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LABEL-FREE CONTINUOUS MICRO CELL SORTER WITH ANTIBODY-IMMOBILIZED OBLIQUE GROOVES

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ABSTRACT

We report development of a novel label-free continuous cell separation method for the extraction of rare cells from a limited amount of sample. The separation method is based on specific adhesion between target cells and antibody immobilized on oblique micro grooves etched into the channel wall. Due to asymmetric adhesive force, cross-flow displacement of the target cells is induced. Label-free lateral separation of micro beads as the cell model has been successfully achieved. Cross-flow displacement of human umbilical-vein endothelial cells (HUVEC) with the CD31 antibody is also demonstrated.

INTRODUCTION

Stem cell therapy is a rapidly evolving biomedical technology, in which multipotent stem cells are cultured in vitro and transplanted to regenerate damaged or deficit tissue. Since use of embryonic stem cells often encounters ethical issues, extraction of adult stem cells such as mesenchymal stem cell (MSC) from terminal blood attracts much attention. However, stem cells are very rare and difficult to identify using their physical properties. Thus, development of an efficient and accurate cell separation method of rare cells from cell mixture is necessary.

Cell separation methods often employ specific binding between cell surface antigen and its corresponding antibody. For example, antibody-conjugated fluorescence molecule and magnetic beads are respectively used in fluorescence activated cell sorting [1-3] and magnetic cell sorting [4-6] for labeling target cells. However, labeling cell is problematic for regenerative medicine because of unexpected effect of the labels in cultivation and transplantation.

In the present study, we propose a continuous label-free cell separation method, where the target cells are separated in the cross-flow direction using the asymmetric antibody pattern on the channel surface.

DESIGN

Miwa et al. [7] has proposed a label-free cell separation method using specific adhesion between target cells and antibody immobilized on the wall. Separation of cell mixture can be accomplished by simply flowing the sample plug through an antibody-immobilized micro channel without any pre-/post-processing. In their device, the target cells rolling on the channel wall are decelerated and separated from the other cells in the direction of main flow. They successfully separate a mixture of human umbilical-vein endothelial cells (HUVEC) and human leukocytes (HL60). However, its throughput remains low, since it is batch process.

Figure 1 shows the schematic of the present cell separation method. At the entrance of the separation channel, cell suspension is hydrodynamically focused to one side of the channel using buffer fluid. The channel wall has oblique grooves with immobilized antibody, which work as adhesive/non-adhesive stripes. When the cells roll on the surface, the target cells are displaced in the cross-flow direction by the asymmetric adhesive force, and collected from the outlet on the other side.

In the present study, it is required that the cells roll along the antibody-immobilized channel wall. Thus, the channel depth is chosen as 40 μm, which is approximately twice the diameter of a typical monocyte. Channel width is 200μm. The oblique grooves 0.2 μm in depth 1 μm in width are etched into the channel surface as the non-adhesive oblique patterns; when the cells located above a groove, the cell surface is displaced from the antibody on the bottom of the groove. The pitch between the grooves is 2 μm.

MICROFABRICATION

Figure 2 shows the fabrication process of the present micro cell separator. Firstly, micro channel structures are etched into a silicon substrate using DRIE with an oxide mask and fluidic ports are ultrasonically drilled (Fig. 2a). The silicon substrate is coated with 2 μm-thick parylene-C (Fig. 2c). Oblique grooves are patterned with EB lithography and etched with CHF_3 plasma into a Pyrex substrate (Fig. 2b). Then, anino-functionalized parylene diX-AM (Kisco) 0.1 μm in thickness is deposited on the Pyrex substrate to form dense amino group on its surface [7]. This is followed by parylene-parylene thermal bonding [8] between silicon and Pyrex substrate in vacuum for an hour (Fig. 2d). The bonding pressure and temperature are respectively 5 MPa and 200˚C. Finally, inlet and outlet ports made of PDMS blocks are firmly

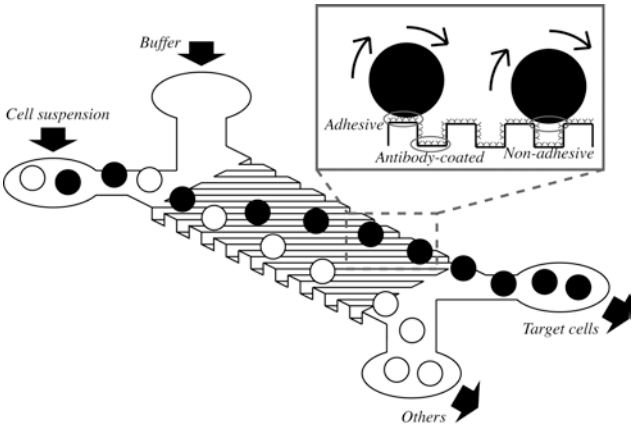

Figure 1: Cell separation principle using oblique antibody patterns.

bound to the silicon substrate after oxygen plasma treatment (Fig. 2e).

