Novel platform for minimizing cell loss on separation process: Droplet-based magnetically activated cell separator

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To reduce the problem of cell loss due to adhesion, one of the basic phenomena in microchannel, we proposed the droplet-based magnetically activated cell separator (DMACS). Based on the platform of the DMACS—which consists of permanent magnets, a coverslip with a circle-shaped boundary, and an injection tube—we could collect magnetically (CD45)-labeled (positive) cells with high purity and minimize cell loss due to adhesion. To compare separation efficiency between the MACS and the DMACS, the total number of cells before and after separation with both the separators was counted by flow cytometry. We could find that the number (3241/59 940) of cells lost in the DMACS is much less than that (22 360/59 940) in the MACS while the efficiency of cell separation in the DMACS (96.07%) is almost the same as that in the MACS (96.72%). Practically, with fluorescent images, it was visually confirmed that the statistical data are reliable. From the viability test by using Hoechst 33 342, it was also demonstrated that there was no cell damage on a gas-liquid interface. Conclusively, DMACS will be a powerful tool to separate rare cells and applicable as a separator, key component of lab-on-a-chip. © *2007 American Institute of Physics.*
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Manipulation and separation of bioparticles such as cell organelles or whole living cells have become the center of attention in the biomedical or clinical research fields as a key function to realize the micrototal analysis systems (μ-TASs). Recent separation technologies combined with the microfluidics can be summarized as follows: miniaturized fluorescence activated cell separation (FACS),[1–4] dielectrophoresis (DEP),[5–8] massively parallel microfabricated sieving device,[9] miniaturized magnetically activated cell separation (MACS),[10–15] and additional manipulations including optics[16,17] and acoustics.[18] Among them, FACS and MACS are, in particular, practically being used.

In the beginning, the separation for cell-based diagnostics or analysis has been remarkably impacted by the FACS system, which has the characteristics such as high performance in cell purity and recovery of rare cells.[19] Although the FACS system has been steadily being used, it requires relatively high cost, difficult sterilization, and need for a large sample volume.[20] To solve some of the drawbacks, the miniaturized FACS systems based on the microelectromechanical system (MEMS) technologies were proposed.[1–4] However, integration of the functions such as optical sensing or sorting gave rise to increasing complexity of the device.[21]

Contrary to the miniaturized FACS systems, there is the other trend based on the DEP technologies, which have in-

