Specific binding and magnetic concentration of CD8+ T-lymphocytes on electrowetting-on-dielectric platform

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In the quest to create a low-power portable lab-on-a-chip system, we demonstrate the specific binding and concentration of human CD8+ T-lymphocytes on an electrowetting-on-dielectric (EWOD)-based digital microfluidic platform using antibody-conjugated magnetic beads (MB-Abs). By using a small quantity of non-ionic surfactant, we enable the human cell-based assays with selective magnetic binding on the EWOD device in an air environment. High binding efficiency ($\sim 92\%$) of specific cells on MB-Abs is achieved due to the intimate contact between the cells and the magnetic beads (MBs) produced by the circulating flow within the small droplet. MBs have been used and cells manipulated in the droplets actuated by EWOD before; reported here is a cell assay of a clinical protocol on the EWOD device in air environment. The present technique can be further extended to capture other types of cells by suitable surface modification on the MBs. © 2010 American Institute of Physics. [doi:10.1063/1.3509457]

I. BACKGROUND AND MOTIVATION

A. EWOD as a lab-on-a-chip platform

Due to its simple design, low-power consumption, and reprogrammable fluid paths, droplet-based or digital microfluidics driven by electrowetting-on-dielectric (EWOD)^{1–5} is an attractive platform to develop microfluidic devices and systems for portable or point-of-care “lab-on-a-chip” applications.⁶ Unlike continuous flow through channels, fluids are handled in the form of individual droplets by the locally applied electric potentials. Power consumption in EWOD (well below 1 mW) is much smaller than typical continuous microfluidic systems.⁷ Moreover, droplet movement is directly controlled by electrical signals, and no other inputs such as thermal, pneumatic, optical, etc., are required. These features make EWOD uniquely suited for battery operation, thus addressing a critical requirement of a portable system. Moving parts such as pumps and valves, which could be failure-prone, are not required for EWOD, enhancing its simplicity and reliability. Unlike “hardwired” channels, the fluid (droplet) path in EWOD is reconfigurable purely through software, allowing the choice between multiple testing operations on the same device using the same system. Economical mass fabrication of EWOD test chips is possible, for example, using Printed Circuit Board (PCB) fabrication⁸ or rapid prototyping.⁹

Despite the various advantages over channel-based continuous microfluidics for a lab-on-a-chip platform, cell-based assays on an EWOD platform have been difficult due to “biofouling” (biomolecular adsorption of cells and proteins) on the hydrophobic EWOD surface. The ability to actuate cell samples on EWOD in an air environment has been demonstrated only recently,¹⁰ opening up the possibility of cell separation assays on EWOD, such as the one reported here.

B. Cell separation on EWOD platform

Target separation is one of the key steps in making EWOD more powerful as a lab-on-a-chip platform for biomedical applications. Magnetic concentration,^{11–14} with its many advantages over other mechanisms (e.g., electrophoretic,¹⁵ dielectrophoretic,¹⁶ and optoelectronic¹⁷), is an attractive option for integration with EWOD. Unlike electric mechanisms, for instance, magnetic interactions are generally unaffected by surface charges, *pH*, or ionic concentration. Magnetic manipulation is possible using an external magnet that is not in direct contact with the fluid, not requiring complex structures or electrical circuitry. The most commonly used approach for magnetic separation is to use superparamagnetic beads, also known as magnetic beads (MBs),¹⁸ having suitable surface modification to achieve specific binding and subsequent isolation of the bound targets such as proteins^{19,20} and cells.^{21,22} Antibody-conjugated magnetic beads (“MB-Abs”) for various such biological targets are now commercially available. Magnetic separation has been used to separate not only the species of interest for detection but also the subpopulations of cells containing the species being detected.²³ For instance, the correlation between gene expression data with disease regulated patterns was found to be much better in the lysate from the isolated subpopulations of cells, as compared to the whole blood.

