

Droplet-based Immunomagnetic Cell Separation

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Accepted 26 July 2007

Abstract

This paper presents a droplet-based immunomagnetic cell sorter with high cell separation efficiency and a microfluidic cell sorter integrated with a microfluidic mixer and reaction chamber that were provided for the serial cell separation. The conventional matrix-based MACS magnetically activated cell sorter separates cells with high separation efficiency, however the tiny gaps of the steel bead matrix can damage or trap the cells. The droplet-based MACS (dMACS) system has a high recovery ratio with comparable high separation efficiency due to its non-matrix structure. The slight agitation of cells in droplet, however, cause high false negative ratio. Therefore, we added a buffer container underneath the droplet, which improved the recovery ratio of negative cells. Agitation of the cell suspension was suppressed, which in turn reduced the false positive and negative ratio to less than 3%. The small volume of the droplet of the dMACS limited its application; therefore we integrated the dMACS system with a microfluidic chip that contained a mixer and a reaction chamber to allow continuous separation of the cell suspension. The droplets of negative cells were serially dripped down to a collection reservoir with the addition of buffer. The cell separation efficiency in the microfluidic chip was not as high as dMACS, however the results demonstrated the feasibility of continuous separation in a microfluidic chip.

Keywords: Cell separator, Droplet, MACS, Microfluidic chip

Introduction

It is often necessary to separate target cells from blood or bone marrow when conducting biomedical

or clinical research. Currently, there are two methods to separate target cells. One category is using their different physical properties including dielectrophoresis, acoustic wave, and centrifugal force¹⁻⁴, whereas the other category is using immunology-based cell separation technique^{5,6}. The former method has demonstrated potential applications; however it is not widely accepted because specific cells often cannot be separated based on only their physical properties. The latter method implements antibody-antigen reactions of the surface-markers, and has been widely used because of its high recovery ratio and throughput, as well as the high purity of the cells it recovers⁷⁻⁹. Macro-sized immunology-based cell sorters, including fluorescence-activated cell sorters (FACS) and magnetically activated cell sorters (MACS) have been commercialized. Although FACS can sort cells using multiple markers and can also provide additional information, such as scattering and counting data, its high price prevents its widespread use in laboratories. Conversely, MACS requires no complex equipment and is suitable for inexpensive cell sorting due to its simple structure and convenient operation. Therefore, MACS is preferable for the prompt selection of target cells, and is already being sold by several companies, including Miltenyi Biotec, Invitrogen, and Stem Cell Technologies.

Because a micro-sized device is suitable for a small volume of cells, microfluidic chips for MACS have been studied by many research groups^{5,10,11}. Most of these chips use a magnetic field in the channel to hold target cells that have been labeled by magnetic beads while non-target cells are washed out. However, the washing flow can drag out the positive cells, which results in an increased ratio of false negative cells. Therefore, we have suggested the use of droplet-based MACS (dMACS), which uses gravitational sedimentation as a negative selection method¹². The use of dMACS was effectively able to sort the target cells because the precipitation of negative cells caused no shear flows on the magnetically selected cells on the top surface. The dMACS system also has no ferromagnetic matrix structure, which reduces the damage to cells caused by the interaction between cells and the matrix structure. Additionally, the configuration of dMACS is so simple that it can be easily combined with a microfluidic circuit for sample preparation.