Figure 3 shows a photo of the present device and a SEM image of the oblique grooves. The oblique pattern locates in the straight part of the channel. Total number of the grooves is 10,000. Sample cell suspension is focused in the center of the channel by buffer fluid to avoid unwanted effect of velocity gradient near the sidewall.

Biomolecule immobilization on the amino group of diX-AM (Fig. 4) is described in Miwa et al [7]. It is performed by successive introduction and incubation of biomolecule solutions with a syringe pump; the diX-AM surface is firstly biotinylated by conjugating NHS-LC-LC-biotin to the surface amines. NHS-LC-LC-biotin is successively dissolved into dimethylsulfoxide and bicine buffered saline (pH 8). The biotin solution is then introduced into the device, and incubated for one hour at 30 ˚C. Streptavidin and biotin-conjugated CD31 antibody solutions, are separately dissolved into PBS (pH 7.4), and successively incubated. Although CD31 is used in the present study, various kinds of proteins and other biomolecules can be immobilized using the present procedure.

EXPERIMENTAL RESULTS

As proof-of-concept, we employ polystyrene micro beads in order to examine performance of the present device. Streptavidin-functionalized surface, which specifically adhere the biotin-coated beads, is formed by conjugation of NHS-LC-LC-biotin and streptavidin on the diX-AM surface. Biotin-coated Yellow and streptavidin-coated Nile Red fluorescence beads (Spherotec) are used respectively as the target and non-target model cells. The diameter of both beads is 7.66±0.33 μm. Under a fluorescent microscope, the beads type can be distinguished by using different filter set. The beads are suspended in PBS (pH 7.4) at the number density of 4×10^6 beads/mL. Because the only difference between the two kinds of beads is the surface chemistry, any difference in the motion of the beads should be attributed to the difference in the adhesive force with the surface antibody.

We characterize the beads motion on the oblique grooves through microscopic measurements. The focal plane of the microscope is set at the bottom wall of the micro channel, and beads images are taken at 25 fps. The beads velocity is calculated from the displacement of the beads center between successive images. The number of beads analyzed is 30 for each beads type.

The flow ratio of the beads mixture and PBS buffer fluid is set 1:1 to focus the beads within 100 μm of the center of the channel, where the flow velocity is almost uniform. The angle between oblique grooves and the main flow is 45˚. Figure 5 shows superimposed sequential images of a biotin-coated bead flowing in the micro channel. The cross flow position gradually shifts to the lower side of the image, which shows the oblique antibody pattern does induce cross-flow adhesive force.

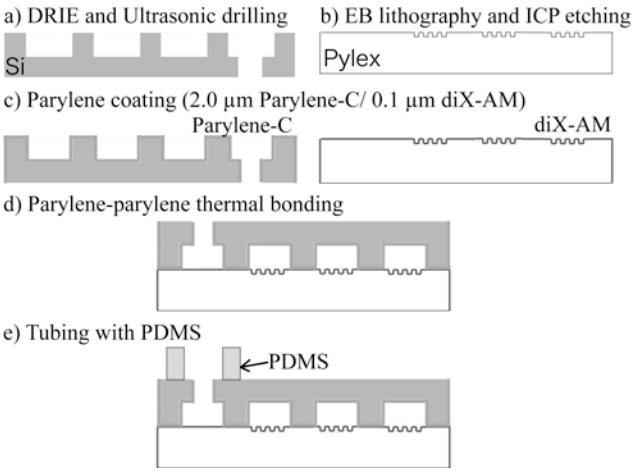

Figure 2: Microfabrication process.