Cytotoxic (CD8+) T-lymphocytes in the human blood [$(2–8) \times 10^5$ cells/ml (Ref. 24)] act as key effectors of the cellular immune response against infections, but also pose clinical challenges, such as rejection of transplanted organs.²⁵ If CD8+ lymphocytes could be isolated from other peripheral blood components and then lysed, the concentration of these cells and their associated proteins could be measured for a noninvasive diagnosis.^{26,27} Protocols for monitoring organ transplants based on such an approach have been developed, e.g., at the UCLA Immunogenetics Center, the patients need to visit the center for the tests. A portable device, such as the one based on EWOD, performing the test would not only obviate the post-transplantation visits but also facilitate early diagnosis and timely treatment.

Figure 1 illustrates the overall scheme for performing the diagnostic assay on EWOD. Droplets containing the MB-Abs and the blood cell (“initial”) samples are merged [Fig. 1(a)] and mixed [Fig. 1(b)] using EWOD actuation. The specificity of anti-CD8 MB-Abs leads to their selective binding to the CD8+ cells, so when a permanent magnet is introduced, the MB-bound CD8+ cells are magnetically collected to one side [Fig. 1(c)]. The droplet is subsequently split so as to reduce CD8– cells (in the form of the “depleted” droplet) while retaining the CD8+ cells in the “collected” droplet [Fig. 1(d)]. Purity of the CD8+ cells can be improved [Fig. 1(e)] by serial dilution, by adding a wash buffer droplet and repeating the steps in Figs. 1(b)–1(d) before they are lysed, and the detection of proteins or mRNA in the lysate is performed [Fig. 1(f)].

In the quest to realize the clinical protocol for organ transplant monitoring on a portable system and extending from a preliminary result,²⁸ we report the specific binding of CD8+ cells (T-lymphocytes) to MB-Abs, followed by the magnetic separation of CD8+ cells from a mixture of CD8+ and CD8– cells, all by electric signals on the EWOD-driven microfluidic chip with the help of a permanent magnet. One of the enabling techniques used for the cell-based assay on EWOD in air environment^{10,17,28} is the addition of a low concentration of nonionic surfactant (viz., Tween 20) to prevent the fouling of EWOD surface^{10,17,28} during the experiment, as discussed later.

The rest of the paper is organized as follows: the materials, equipment, and techniques used in the experiment have been described in Sec. II, following which the Experimental results are presented in Sec. III. A greater discussion on certain aspects of the methods and the experimental results is provided in Sec. IV to relate the current work with the overall objective and previous reports.

II. MATERIALS AND METHODS

A. Cell sample preparation

IRB clearance from the UCLA Institutional Review Board was obtained by all the laboratories involved in this work. All experiments were performed in facilities approved by, and by personnel trained through, the UCLA Department of Environmental Health and Safety’s Biosafety program.

FIG. 1. Schematic of the overall assay for transplant rejection monitoring envisioned on EWOD device. (a) Droplets containing the sample and MB-Abs are merged and (b) mixed so as to bind the target (CD8+) cells to the MBs. (c) The MBs and MB-bound cells are collected with a magnet. (d) Droplet is split to collect the MB-bound cells (in collected droplet), while removing some of the nontarget (CD8-) cells (in depleted droplet). (e) In the future, the steps of (b)–(d) can be repeated to improve the purity of CD8+ cells, and (f) the collected CD8+ cells can then be lysed chemically or electrically (not shown) before the mRNA or proteins in the lysate can be detected.

FIG. 2. Schematic cross section of the EWOD device. EWOD actuation electrodes are patterned from the ITO layer of the “Active” chip. Contact pads are formed with Cr/Au (outside the given field of view and not shown). Electrodes are coated with silicon nitride as dielectric and Cytosol® as hydrophobic coating. Thinner silicon nitride and Cytosol® layers are deposited on the ITO-coated “Reference” chip, connected to the ground. The two chips are separated by a double-sided adhesive spacer.