In this paper, we describe a new dMACS system

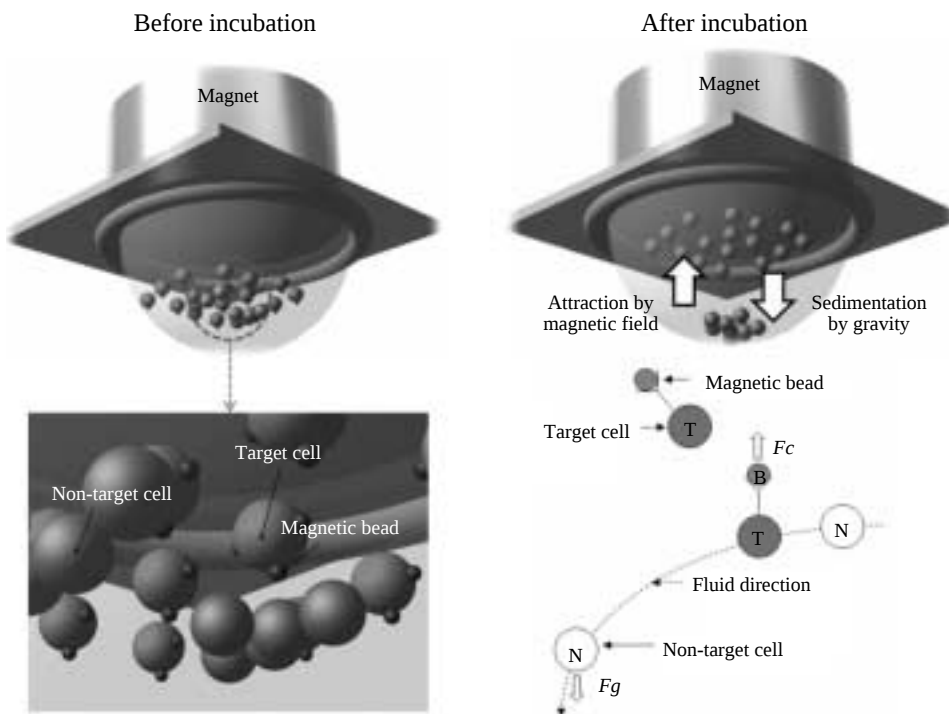


Figure 1. The separation principle of droplet-based MACS: The target cells were attracted by magnetic force (F_c) and non-target cells were precipitated to the bottom of the droplet by gravity (F_g).

that was designed to decrease the ratio of false negative cells, and its subsequent integration with a microfluidic chip to increase its throughput by continuously supplying the cell suspension. The reaction chamber was also accompanied by a serpentine mixer to improve the mixing of cells and magnetic particles^{13,14}.

Principle of Droplet-based MACS

Droplet-based Cell Separation

In droplet based cell separation, the positive cells (target cells) were attached to antibody-coated magnetic nanoparticles that could not react with the magnetic particles. The cell suspension mixture was then formed into a hanging droplet under a magnet using a micropipette. While the magnetic field was applied, the positive cells were attracted to the top surface by the magnetic force in droplet¹⁵, and the negative cells are precipitated down by gravity (Figure 1). If the droplet was divided into the upper and lower part, the mixed cell suspension could then be separated. The methods of droplet separation included dripping of the droplet¹⁵ and dividing the droplet by shear or external force. The force acting on a single magnetic bead, F_m , can be described by equation (1)¹⁶

$$F_m = \frac{1}{2} \frac{V_m \Delta\chi}{\mu_0} \nabla B^2 \quad (1)$$

where $\mathbf{B}$ is the external magnetic field, V_m is the volume of the particle, and $\Delta\chi$ is the difference in susceptibility between the magnetic bead and the surrounding medium ($\Delta\chi = \chi_m - \chi_f$). Considering that a number of magnetic beads are attached to the cell surface, the actual magnetic force acting on a labeled cell, F_c , can be represented by equation (2).

$$F_c = A_c \alpha \beta F_m \quad (2)$$

where A_c is the surface area of the cell, α is the number of specific cell surface markers per membrane surface area, and β is the number of antibody magnetic bead complexes bound per receptor. The magnetic field strength and the susceptibility of the magnetic bead were the main parameters used to control the force on the cells in the suspension medium. When the droplet was suspended by the micropipette, the turbulent flow in the droplet swirls the cells, which resulted in some of the target cells becoming attached to the magnet and the negative cells being rotated along the stream line. When the flow was weakened, the remnant target cells moved up against the hydrodynamic flow and the negative cells were precipitated to the bottom of the droplet by gravity. After incubation, the positive and negative fractions could be separated by cutting the droplet in two. Thus, the droplet-based cell separation requires very careful manipulation to ensure that no agitation of the separated cells occurred because even a slight distur-