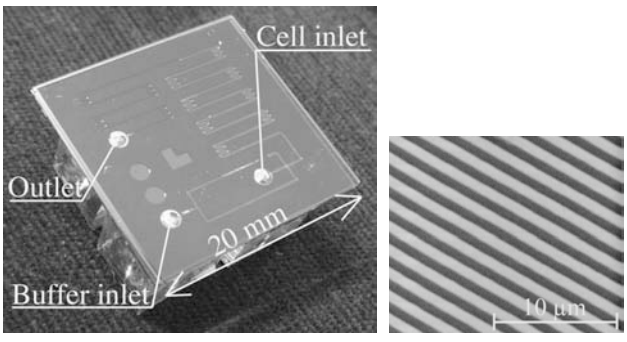

Figure 3: (a) Photo of the present device, (b) SEM image of the oblique grooves.

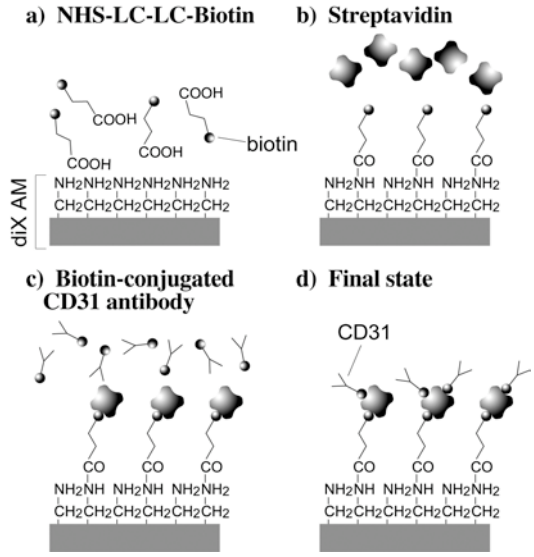

Figure 4: Schematic of the CD31 antibody immobilization process on diX-AM surface [7].

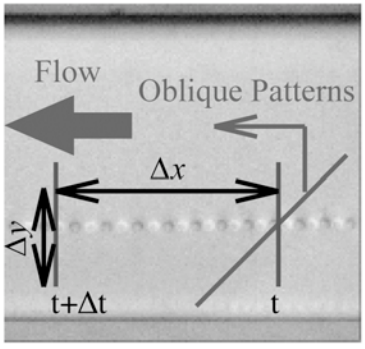

Figure 5: Microscopic measurement of micro beads velocity and displacement.

Figure 6 shows the streamwise velocity of beads u at by the bulk mean velocity u_b of 0.5 mm/s. All the beads are almost uniformly distributed in the cross-flow direction within the middle half of the channel. While the velocity of the streptavidin-coated beads is somewhat larger than u_b, the biotin-coated beads travels at about 0.4 u_b, showing effective deceleration due to the biotin-streptavidin interaction.

Figure 7 shows the cross-flow displacement Δy versus the beads traveling speed u. The biotin-coated beads are displaced in the cross-flow direction, and the mean value is 0.8% of the traveling length Δx. On the other hand, the cross-flow displacement of streptavidin-coated beads is less than 0.2%, and scattered around Δy = 0. Therefore, the adhesive force on the oblique patterns should be responsible for the cross-flow displacement, and the effect of the secondary flow or flow inside the grooves is negligible.

Figure 8 shows the mean cross-flow displacement for different bulk mean velocities. The mean cross-flow displacement is about 0.8% for all the cases examined, and independent of the flow velocity. This result indicates that the cell separation using the oblique antibody pattern can be accomplished in a wide range of flow velocity.

The beads separation is demonstrated using a beads mixture. The separation performance is evaluated by counting the number of beads passing the measurement volume. The length of patterned area is 20 mm (10,000 grooves). The biotin- and streptavidin-coated beads are mixed at 1:1 with 2 x 10^4 beads/mL total concentration. The flow ratio of beads mixture and the PBS buffer fluid is set 1:2.5 to focus the beads within 50 μm of center of the channel. The bulk mean velocity is 1.0 mm/s.

Figure 9 shows histograms of cross-flow position of the beads. The sample beads are successively separated into each type of the beads after 10,000 oblique grooves, showing the effectiveness of present cell separation method.

Finally, we employ human umbilical vein endothelial cells (HUVEC) to evaluate performance of the present device for actual cells. HUVEC has mean diameter of 20 μm and nearly 100% expression of CD31 (Fig. 10).