Human whole blood was obtained using venipuncture at the UCLA Immunogenetics Center. Lymphocytes were isolated from the whole blood using standard hematological procedures. Peripheral blood mononuclear cells were separated over a Ficoll-Hypaque gradient. Lymphocytes were obtained after macrophage depletion by adherence to a plastic flask. CD8⁺ and CD8[−] lymphocytes were separated using anti-CD8 MB-Ab [Dynabeads CD8 Positive Isolation Kit from Invitrogen Inc. (Ref. 29)] before being detached from the MBs using the DETACHaBEAD® product (part of the kit). For long-term storage, cells were frozen at -80°C in a medium containing 10% dimethyl sulfoxide (DMSO) in fetal bovine serum (FBS). After thawing, cells were stored and transported in RPMI medium with 10% human blood type AB serum. Just before EWOD experiments, the cells were spun down and resuspended in a serum-free buffer containing low concentration (0.01%–0.015% w/v) of surfactant Tween 20. For visualization, the CD8⁺ or CD8[−] cells were stained with either carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) or 5-chloromethylfluorescein diacetate (CMFDA), also known as CellTracker® Green dye from Invitrogen Inc. Both these dyes are fluorescein-based membrane-permeant dyes, containing diester groups that need to be cleaved by esterases within the cells for them to fluoresce.³⁰ The two dyes, CFDA-SE and CMFDA, were used interchangeably (based on the availability) as the properties of both these dyes are virtually identical for short-term experiments that do not involve proliferation studies, such as those in the present report. Although other dyes were considered, they were not used in the present report (further discussed in Sec. IV).

B. EWOD device fabrication

Typical UCLA EWOD fabrication processes³¹ were used to prepare the device (Fig. 2). EWOD electrodes were defined from a 1400 Å indium tin oxide (ITO) layer on a 700 μm thick glass substrate (TechGophers Inc.), named “Active” in the figure. Cr/Au ($\sim 100/1000$ Å) was deposited and patterned to define the contact pads for better electrical contact. Next, a Si₃N₄ layer (~ 1 μm) was deposited using plasma-enhanced chemical vapor deposition (PECVD) and patterned to define the dielectric layer. A Cytosol® (Asahi Inc.) layer (~ 1 μm) was spin-coated on top and annealed at 200 °C to make the surface hydrophobic. 1.1 mm thick glass substrates coated with ITO (1400 Å) (Delta Technologies Inc.) were used to fabricate the “Reference” substrate. A thinner PECVD Si₃N₄ layer (~ 1000 Å) was deposited and patterned on it to expose the ITO for electrical ground connection, followed by Cytosol® spin-coating and annealing (~ 1000 Å). A double-sided tape (~ 110 μm thick) was used as the spacer between the substrates.

FIG. 3. Confirmation of CD8+ cell binding to MBs, performed at macroscale (not using microfluidics) under EWOD-like conditions. Presented are flow-cytometry data obtained for lymphocyte distribution before and after magnetic separation using antibodies (for CD8 and CD3 membrane proteins) labeled with fluorescent dyes (APC and PerCP, respectively). In each figure, dots on the upper right quadrants, which have high APC as well as PerCP intensity, indicate CD8+ T-lymphocytes, while those on the lower right quadrants, having high PerCP but low APC intensity, indicate CD8- T-lymphocytes. (a) Before separation. (b) After magnetic separation for MB-cell binding done at 4 °C as per protocol, (c) After magnetic separation for MB-cell binding at room temperature in serum-free buffer containing Tween 20, as used during EWOD experiments. In both (b) and (c), most (>95%) of the cells collected are CD8+ T-lymphocytes, indicating that the collection efficiency under the EWOD-like conditions is similar to that under the protocol.

C. Device actuation and image capture

Droplet actuation was achieved by applying voltage (70–80 V_{ac}, 1 kHz) to EWOD electrodes. Electronic control for the actuation sequence was controlled using LABVIEW (National Instruments Inc.) with the help of a digital I/O device (DAQPad 6507, National Instruments), which allows 48 independent EWOD contact pads to be individually addressed. All the electrodes were kept grounded by default. Droplet movement was achieved by turning on (one or two) electrodes at the advancing edge of the droplet, while keeping those at the trailing edge of the droplet grounded. Mixing was achieved by moving the droplet around a circular path of the electrodes. Droplet cutting was achieved by turning on the electrodes at both the edges of the droplet, while keeping the middle few electrodes grounded.