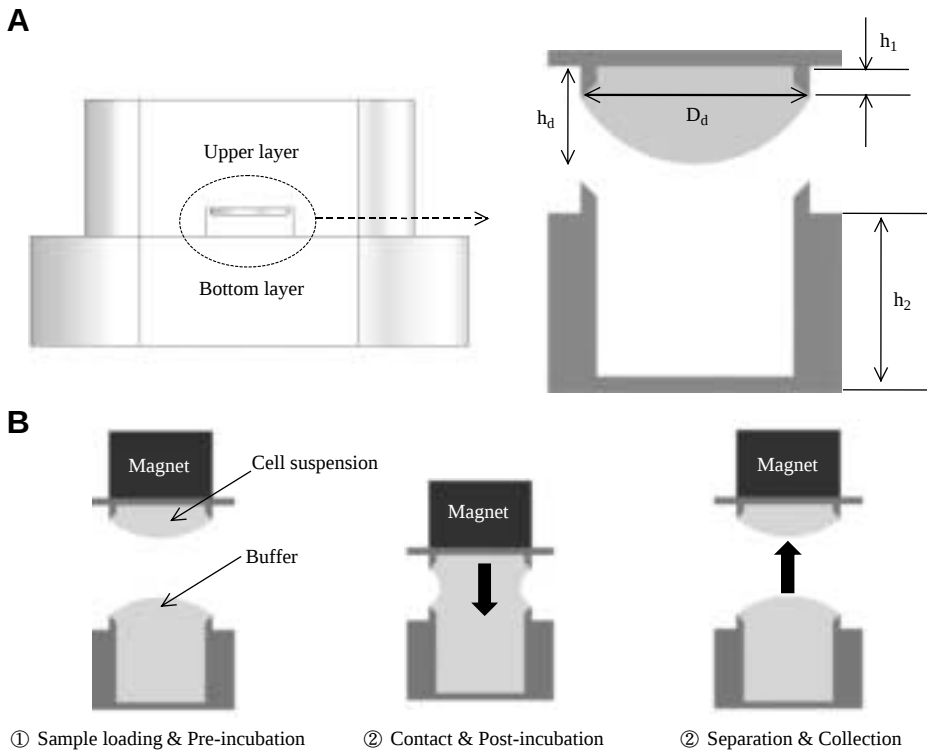


Figure 2. The suggested droplet-based MACS and the cell separation protocol: (A) The structure of the dMACS and the geometric parameters used to optimize the separation efficiency (B) the cell separation protocol using double layers.

bance could cause re-mixing of cells. Dripping of the droplet by increasing its weight may also cause some turbulence within the droplet unless the feeding flows are carefully controlled.

Therefore, we modified the dripping cell separation method¹⁵ by using two droplets located in the upper and lower layer, respectively (Figure 2). The upper container was filled with the cell suspension and the lower container was filled with buffer. Each container then formed a droplet with surface tension, the size of which depended on the volume of fluid injected by the pipette. Before the two droplets were combined, a magnetic force was applied to the hanging droplet in the upper container for pre-incubation while the cells were separated. The upper droplet was then merged into the lower droplet by contacting the upper and lower layers. The two fluids were then mixed, and gravity precipitated the negative cells into the lower container. After incubation, the fluid was divided into two drops by detaching the upper plate from lower panel. This configuration has enough distance between the positive and negative cell region to prevent external disturbance.

Continuous Cell Separation in the Microfluidic Chip

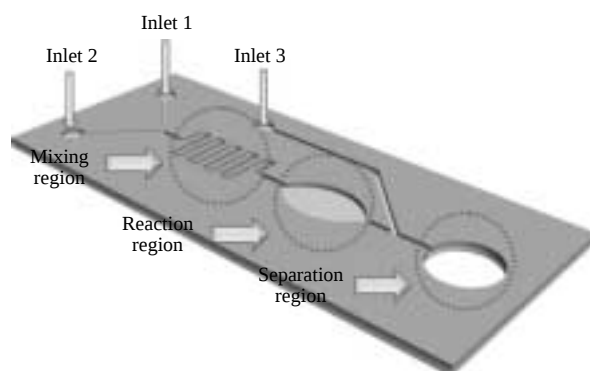
Figure 3A shows the operation of the continuous microfluidic MACS device and Figure 3B shows the

fabricated device and experimental setup. First, the cell suspension and magnetic bead suspension were injected into inlet 1 and 2, respectively. When the injected fluids flowed through the mixing region, the magnetic beads and cells were mixed. Next, the flow was stopped to allow interaction of the cells and beads for 10 min in the reaction region. When the reaction was completed, the pre-determined volume of fluid was transported to the separation region to form a droplet. At that time, the magnetic field was applied to the droplet to separate the positive and negative cells. After 10 min of incubation, the buffer solution was then injected from inlet 3 to detach the portion of the droplet containing the negative cells. This separation could be processed continuously until the injected fluids were consumed.