The cells are stained with SYTO24 and suspended in

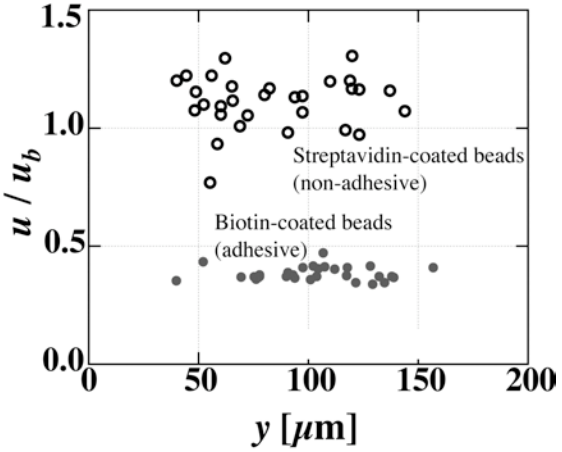

Figure 6: Streamwise velocities of individual beads at u_b = 0.5 mm/s.

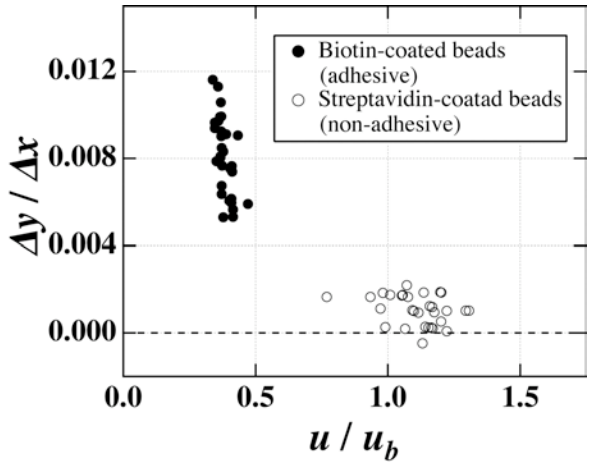

Figure 7: The cross-flow displacement versus the beads velocity at u_b = 0.5 mm/s.

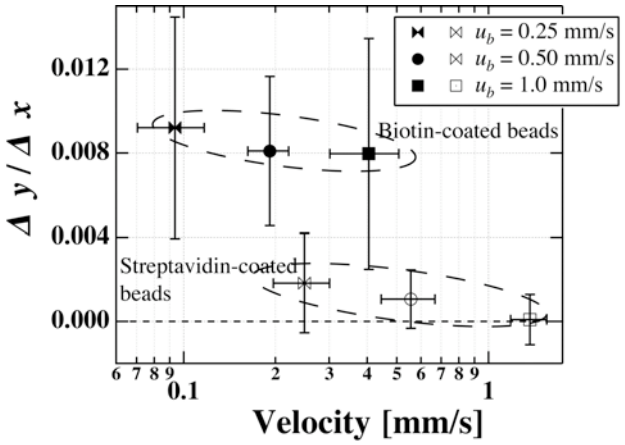

Figure 8: Dependence of the mean cross-flow displacement on the flow velocity. The error bars correspond to twice the standard deviation for n = 30.

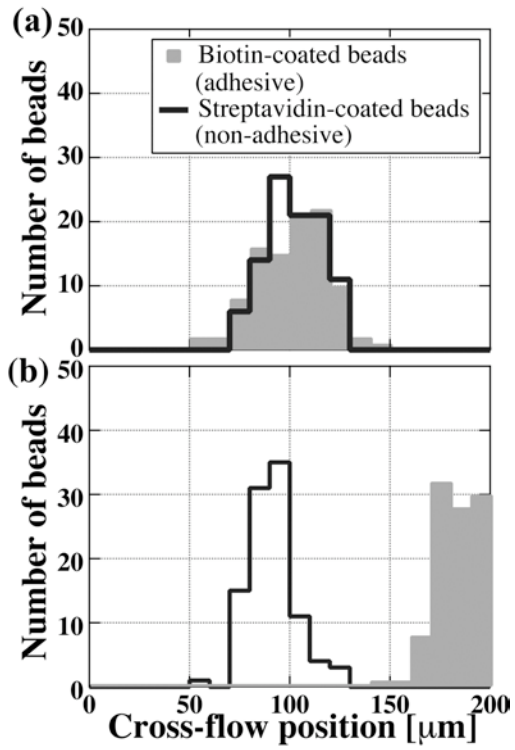

Figure 9: Histograms of the cross-flow position of the beads at u_b = 1.0 mm/s. (a) At the inlet, (b) After passing through 10,000 oblique streptavidin patterns.