Magnetic force was provided using a powerful rare-earth magnet [NdFeB, 0.5 in. in diameter, 0.5 in. thick, and surface magnetic field strength of 0.5–1 T (Ref. 12)] placed on top of the EWOD substrate (Fig. 2). The device was mounted on an inverted fluorescence microscope (Nikon TE-2000U) for visualization. A video camera (Panasonic KR-222) was used to capture the droplet actuation movies, while bright-field and fluorescence still images were taken using a cooled CCD camera (Photometrics Coolsnap EZ).

III. EXPERIMENTAL RESULTS

A. Test of protocol under EWOD conditions

Before doing the experiments on EWOD, conventional laboratory techniques were used to confirm key steps in the assay. Flow-cytometry measurements were performed to determine the isolation efficiency before [Fig. 3(a)] and after [Fig. 3(b)] cell separation according to the protocol,²⁹ i.e., at 2–8 °C, and in phosphate buffer saline (PBS) with 0.1% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid (EDTA). To ensure that MB-cell binding will also occur on the EWOD device, cell separation was also performed for binding conditions similar to EWOD [Fig. 3(c)], i.e., at room temperature, and in the serum-free buffer containing EDTA and Tween 20. CD8+ isolation efficiency was similarly high (>95%) under both conditions, suggesting that the MB-CD8 binding is not significantly affected by the conditions presented on a typical EWOD device.

B. Binding cells and MBs on chip by EWOD operation

Figure 4 shows the steps to evaluate the binding of cells with MBs by EWOD operation on the EWOD device in the schematic representation [Figs. 4(a)–4(j)]. To show the comparison between the binding of CD8+ [Fig. 4(a)–4(e)] and CD8- [Figs. 4(f)–4(j)] cells to the CD8- specific MB-Abs, the two types of cells were isolated before EWOD experiments, using the cell isolation

FIG. 4. Schematic representation comparing the binding of cells [CD8+: (a)–(e); CD8–: (f)–(j)] to MBs conjugated with anti-CD8 antibodies (MB-Abs), followed by magnetic collection on the EWOD device. Droplets containing the MB-Abs and cells [CD8+: (a) and (b); CD8–: (f) and (g)] are merged and mixed. The circulating flow inside the droplet leads to high interaction between the CD8+ cells and the MB-Abs specific to them (c). However, despite the high interaction between the CD8– cells and the MB-Abs, there is little binding (d). After the droplet is in position [CD8+: (c); CD8–: (h)], a magnet is introduced, collecting the MBs and the cells bound to them to the left edge of the droplet [CD8+: (d); CD8–: (i)]. The droplet is subsequently cut, collecting the MBs and the MB-bound cells in the left (collected) droplet, and leaving the right (depleted) droplet with only unbound cells [CD8+: (e); CD8–: (j)]. Bright-field image sequence [(k)–(o)] showing that the corresponding steps look virtually identical for the two cases.

protocol from Invitrogen Inc.²⁹ (as per the product manual for the CD8 Positive Isolation Kit, “isolated cells are...phenotypically unaltered and suitable for any downstream applications including... functional studies and cell culture”), and independently stained with a fluorescent dye explained later (in Sec. IV). Samples of each cell type were taken through the same steps on the EWOD device. In each case, droplets (~ 500 nl), one containing MB-Abs ($\sim 10^7$ /ml) and another containing fluorescently stained cells ($\sim 10^5$ /ml), were merged using EWOD [Figs. 4(a) and 4(b)]. The combined droplet was moved repeatedly over a circular path of the electrodes to allow MB-cell mixing. During the mixing, the specificity of the anti-CD8 antibody on the MB-Abs was expected to cause their preferential binding to the CD8+ cells [Figs. 4(b) and 4(c)] over CD8– cells [Figs. 4(g) and 4(h)]. After about 8–10 min, a permanent magnet is introduced on the left, attracting most of the MB-Abs to the left end of the droplet [Figs. 4(d) and 4(i)]. Earlier reports of MB separation on EWOD used meniscus-assistance¹² or performed separation immediately after sample introduction¹³ so as to overcome the lower collection efficiency due to surface adhesion forces. In the present report, we added a low concentration of surfactant as a chemical means of reducing the surface adhesion of the particles. The droplet is subsequently cut to form the collected droplet (left) and the depleted droplet (right) [Figs. 4(e) and 4(j)]. Figures 4(k)–4(o) show the corresponding bright-field image sequence for the above steps, which looks virtually identical for the CD8+ and CD8– cases.