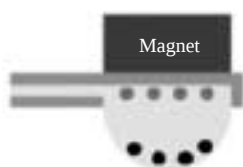
Results and Discussion

Geometric Optimization of Droplet-based MACS

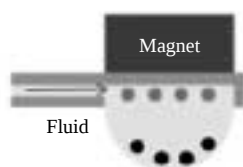
The most important condition required for maximization of the separation efficiency of droplet-based MACS was the minimization of the effect of turbulent flow in the droplet when it is being separated. Since the disturbing flow occurs at the separation boundary, both positive and negative cells should be

A

1. Polarization



2. Fluid supply



3. Separation

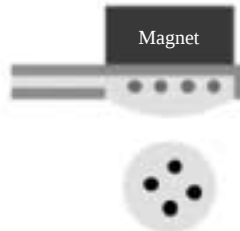
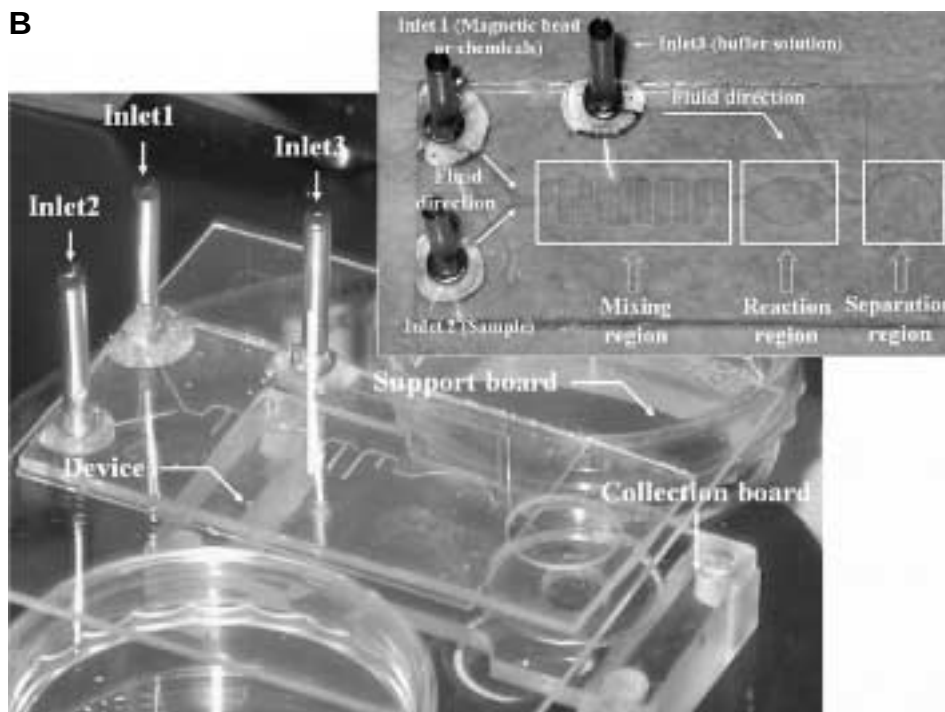
**B**

Figure 3. The microfluidic device used for the continuous separation of droplet-based dMACS: (A) The schematic of the proposed device and working principle (B) the image of the fabricated cell separator and the experimental setup.

far from the separation boundary of the droplet, however, too great a distance causes a weak magnetic force, which decreases the negative cell separation efficiency. Therefore, geometric optimization was performed by varying the heights of the containers.

The diameter (D_d) of the droplet was fixed at 6 mm to ensure that the droplet remained stable while attaching or detaching the upper layer. The depth of the droplet (h_d) was determined by the injected volume, however the maximum depth was 3 mm because the