PBS (pH 7.4) at the number density of 2 x 10^6 cells/mL, which corresponds to the volume concentration of approximately 0.25 %. The sample cell suspension is continuously introduced with a syringe pump. The bulk mean velocity is 1.0 mm/s. The flow ratio of beads mixture and the PBS buffer fluid is set 1:2.5.

Figure 9 shows histograms of the cross-flow positions of HUVEC before and after passing the CD31 oblique patterns. The histogram downstream the patterns is shifted in the cross-flow direction, but their displacement is smaller than the beads. Longer channel should be necessary for complete separation of HUVEC, partially due to small deceleration of the cells. Figure 12 shows HUVEC velocity near the outlet. Based on Miwa et al. [7], HUVEC are expected to decelerate to 0.6 u_b in antibody immobilized micro channel. However, many cells travel faster than 0.6 u_b in the present device.

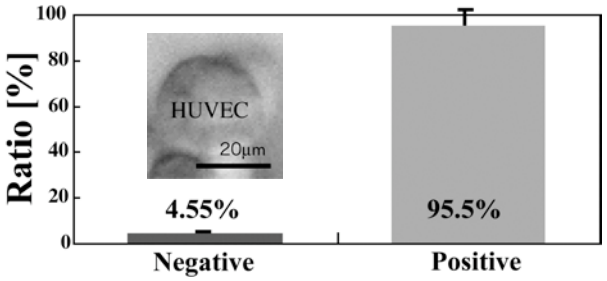

Figure 10: CD31 expression of HUVEC [5].

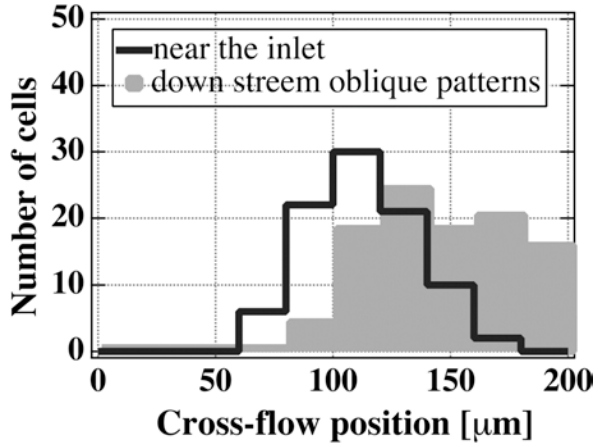

Figure 11: Histograms of cross-flow positions of HUVEC at u_b = 1.0 mm/s. (a) Near the inlet, (b) After passing through 10,000 oblique CD31 patterns.

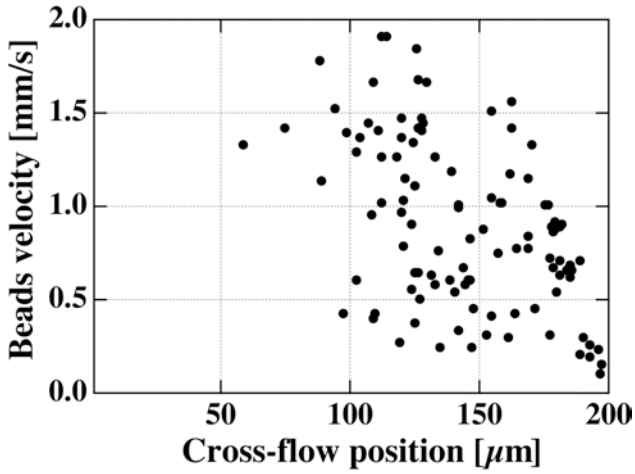

Figure 12: Traveling velocity of HUVEC versus the cross-flow position near the outlet.

CONCLUSIONS

A novel label-free continuous cell separation method has been developed for accurate extraction of rare cells from a limited amount of sample. The separation method is based on specific adhesion between target cells and oblique antibody patterns, which are realized through immobilization of antibodies on oblique micro grooves etched into the channel wall. It is found that, on the streptavidin-coated surface, biotin-coated beads are decelerated by 60 % due to the specific adhesion. The adhesion force induces cross-flow motion of the beads, and the cross-flow displacement of about 0.8 % of the beads' traveling distance is achieved. Complete separation of beads mixture in a 20 mm-long micro channel is also demonstrated. When HUVEC is employed as the target cell with the CD31 antibody, cross-flow distribution of HUVEC is also shifted in the cross-flow direction.

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