Figure 5 shows the comparison between the cases of CD8+ cells [Figs. 5(a)–5(c)] and CD8– cells [Figs. 5(d)–5(f)]. Fluorescence images (superposed with the corresponding bright-field images to show the nonfluorescent features such as the droplet meniscus) are shown for the initial [Figs. 5(a) and 5(d)], the collected [Figs. 5(b) and 5(e)], and the depleted [Figs. 5(c) and 5(f)] droplets in each case. Cell collection efficiency is estimated by manually counting the fluorescent cells in each of these droplets.

In both cases, ~ 85 cells [Figs. 5(a) and 5(d)] were counted in each of the initial droplets. For CD8+ cells, ~ 81 cells were counted in the collected droplet [Fig. 5(b)], while ~ 2 cells were

FIG. 5. Comparison between binding of (fluorescently stained) CD8+ cells and (fluorescently stained) CD8− cells to the magnetic beads conjugated with anti-CD8 antibodies on the EWOD device. [(a)–(c)] CD8+ cells: (a) the initial sample contained ~ 88 cells. After MB-cell binding and magnetic collection, (b) collected droplet contained ~ 81 cells, while (c) depleted droplet contained only ~ 2 cells. Moreover, the fluorescence pattern appears over the MB-Abs in (b) the collected droplet, suggesting binding of the CD8+ cells to the MBs. [(d)–(f)] CD8− cells: (d) the initial sample contained ~ 81 cells. After MB-cell binding and magnetic collection, (e) collected droplet contained ~ 49 cells, while (f) depleted droplet contained ~ 29 cells. Very little fluorescence is seen over the MBs in (e), suggesting little binding between the CD8− cells and the MB-Abs.

counted in the depleted droplet [Fig. 5(c)]. For CD8− cells, in contrast, ~ 49 cells were counted in the collected droplet [Fig. 5(e)], while ~ 29 cells were counted in the depleted droplet [Fig. 5(f)]. The results are summarized in Table I and further discussed in Sec. IV.

C. Separation of CD8+ and CD8− cells on chip by EWOD

Figure 6 shows the ability to separate CD8+ from CD8− cells in the form of schematic representation [Figs. 6(a)–6(d)] and an image sequence [Figs. 6(e)–6(h)]. Figures 6(e)–6(h) are fluorescence images superposed with bright-field images to see the nonfluorescent features such as droplet meniscus in addition to the fluorescent cells. The initial sample ($\sim 1.5 \mu\text{L}$) contains fluorescently stained CD8+ cells conjugated with MBs, mixed with unlabeled CD8− cells ($\sim 10^5/\text{ml}$ in total) [Figs. 6(a) and 6(b)]. Upon the introduction of a magnet, the MB-CD8+ cells move to the left meniscus [Figs. 6(c) and 6(d)]. Fluorescence can be observed where MBs are collected (more clearly seen in the inset). The droplet is then cut using EWOD so that most MB-bound CD8+ cells are now collected in the left droplet [Figs. 6(e)–6(i)]. The number of fluorescent spots (only CD8+ cells) in the initial, collected, and depleted droplets is compared. Starting with ~ 85 CD8+ cells in the initial droplet, most (conservatively $\sim 90\%$) of these CD8+ cells were magnetically collected in the collected droplet, while very few CD8+ cells ($< 5\%$)

TABLE I. Summary of the results comparing the binding of CD8+ and CD8− T lymphocytes to anti-CD8 MB-Abs on EWOD.