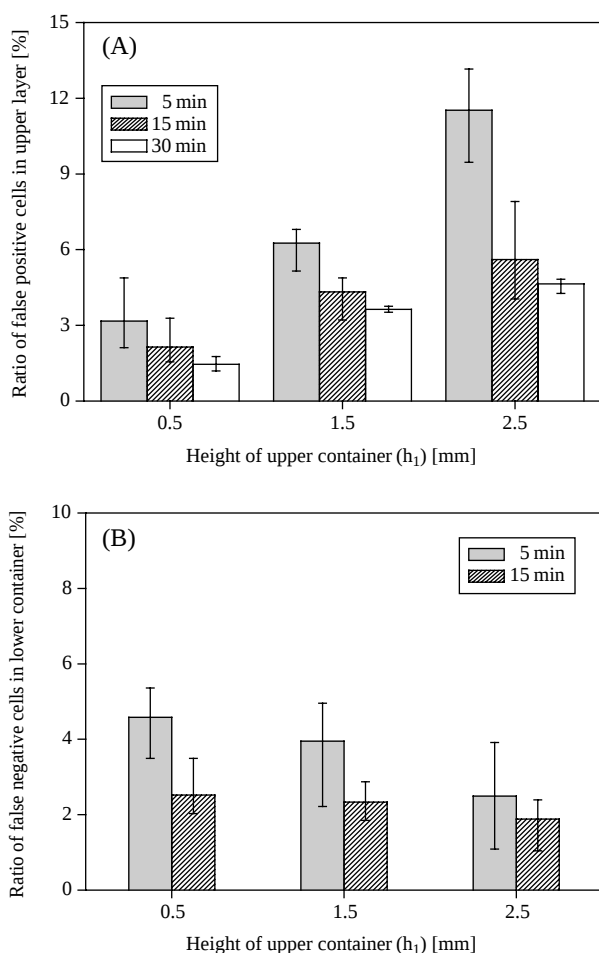


Figure 4. The separation performance of dMACS when the height of the upper container was changed: The ratio of false positive cells in the upper layer (A) and the ratio of false negative cells (B) in the lower container when the height of the upper container (h_1) and the incubation time were adjusted.

extent of the magnetic force was limited. Therefore, the droplet volume ranged from 40 to 60 μL , depending on the height of the upper container (h_1). Thus, the controllable geometric parameters involved in optimization of the performance were the height of the upper container for the hanging droplet (h_1), and the depth of bottom layer for the buffer reservoir (h_2). The separation performance was evaluated by determining the false positive and false negative ratio of cells, which are represented by the percentage of negative cells present in the upper layer and positive cells present in the bottom container, respectively. When h_1 was changed from 0.5 mm to 2.5 mm, the false positive ratio was increased and the false negative ratio was decreased after an incubation time of 5 min (Figure 4). However, when the incubation time

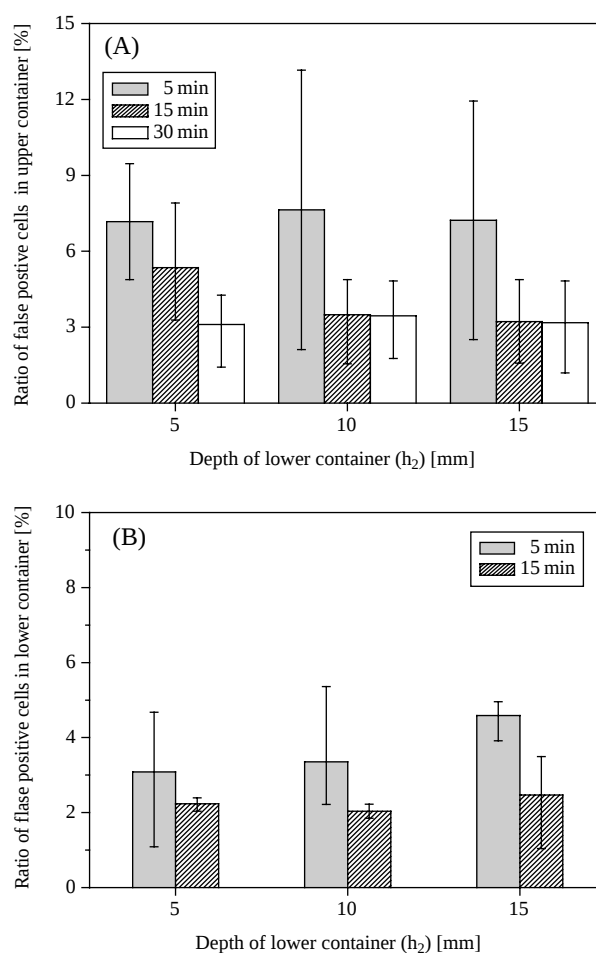


Figure 5. The separation performance of dMACS when the height of the lower container was changed: The ratio of false positive cells in the upper layer (A) and the ratio of false positive cells (B) in the lower container when the depth of the lower container (h_2) and the incubation time were changed.