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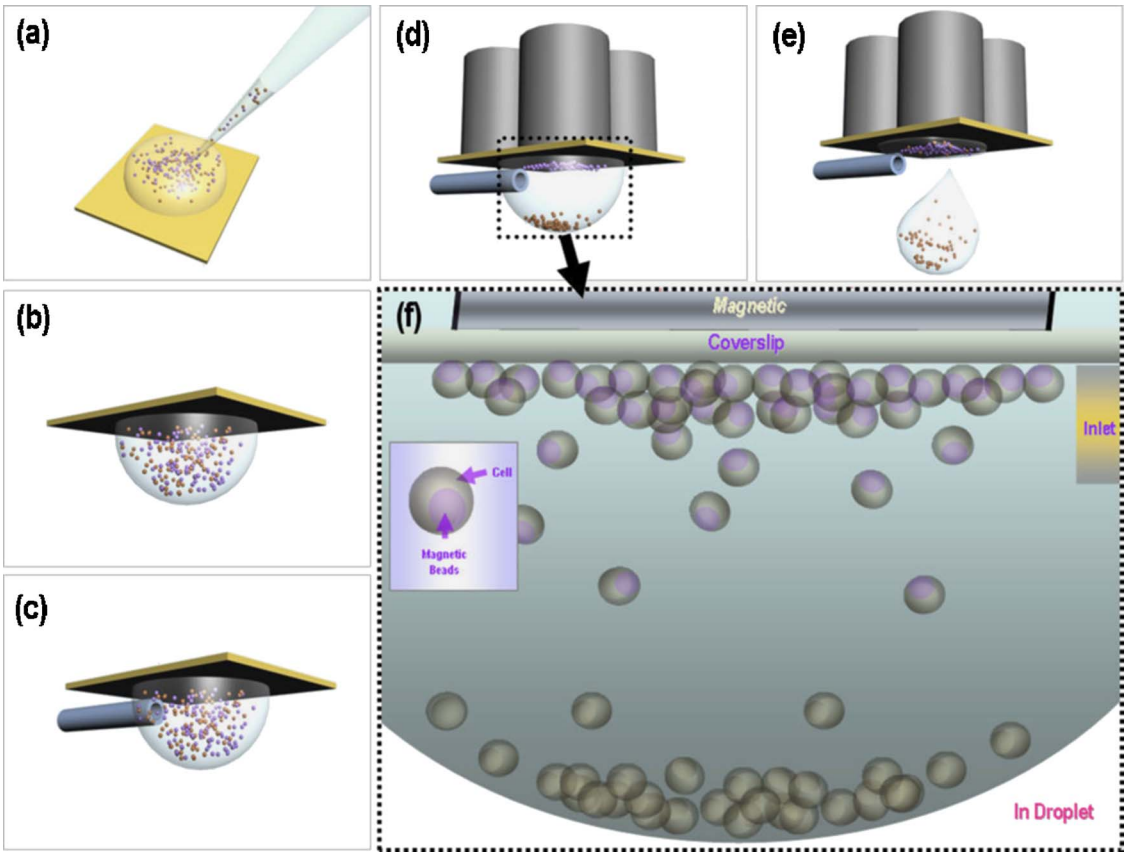

FIG. 1. (Color online) Schematic illustration for separation process. (a) Loading cell suspension. (b) Turning over the coverslip. (c) Locating an inlet to inject a buffer solution and (d) a magnet. (e) Detaching the bottom of a droplet due to buffer injection. (f) Detailed cell arrangement in droplet of (d).

duced many researchers to develop chip applications probably owing to the relatively facile engineering of the electric fields and interface to integrated chip.[3] However, the problems to be solved such as cell viability due to electropermeabilization[22] or cell loss due to adhesion[23] have been reported. Although the recent study of DEP-trapped neural cells did not show any morphological cell damages,[24] it was also found that generation of an electric field might result in local heating, which led to fluid flow that whirls the cells round.[25] Practically, Urban *et al.*[26] showed that electric fields in conducting media induce Joule heating related to the risk of thermal cell damage.

To avoid the cell damages induced by the electric fields, the size-dependent separation based on hydrodynamic force[21] has also been contrived. However, it is not a useful strategy for all kinds of cells which have the different characteristics but the same size. In addition, this approach is inapplicable to rare cell separations in a small amount of samples, because most applications adopt the continuous flow control.

Contrary to the above-mentioned methods (the miniaturized FACS systems and DEP-based systems), there is another trend based on the magnetic force, which is economical, no requirement for a large sample volume, and no Joule heating related to the risk of thermal cell damage. Moreover, for the very simple reason that can exactly separate a large amount of target cells within a short time, various products (such as MACS® of Miltenyi Biotec, Dynal MPC® of Invitrogen, MagCellect™ of R&D system, EasySep® of StemCell Technologies, BD IMag™ of BD Biosciences, etc.) have been commercially employed, although their purity and recovery had large variances.[2] To minimize the variances, several researches by using the miniaturized MACS systems have already been reported.[10–14] Especially, the performance of paramagnetic capture (PMC) mode magnetophoretic microseparators[15] was dramatically demonstrated by compar-