Type of cells	No. in initial droplet	No. in collected droplet	No. in depleted droplet	% cells in collected droplet	% cells in depleted droplet	Volume % of collected droplet	Volume % of depleted droplet
CD8+	88 ± 2	81 ± 4	≤ 2	~ 92.1	~ 2.2	45.8	54.2
CD8−	81 ± 2	49 ± 3	29 ± 1	~ 60.4	~ 35.8	57.2	42.8

FIG. 6. Schematic representation [(a), (c), (e), and (g)] and superposed image sequence [(b), (d), (f), (h), and (i)] showing the separation of MB-bound and fluorescently labeled CD8+ cells from the unlabeled CD8- cells. The sample is placed on the EWOD device [(a) and (b)], and a magnet is introduced [(c) and (d)] to collect the MB-bound fluorescent CD8+ cells to the left [(d), inset]. With the magnet in place, EWOD microfluidic operations are used to stretch the droplet to the left [(e) and (f)]. Droplet is split by EWOD so as to magnetically collect the fluorescent CD8+ cells in the collected droplet [(g), (h), and (i)]. (Each pair of inset shows zoomed-in bright-field and fluorescence images of certain regions of the droplets.) As indicated by the fluorescence concentrated over the MBs at the left edge [(h), lower insets], most (conservatively $\sim 90\%$) of the fluorescently stained CD8+ cells are in the collected droplet. Unstained CD8- cells are divided between the collected [(h), upper insets] and depleted droplets [(i), insets], similar to Figs. 5(d)–5(f). On the other hand, the lack of fluorescence [(i), insets] indicates that few ($<5\%$) CD8+ cells are in the depleted droplet.

were left behind in the depleted droplet similar to the case in Figs. 5(a)–5(c). The unstained CD8- cells can be spotted in both collected and depleted droplets, which is similar to the results in Figs. 5(d)–5(f) (where the CD8- cells were stained to aid counting).

IV. DISCUSSION OF THE RESULTS

The main objective of the present work is to show the specific binding of CD8+ cells to MB-Abs and their subsequent magnetic concentration, all on the EWOD device by a sequential array of EWOD voltages. After verifying that the binding assay in the tube was not affected by the typical conditions encountered on the EWOD device, the MB-cell binding was tested on the EWOD device.

A. Further discussion of the experimental results

The results for the binding of both types of cells (CD8+ and CD8−) with MB-Abs are shown in Fig. 5 and summarized in Table I. In both cases, the decreased surface adhesion due to the added surfactant led to the accumulation of nearly all the MBs (>99%) to the left upon the introduction of the magnet, followed by the droplet splitting. However, the results for the cell collection were markedly different in the two cases. For the CD8+ cells case [Figs. 5(a)–5(c)], after the mixing and magnetic collection, about 92% of the cells was collected with the MBs in the collected droplet [Fig. 5(b)], while very few (~2%) remained in the depleted droplet [Fig. 5(c)]. (Some cells in the collected droplets may be hidden behind MBs, so the actual collection efficiency is expected to be higher. The counting error is conservatively estimated to be $\pm 5\%$ in the case of collected droplets and $\pm 2\%$ in other cases, i.e., initial and depleted droplets.) Moreover, the fluorescence pattern appeared to follow the pattern of the MBs distributed in the collected droplet [Fig. 5(b)], suggesting that the cells were bound to the MBs. The high collection efficiency is attributed to the high interaction between MBs and cells confined in the droplet with the circulating flow inside it,^{32–35} as compared to the usual flow through microfluidic systems typically having little mixing. Further optimization of the actuation steps, relative concentration, and time of incubation is expected to bring about shorter step duration and fewer MBs per cell without loss in collection efficiency.