was 15 min, the false positive ratio was less than 3%, regardless of h_1 . Therefore, a length of 0.5 mm was selected for h_1 since this allowed application of the strongest magnetic force with the shortest upper container. As the depth of bottom layer was changed from 5 mm to 15 mm (Figure 5), the false positive ratio was less than 3% when h_2 was greater than 10 mm and the incubation time was 15 min. Additionally, the false negative ratio seemed to increase as h_2 increased, however it was not seriously affected by variation of h_2 when the incubation time was greater than 10 min. Therefore, 10 mm was selected as the optimal depth. The recovery ratio of the optimized device was greater than 97% for both positive and negative cells (Figure 6). The high recovery ratio is a feature unique to droplet-based MACS because it causes less damage

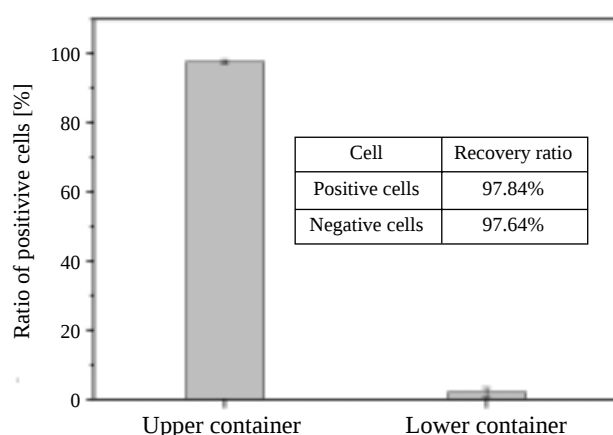


Figure 6. The ratio of positive cells in the upper and lower layer: The mixture of Jurkat cells was separated in an optimized dMACS. The positive cells were Jurkat cells tagged with magnetic beads. The recovery ratio was the number of collected cells divided by the number of injected cells.

than the commercial MACS (Miltenyi Biotec) because no steel bead matrix structure is present.

Mixing of Beads and Cells in the Microfluidic Chip

The mixing of magnetic beads and cells was required to ensure that an efficient reaction occurred. Because the particles could not be easily mixed at a low Reynolds number (Re) due to the low diffusion, we implemented a 2-step serpentine mixer that used the surface tension of the PDMS, which is described in detail in an earlier report¹⁷. When a suspension of 5 μm beads and a buffer solution were injected, the mixing efficiency of the particle suspension, which was determined using equation (3), was plotted (Figure 6).

Mixing efficiency [%]

$$= \frac{2 \text{ the number of beads in section A}}{\text{the number of beads in reaction region}} \times 100 \quad (3)$$

Efficient mixing results in even distribution of the micro beads throughout the reaction region, which would provide a mixing efficiency of 100%. The mixing efficiency monotonically increased with the Re and reached almost 100% when the Re was greater than 16.

Separation using the Microfluidic Chip

We evaluated the microfluidic dMACS to determine if it could separate a mixture of 5 μm polystyrene beads and 1 μm magnetic beads. After mixing was completed, the fluid was moved into the separation region to form a 60 μL droplet, and then a