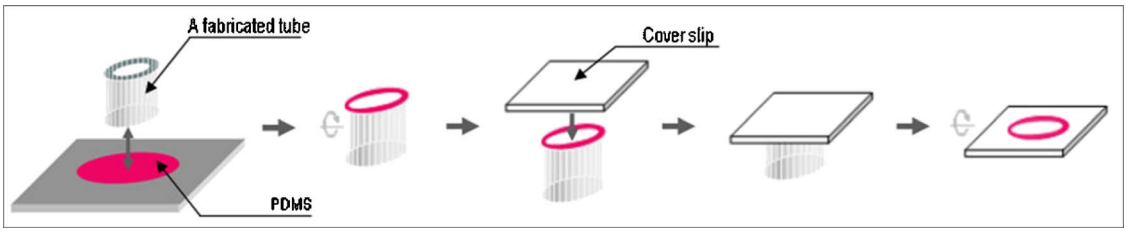

FIG. 2. (Color online) Schematic drawing of imprinting process on a coverslip.

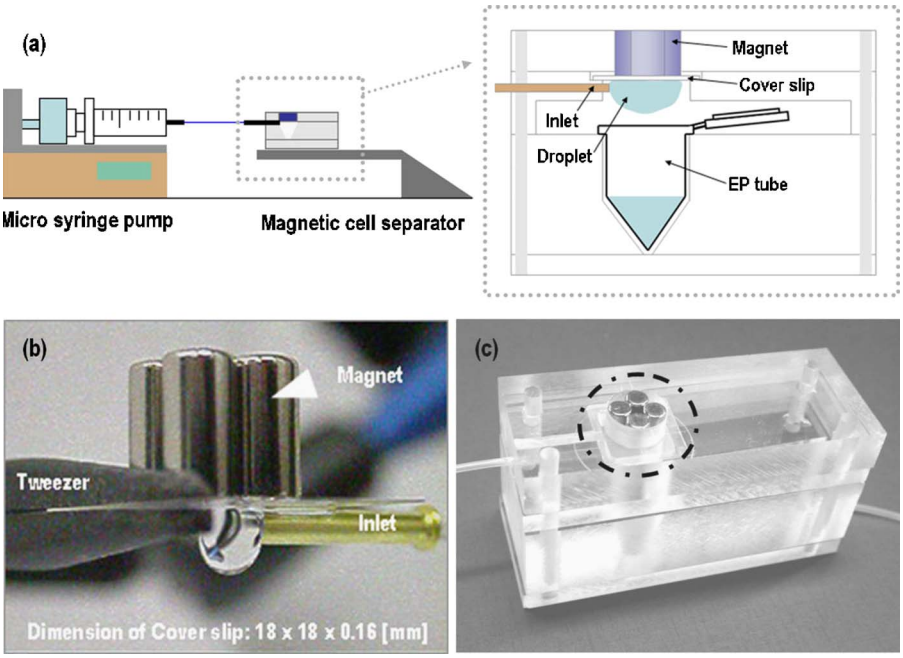

FIG. 3. (Color online) Experimental setup. (a) Configuration of cell separation system; to effectively handle a droplet which is attached to the fabricated coverslip, we contrived a DMACS tool. A dripped cell suspension was collected by FACS tube or EP tube. (b) Working scheme for the buffer injection; this shows a process for detaching a droplet, unveiled at (c) the integrated DMACS tool.

ing their separation efficiency. There is, however, latent problem such as cell (or bead) loss due to adhesion on microchannel. In most cases, a miss not to notice this point has occurred. Although coating materials (e.g., Pluronic-F108 surfactant) for reducing the adhesion phenomena are generally used, a critical parameter is a running time from cell separation to recovery. Practically, an increase in the adhesion phenomenon always stemmed from the lapsed experimental time, because the injection flow required for optimal movement of cells in microchannel is too slow to obtain sufficient cell fraction for cell-based analysis within a short time.

