and analyzed with this new structure.

This system allows us to select target cells from large samples for single molecule analysis. After improving and optimizing the structure and pre-treatment, there will be applications for the device in the medical and biological field for single cell and single molecule analysis.

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