On the other hand, after the same set of mixing with EWOD, magnetic collection, and droplet splitting steps were performed, there was no concentration of the fluorescence signal over the MBs [Fig. 5(e)], which indicates that there was little binding of the CD8− cells to the MBs. The distribution of the cells between the collected and the depleted droplets was roughly proportional to the volumes of the two droplets (Table I), as would be expected for nonspecific species (Some deviation from the exact proportionality to the volumes was also expected due to the hydrodynamically driven transport during the droplet splitting operation.³⁵)

In summary, while CD8+ cells are concentrated over the collected MBs in the collected droplet alone, the CD8− cells show no concentration over the collected MBs and get distributed between the collected and the depleted droplets. The results indicate that if anti-CD8 antibody-conjugated MBs are added to a sample containing CD8+ and CD8− cells, the MBs will be bound specifically to the CD8+ cells and not to the CD8− cells during the same operations, enabling their magnetic concentration on EWOD. As shown in Fig. 6, the MB-bound CD8+ cells could be magnetically collected in the collected droplet, while the CD8− cells (and other nontarget species) were removed via depleted droplets. Although only a sample set of experimental data is presented here, similar binding efficiency of CD8+ cells to the MB-Abs followed by their magnetic collection, and the lack of binding of CD8− cells to the MB-Abs was observed during repeated experiments.

By adding wash buffer droplets, this process can be repeated to serially dilute the nontarget species¹² while retaining the target species, viz., CD8+ cells [Fig. 1(e)]. An alternative technique to improve the purity of the target species could be to use a modified electrode layout to form slender fluidic conduits.³⁶

B. Discussion of the techniques used

The ideal way to show that the MBs bind specifically to CD8+ cells and not to CD8− cells would be to have two different dyes, one for each cell type, merge the mixed cells and MBs, and show the collection of only CD8+ cells on the MBs. The two dyes should be membrane-permeable and not surface binding so that they do not affect the binding of cells to the MBs. Also, they should have no spectral overlap (of the fluorescence excitation/emission wavelengths) with each other so as to avoid the false appearance of one in the other's image. Moreover, to avoid the nonspecific staining other than the cells (e.g., dirt, particles, etc.), it is desirable to use dyes that require enzymatic cleavage to fluoresce.

As mentioned before (in Sec. II), CFDA-SE (Ref. 37) and CMFDA are fluorescein-based membrane-permeant green fluorescent dyes containing diacetate groups that require enzymatic

cleavage by an esterase present inside the cells to fluoresce.³⁰ These properties are ideally suited for the present application to achieve specific fluorescent labeling of only the cells without interfering with the MB-binding sites on the membrane. However, it was hard to find a second dye having these characteristics as well as no spectral overlap with the above-mentioned dyes. For example, although the CellTracker® Red dye (CMPTX) has no overlap with the green dyes' spectra, it does not need enzymatic cleavage to fluoresce and had a greater tendency for nonspecific staining despite washing. Moreover, the autofluorescence from the MBs (Ref. 38) was found to interfere a lot more with the fluorescence signal of the red dye as compared to the green fluorescence signal, making it harder to quantify the red-labeled species. On the other hand, the CellTracker CMRA orange also does not fluoresce without enzymatic cleavage, but has too much spectral overlap with the green dyes' spectra.

Alternative labels having sharper and/or tunable absorption and emission spectra, such as quantum dots,³⁹ may be explored in the future to overcome the challenges due to undesirable fluorescence and expand the choice of label pairs that satisfy the above criteria. However, the introduction of quantum dot labels into cells is still under early investigation and not yet a well-established technique, with only very low intracellular levels being currently attainable.⁴⁰ During the present experiments, therefore, we only used the conventional green (and not the red) fluorescent dye, separately performing the same steps with stained CD8+ and stained CD8− cells to investigate the difference (Fig. 4) and hence the specificity of magnetic collection.