Therefore, as an alternative approach to minimize the various limitations (requirement of large sample volume, cell damage, and cell loss) of different cell separator, we previously proposed a novel platform—a droplet-based magnetically activated cell separator (DMACS) for rare cell separation on a coverslip without microchannel.[27,28] However, irregular injection due to "stick and slip" phenomena in micropipette led to a discrete flow and then a decrease of separation efficiency of the DMACS. In this article, efficiency of cell separation was reevaluated under the exact flow control with microsyringe pump. With the total number of the cells before and after separation, a degree of cell loss by MiniMACS system and the DMACS was examined and compared.

To perform cell separation based on the novel platform, we followed the procedure shown in the schematic presentation (Fig. 1). First of all, using a micropipette (i.e., ~20–100 μl), a portion of mixed cell suspension was attached to the glass coverslip [Fig. 1(a)]. After the coverslip was turned over, "a hanging droplet" was made as the cell suspension stuck to the bottom of the coverslip [Fig. 1(b)]. To divide the droplet including labeled and unlabeled cells into two fractions, an inlet connected to a microsyringe (i.e., 250 μl) was installed at the side of the hanging droplet [Fig. 1(c)]. After placing a permanent magnet on the coverslip during a specified period of time [Fig. 1(d)], the solution was added by the microsyringe. At that time, cells are arranged as shown in Fig. 1(f). Each cell in the hanging droplet moved as follows. The magnetically labeled cells were attached to the surfaces of the coverslip by magnetic force. The unlabeled cells settled down to the bottom side of the hanging droplet under the influence of the gravity. Then, the droplet was detached from the coverslip due to an increase of the weight of inflow [Fig. 1(e)]. At this moment, a small amount of the solution attached on the coverslip was named "positive fraction," and the dripped solution was named "negative fraction." Most cells in the positive fraction were the labeled cells (positive cells) which are treated by immunomagnetic labeling process using a biotinylated anti-CD45 antibody solution. Most cells in the negative fraction were the immunomagnetic unlabeled cells (negative cells). Finally, each of the fractions was collected by micropipette.

To attach a droplet in a limited circle area (6 mm diameter) on a coverslip with hydrophilic characteristics, poly(dimethylsiloxane) (PDMS), which is one of the hydrophobic and biocompatible materials, was used.[29] A small amount of PDMS solution was drop dispensed on the substrate and the fabricated tube of 6 mm in diameter was stamped on the substrate to smear PDMS solution. Subsequently, it was imprinted for a circle-shaped boundary on the coverslip. To cure the imprinted coverslip, it was baked at 70 °C for 1 h (Fig. 2).

Adult ICR mice were sacrificed, and their femurs and tibias were removed aseptically. Marrow cavities were flushed with phosphate-buffered saline (PBS, *p*H 7.4) with 0.5% bovine serum albumin (PBS/BSA) using a 26-gauge needle attached to a microsyringe. Single cell suspensions were prepared by repeated pipetting. The recovered bone marrow cells (BMCs) were passed through a 30 μm nylon mesh (MACS® Pre-Separation Filter; Miltenyi Biotec, Inc., Bergisch Gladbach, Germany) to remove large cell masses. The cells were washed twice in PBS/BSA, before estimating the number of cells using a hemocytometer. The cells were resuspended in PBS/BSA at a final concentration of 10^7 cells ml^{-1} before immunomagnetic labeling.

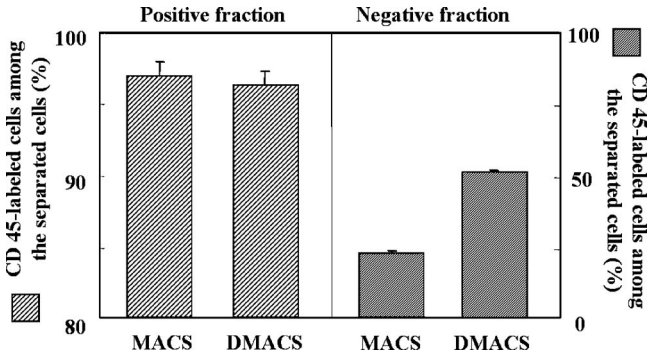

FIG. 4. Comparison of the separation efficiency in the MACS and the DMACS (where "positive fraction" means a relative ratio of the positive cells included in the positive fraction after cell separation and "negative fraction" means a relative ratio of the positive cell included in the negative fraction after cell separation).