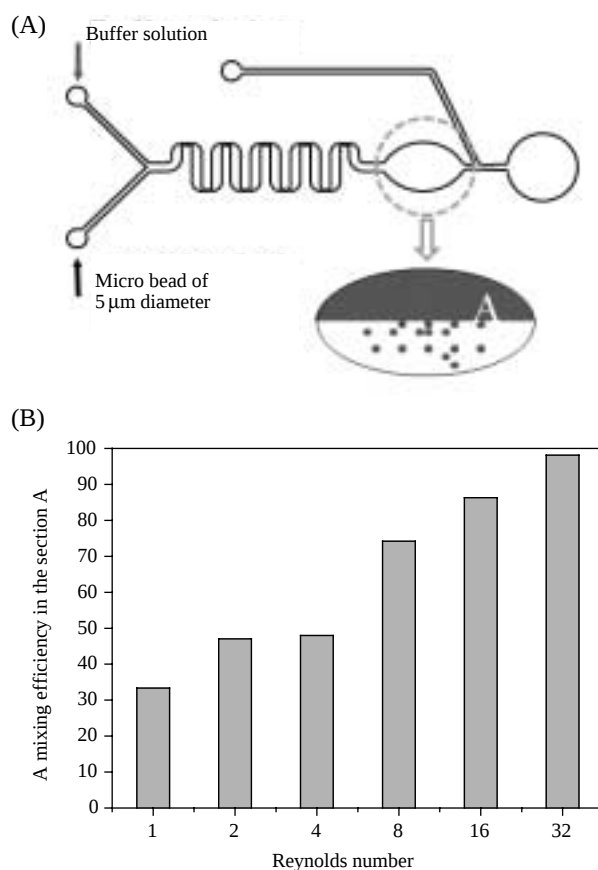


Figure 7. The mixing efficiency at various flow rates: (A) The mixing efficiency was evaluated based on the distribution of injected beads throughout the reaction area. (B) The mixing efficiency was plotted as a function of injected flow rates.

magnetic field of 0.7 T was applied. After 10 min, the buffer solution was injected from inlet 3 at various flow rates below 60 $\mu\text{L}/\text{min}$ ($\text{Re} < 64$). When the droplet was fully grown, it was dripped down into a collection chamber. After eliminating the magnetic field, the buffer solution was again injected to collect the magnetic beads into another collection chamber. Images of the collected magnetic and polystyrene beads are presented in Figure 7A. The number of beads was counted using hemacytometer, and it was determined that the magnetic beads could be successfully separated at a flow rate of 30 $\mu\text{L}/\text{min}$. The separation efficiency was greater than 90% when the flow rate was less than 30 $\mu\text{L}/\text{min}$, however serious turbulence caused a lower separation efficiency at 60 $\mu\text{L}/\text{min}$. However, the false negative ratio was less than 10% at all flow rates, with a higher ratio being observed at higher flow rates.

Next, the microfluidic device was used to separate

Table 1. The separation efficiency of the mixture of Jurkat cells and K562 cells when the flow rate of the dripping fluid was changed.

Flow rate	Ratio of positive cells in upper container	Ratio of negative cells in lower container
7.5 $\mu\text{L}/\text{min}$	84.9%	52%
15 $\mu\text{L}/\text{min}$	89.45%	40%
30 $\mu\text{L}/\text{min}$	73.75%	35%
60 $\mu\text{L}/\text{min}$	64%	43%

a mixture of cells. Jurkat cells were used as the positive cells and K562 cells were used as the negative cells. Magnetic nanobeads and the mixture of cells were injected into inlet 1 and 2, respectively, at a flow rate was 60 $\mu\text{L}/\text{min}$ ($\text{Re}=32$) to ensure complete mixing. The fluids passed through the mixing region and arrived at the reaction region, at which point the flow was stopped for 10 min to allow the reaction of the magnetic particles and Jurkat cells to proceed. The cells were then separated using the same protocol that was used to separate the magnetic beads from the polystyrene beads. As shown in Table 1, the separation efficiency of the Jurkat cells (positive) was greater than 80% when the flow rate of the buffer was 15 $\mu\text{L}/\text{min}$ or less. However, the separation efficiency was less than that of the previous bead separation because the magnetic force on the nanoparticles was smaller than it was on the 1 μm magnetic bead and the shearing effect of the injected flow on the cells was higher. As expected, the negative separation efficiency was lower than that of the dMACS because the flow from the microfluidic channel sweeps out some of the positive cells attached on the magnet. In addition, the injection flow should be located on the bottom side of the droplet to decrease the false positive ratio¹⁵. Adding a shallow chamber to the upper slab of the chip should greatly improve this error, and based on the results of the optimization experiment, a chamber of approximately 0.5 mm would be necessary to achieve the optimum results.