Since most of the EWOD actuation voltage drops across the dielectric layers, the applied voltage is not expected to have a significant effect on cell vitality.¹⁰ To achieve the EWOD manipulation of cell samples in an air environment or to enhance the collection efficiency of MBs, the process was helped by the addition of nonionic surfactant, in this case Tween 20. Although known to be lethal in high concentrations ($\sim 0.05\%$ w/v), cell viability on EWOD in air environment with nonionic surfactant additives has been demonstrated in reports, where cell viability studies were explicitly carried out.¹⁰ Although cell viability data are not explicitly reported here, the dyes (CFDA-SE and CMFDA) used are “living cell dyes” (typically used for cell proliferation studies³⁰) since they require enzymatic cleavage occurring inside living cells to fluoresce. The concentration of Tween 20 used in the present results did not seem to have a noticeable effect on cell viability, at least for the duration of the relatively short experiments, as suggested by the continued fluorescence in cells several minutes after the experiment. (Since the present application requires the eventual lysis of cells, their long-term vitality is not a major concern.) Other nonionic surfactants such as pluronic F68 have also been used to actuate cell samples, while being quite “gentle” to the cells,¹⁰ and may be used instead to ensure better cell vitality. Also, due to the concern that the protein albumin present in the serum is notorious for irreversibly fouling⁴¹ the hydrophobic EWOD surface, we avoided its use in the present EWOD experiments. However, it has been reported that the media containing up to 10% fetal bovine serum w/v, which is much more favorable for cell survival, could be actuated using pluronic surfactant F68 in the sample.¹⁰ The use of pluronic F68 instead of Tween 20 in the future experiments therefore appears quite promising. However, in our preliminary experiments, we found increased nonspecific binding to the MBs when we replaced Tween 20 with pluronic F68. Further investigation would be needed to find an optimized protocol for the assay using pluronic F68.

All experiments in this report were performed using air as the surrounding medium. When the device is immersed in oil^{1,42,43} rather than dry in air,^{3,31,44,45} a thin layer of oil present between the hydrophobic device surface and the aqueous droplet⁷ greatly reduces the resistance against droplet sliding, making most of the basic EWOD operations easier. The thin oil layer also separates the particles in the droplet from the device surface, preventing their adhesion on the surface.^{7,46} In addition, the surrounding oil helps reduce evaporation of the EWOD droplets, helping to maintain their size and concentration. Despite the conveniences, there have been some concerns regarding the use of silicone oil, particularly in biological applications,¹² leading to the choice of air as the surrounding medium. Droplet evaporation on the EWOD device in the air environment can be minimized by sealing the gap between the device chips as simple as using a sealing tape.⁴⁷ Although not utilized in the present experiments, which were relatively short (~ 15 min), signifi-

cant prevention of evaporation during much longer experiments (a few hours, as opposed to several minutes in our case) with cells can be achieved through the use of a humidified environment.¹⁰

V. CONCLUSIONS

We have shown the selective binding of CD8+ cells to magnetic beads and their subsequent magnetic collection on the EWOD device using pure electric signals as important steps toward cell-based assays on a portable lab-on-a-chip device. The circulating flow inside the droplet led to excellent mixing and a high collection efficiency. Specificity of binding was demonstrated by comparing results for CD8+ and CD8− cells. Although shown here for the specific selection of CD8+ cells, which are important for monitoring organ transplant, the same technique can be extended to concentrate other cell subpopulations (e.g., CD4+ lymphocytes,⁴⁸ pathogenic bacteria,⁴⁹ tumor cells,⁵⁰ etc.), often associated with specific diseases such as cancer⁵¹ and multiple myeloma,⁵² by using the appropriate surface modification on the MBs.

Future work will focus on starting with less preprocessed blood derivatives as samples so as to move closer to the laboratory protocol used to monitor organ transplant rejection. Integration of other functions, such as cell-lysis and protein detection, will also be necessary for the complete diagnostic device.

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Summary of acronyms

CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CMFDA	5-chloromethylfluorescein diacetate
FBS	Fetal bovine serum
IRB	Institutional Review Board
ITO	Indium tin oxide
MB	Magnetic bead
MB-Ab	Magnetic bead conjugated with antibody
PECVD	Plasma-enhanced chemical vapor deposition
RPMI medium	Roswell Park Memorial Institute medium
EDTA	Ethylenediaminetetraacetic acid
PBS	Phosphate buffered saline

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