Each cell mixture for both cell separators and separating buffers was prepared as described in the manual.[30] Briefly, the suspended BMCs were incubated for 10 min in a biotinylated anti-CD45 antibody solution (1:500; Pharmingen, San Diego, CA). The cells were washed twice with PBS/BSA and resuspended in 80 μl PBS/BSA before the addition of 20 μl of streptavidin-conjugated microbead solution (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany). The cell mixture was incubated at 4 °C for 15 min to allow biotin-streptavidin interactions. For the flow cytometry analysis of magnetically labeled cells, streptavidin-PE-Texas Red (1:500; Pharmingen, San Diego, CA) was added, and the cells were incubated at 4 °C for 15 min in dark.

The system configuration of the DMACS is presented in Fig. 3(a). To precisely drop a hanging droplet on a coverslip, a microsyringe pump (VIT-FIT, Lambda Co., Switzerland) and a microsyringe (250 μl, 1725RN, Hamilton Co., Reno, NV) with a 22-gauge Medicut needle were used. The DMACS comprises the following components. The first layer includes a permanent magnet of a cylindrical type made of Nd–Fe–B with a surface field of 0.45 T, which is measured by Tesla meter (TM-601, Kanetec Co., Ltd., Japan).[31] The second layer is an imprinted coverslip stand and has an inlet for buffer injection. The third layer is an EP tube (Ependorf Scientific, Inc., Westbury, NY) stand, which collects a dripped cell suspension. Each layer was fixed by a bolt at the edge of the DMACS. Figure 3(b) shows an increasing size of a hanging droplet due to buffer injection. This process is also realized in an integrated structure [Fig. 3(c)].

To compare the rate of the cell adhesion and separation efficiency in each, case control group, the MACS group, and the DMACS group, three samples were prepared with a cell density of 6.566×10^6 cells/70 μl. One of the prepared samples was not separated as a control group, the other sample was separated by MS column of MACS system,[30] and another sample was separated by DMACS system. To obtain each fraction, PBS/BSA solution was added to the droplet at a rate of 100 μl min^{-1}. A negative fraction was collected directly into a FACS test tube to minimize the cell loss. Separated cells were collected by centrifugation (700g,5 min) and then resuspended in a 2 ml volume of PBS/BSA.

The CD45+ cells labeled with Texas Red-conjugated antimouse were analyzed and separated using FACS Calibur (Becton Dickinson, San Jose, CA). Standard gate based on the staining profiles of the negative control with the primary antibody omitted was established. The labeled cells were gated out according to appropriate filter block for Texas Red dye. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter and side scatter as described.[32,33] Between samples, a solution of 10%/0.5N NaOH was flushed through the flow cytometry tubing for 10 min to clear adherent cells from the prior sort. Suspended cells were read for 30 s at a rate of 3000 cells s^{-1} to avoid the possibilities of differences during the analysis. The raw data were assessed by using BD CellQuest™ (BD Biosciences, Mountain View, CA) and the WINMDI 2.8 (http://facs.scripps.edu/software.html; Joseph Trotter, Scripps Research Institute, La Jolla, CA) software.