Conclusions

The buffer container under the droplet of the cell suspension was effective at decreasing the false positive ratio in the lower container. The distance between the positive and negative cells was optimized by adjusting the height of the two containers to enhance the separation efficiency. The observed recovery ratio of greater than 95% was better than that of the commercial MACS system, which is based on a steel matrix. The droplet-based magnetic cell separation was integrated into a microfluidic chip to allow

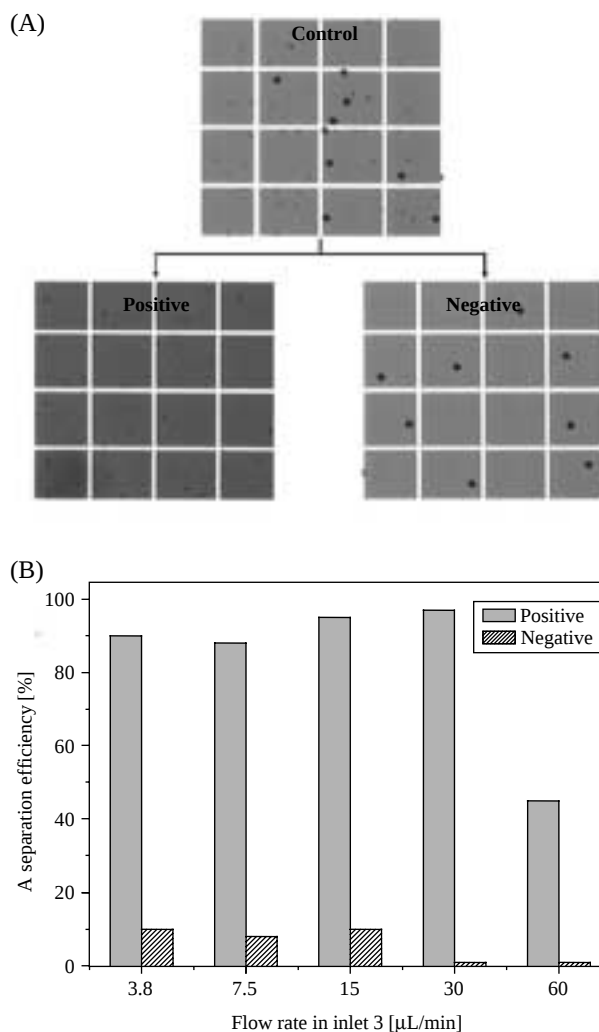


Figure 8. The separation efficiency of the mixture of polystyrene beads and magnetic beads: (A) The images of the hematocytometer before and after the separation, where 'control' indicates the mixture of beads before separation, 'positive' indicates the 1 μm magnetic beads, and 'negative' indicates the 5 μm polystyrene beads after separation (B) the ratio of magnetic beads (separation efficiency) with the change of flow rate.

continuous operation to overcome the limited throughput of the droplet-based MACS system. Although the separation efficiency in the negative fraction was not satisfactory, the feasibility of this system was confirmed based on the separation of magnetic beads and magnetically labeled cells. If the droplets were separated using a buffer container or the shape of the positive chamber was modified, the efficiency could be improved. Since the droplet-based MACS system has little cell loss, high separation efficiency, and small dimensions, it may be a useful biomedical instrument for many cell-based experiments.

Material and Methods

Fabrication of the Microfluidic Cell Separator

The fabrication method used to make a barrier structure using the surface tension of PDMS was fully described in an earlier report¹⁷. Briefly, after double coating a photoresist with SU-8, the SU-8 was patterned to fabricate a master mold that was 500 μm deep. PDMS was then poured onto the master mold and cured at 80°C for 60 min to fabricate the PDMS channel layer. Liquid PDMS was then coated onto the silicon substrate and cured at 80°C for 20 min to make a substrate layer. Next, the substrate layer was coated with liquid PDMS and a PDMS channel layer was added to the coated substrate layer. The coated liquid PDMS then intruded into the microchannel by capillary effect. At that time, a double depth was formed by the difference of the channel width, resulting in a structure that was an appropriate micro mixer. The barrier structure enhanced the mixing ability of the mixer by generating chaotic advection.

Culture of Cells and Magnetic Beads

The Jurkat cells and K562 cells (ATCC) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-Invitrogen, NY, USA), 50 $\mu\text{g}/\text{mL}$ penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 1 mM sodium pyruvate, and 20 mM Hepes buffer (all of which were obtained from Invitrogen, Carlsbad, CA). The cells were maintained at 37°C in a humidified atmosphere comprised of 5% CO_2 and passaged at 2- or 3-day intervals. The magnetic beads (50 nm) and microbeads that were used for separation (1 μm) were purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany) and DynalBeads (Invitrogen, Brown Deer, WI), respectively.

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