Under the exact flow control by microsyringe pump, the experiment to evaluate separation efficiency was repeated several times. The analysis result is shown in Fig. 4. In the case of the ratio of the positive cells in the positive fraction, cell separation efficiency in the MACS (96.72%) and the DMACS (96.07%) was almost the same. However, in the event of the ratio of the positive cells in the negative fraction, the ratio in the DMACS (50.31%) was included about two times as much as that in the MACS (21.97%), because

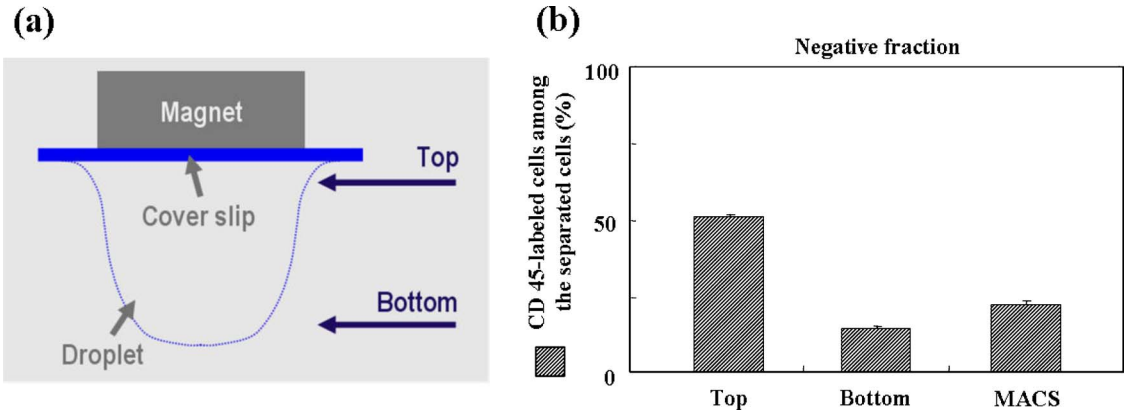

FIG. 5. (Color online) Buffer injection position (left) and separation efficiency (right).

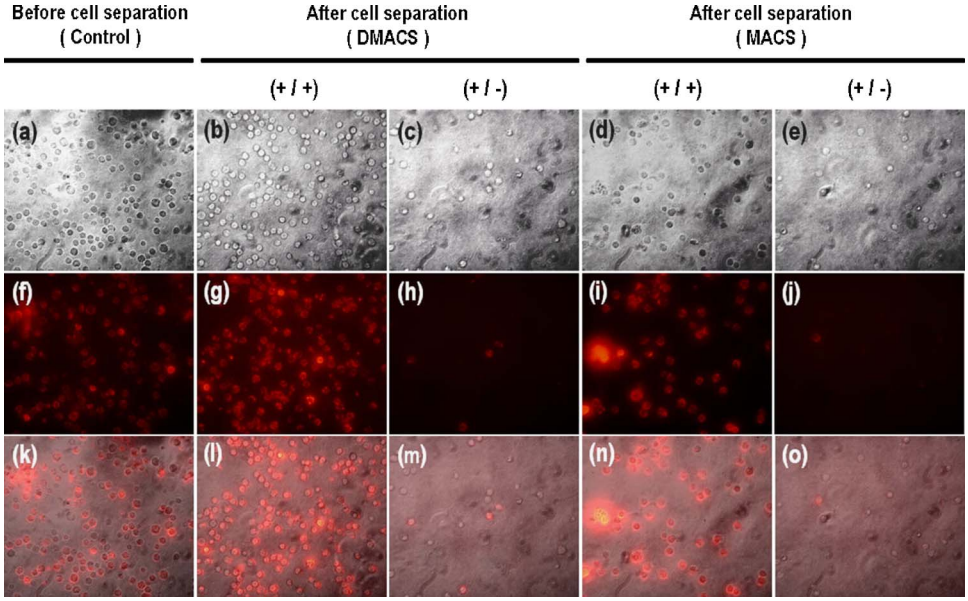

FIG. 6. (Color online) Demonstration of the positive cells included in each fraction: The top row, entire cells at the bright field, (a)–(e). The middle row, positive cells labeled by streptavidin-PE-Texas Red, (f)–(j). The bottom row, images merged with the top and middle rows, (k)–(o). The (+/+) means positive cells in the positive fraction as CD45 positively selected BMCs and (+/−) means positive cells in the negative fraction as CD45 depleted BMCs, (k) BMCs before separation, (l) CD45 positively selected BMCs with DMACS, (m) CD45 positively selected BMCs with MACS, (n) CD45 depleted BMCs with DMACS, and (o) CD45 depleted BMCs with MACS.

of the following reason: when the position of needle for buffer injection was underneath the coverslip (named as "Top"), a portion of the positive cells collected on the coverslip by magnetic force was moved to the bottom side of droplet during the injection. On the process, the positive cells detached from the coverslip by the flow were mixed with the negative cells gathered at the bottom side by gravitation. That droplet swelled by buffer inflow was immediately dropped. Therefore, a portion of positive cells is included in the dripped solution (negative fraction). We supposed that this problem (some more positive cells were found at the negative fraction in the DMACS) comes from injection position [Fig. 5(a)]. Based on that assumption, change in separation efficiency according to its position is investigated and presented in Fig. 5(b). When the position of needle for buffer injection was near the bottom of hanging droplet (named as "Bottom"), the ratio (13.91%) of the positive cells in the negative fraction was much less than that (21.97%) in the MACS, because movement of positive cells stemmed from injection flow could be decreased. Therefore, we confirmed that change in injection position could affect separation efficiency of the DMACS as a primary factor for the negative fraction.

To visually evaluate the real population of positive cells in each fraction, the images of positive cell distribution in each fraction before and after separation were compared (Fig. 6). The positive cells which appeared as "red spot" in each fraction were utilized as indicator for comparison of both separators. Comparing the MACS with the DMACS in the case of the positive fraction, the total number of cells including the positive (CD45 positively selected BMCs with the DMACS) and negative cells was much more than that in the MACS [Figs. 6(l) and 6(n)] although separation efficiency (positive fraction) of both MACS and DMACS was almost the same. Comparing the MACS with the DMACS in the case of the negative fraction (means CD45 depleted BMCs), the total number of the cells including the positive and negative cells was, also, more than that in the MACS [Figs. 6(m) and 6(o)]. It could be said that cell separation with the MACS was accompanied by cell loss, because the MACS column had a matrix structure leading to cell adhesion.

To examine the number of cells adhered to separator on the process, the entire cells before (Before) and after (After) separation as shown in Table I was counted by flow cytometry system and numerically compared. To evaluate a degree of cell loss in each separator, i.e., MACS and DMACS, the total number of cells before separation was statistically fixed as the same number, 59 940. In the event of the entire cells after separation, the total number of cells in the positive fraction was added to that of the cells in the negative fraction. As shown in Table I, there is a relative difference on the total number of the cells between the Before and the After, according to the separator. Although the number of cells before separation was 59 940, the numbers of cells after separation were changed as follows: 37 580 by MACS column and 56 699 by DMACS. When the number of cell loss was counted, those in the MACS and the DMACS are 22 360 and

TABLE I. Cell loss due to adhesion and the calculated contact surface area. Percentage of loss=(100 ×loss/control): "Control" means the total number of cells "Before" separation. A contact surface area* means summation of the area in the separator to which cells can attach.

	Total number of cells				
	Before	After	Loss	Percentage of loss (%)	A contact surface area (mm^2)
MACS	59 940	37 580	22 360	37.31	259.81 π
DMACS	59 940	56 699	3 241	5.41	9π

3241, respectively. A percentage of cell loss in the MACS was seven times higher than that in the DMACS; a ratio in the MACS was 37.31%, while that in the DMACS was 5.41%. This may be caused by the following reason: in the case of the MACS, the gaps in the highly magnetized small steel bead matrix geometrically obstruct the cell recovery. In other words, narrow gaps between the beads can lead to cell adhesion, consequently, interrupt cell movement. On the contrary, cell separation of the DMACS was realized on the open space without a confined area between small steel beads. Therefore, it was confirmed that cell separation with the DMACS could significantly reduce cell loss.

To explain the difference related to geometrical structure of each separator, we considered that surface area is an index for comparing a cell separation system because it might lead to cell loss based on the adhesion. Each surface area of the MACS and the DMACS means the entire surface area of small steel beads loaded in column and surface area on a coverslip to which a droplet sticks, respectively. Practically, the surface area of the MACS and the DMACS to which cells can be attached was quite different. The calculated total surface areas of attracting positive cells in cell suspension were about 259.81π (MACS) and 9π (DMACS), respectively (Table I). Comparing difference of the surface area between both separators, that of the MACS was about 29 times larger than that of DMACS. From the result, it could be said that the MACS with a matrix structure of multilayers could supply more chance that the cells could attach to magnetized beads and could minimize the number of the positive cells in the negative fraction. However, its geometrical structure simultaneously led to cell loss based on the adhesion.

In the case of the platform using a microdroplet, it has been reported that the gas-liquid interface would affect cell viability; most cells would be killed if there are no agents or protective additives in medium or buffer.[34,35] In addition, bubbling in medium or buffer leads to critical damage in cells.[36,37] Therefore, we carried out additional viability test, although there was none of cell deformation such as distortion or rupture of cell membrane due to lysis on the cell images of the bright field, as shown in Fig. 6(a). We collected entire cells in the negative fraction and examined cell viability on the DMACS platform using gas-liquid interface. As shown in Figs. 7(a) and 7(b), it was confirmed that most cells are viable since those are stained by Hoechst 33 342.

In this article, we proposed that the DMACS is a novel platform having high performance in cell purity as much as the conventional MACS, reducing the CD45-labeled cell loss due to adhesion during the separation process. Especially, superiority of the DMACS was demonstrated in the sense that the number (3241/59 940) of cells lost in the DMACS is much less than that (22 360/59 940) in the MACS. From these results, it was found that not only separation efficiency but also cell loss has to be considered as one of the schemes to evaluate separator.

Therefore, we confirm that the proposed DMACS can be successfully employed for the rare cell separation in microdroplet since we can significantly reduce cell loss without losing separation efficiency, using a 70 μl droplet of cell suspension. In addition, we expect that it is applicable to lab-on-a-chip (LOC) as one of the main functions.

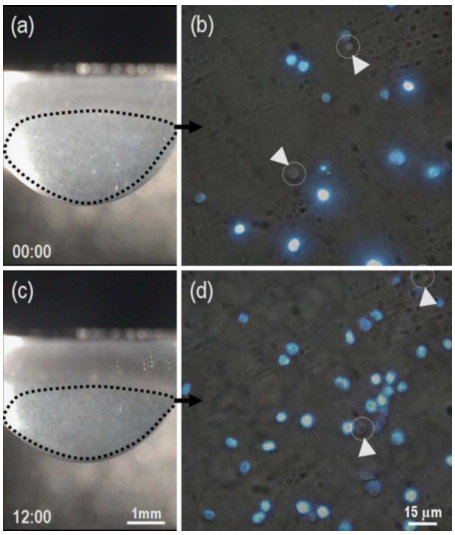

FIG. 7. (Color online) Cell viability of the DMACS platform based on gas-liquid interface; to examine the effect of gas-liquid interface, cells obtained from the negative fraction (presented by the black-dotted line) were dyed by Hoechst 33 342, which allows viable cells to be blue color in fluorescent field. This figure shows a hanging droplet on (a) initiation (time=0 min) and (c) completion (time=12 min) of cell separation at bright field. Results of cell viability in each case are (b) and (d), respectively. To effectively present the results, we merged (gray-colored) entire cells at bright field with (blue-colored) viable cells at fluorescent field. The 00:00 (or 12:00) in (a) [or (c)] means the time elapsed (minute:second). The arrows in (b) and (d) indicate dead cells.

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