Electronic Supplementary Information

Parallel Affinity-based Isolation of Leukocyte Subsets using Microfluidics: Application for Stroke Diagnosis

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EXPERIMENTAL

**Contact angle measurements.** Sessile drop contact angle measurements were performed using a VCA 2000 contact angle system (AST Products). Two µL of 18 MΩ-cm deionized water was dispensed onto substrates, images were collected and contact angles were measured using the manufacturer’s software. Five measurements were averaged for all reported values.

**Fluorescence imaging.** Cy3-labeled oligonucleotides (40 µM) modified with an amino group at their 5’ end were suspended in PBS buffer (100 mM, pH = 7.4) containing EDC (20 mg/mL) and incubated overnight at 4°C within the appropriately activated microfluidic device. The chip was then rinsed with ~2 mL of 0.1% SDS and PBS to remove unreacted molecules. All images were acquired (230 ms acquisition time) with a 200M inverted microscope (Zeiss) that contained an XBO 75 W Xe arc lamp, a single band filter set (Omega Optical) and a Cascade 1 K EMCCD camera (Photometrics). Images were analyzed using Image-J software.

**Quantification of carboxylic acid surface densities.** An incubation chamber (BioRad) was glued to the substrate’s surface and filled with 0.1% (w/v) toluidine blue O (TBO) in carbonate buffer (50 mM, pH = 10.5). After 15 min, the substrate was submersed in the same buffer for 15 min and air dried. TBO was desorbed using 40% acetic acid (d = 1.0196 g mL⁻¹), collected in a pre-weighed microfuge tube and analyzed with an Ultrospec 4000 UV/Vis spectrophotometer (Pharmacia Biotech) against a 40% acetic acid blank.

**Microfluidic device fabrication and assembly.** Microfluidic devices were hot embossed into COC or PMMA substrates via micro-replication from a metal mold master as previously reported.¹ Two architectures (see Figure S1) were tested in these studies, both possessing four independent cell isolation beds comprised of parallel arrays of curvilinear cell isolation channels with an 8 x 13 mm² footprint.² Prior to device assembly, inlet and outlet reservoirs (d = 0.85 mm) were drilled into both chip designs followed by cleaning with 10% Micro-90 and IPA and then drying at 60°C. A 250 µm thick cover plate and the device substrate were activated for 15 min in a home-built UV chamber equipped with a low pressure Hg lamp (22 mW/cm² at 254 nm) to generate carboxylic acid scaffolds on the microchannel surfaces.³ Thermal fusion bonding of the cover plate to the substrate was performed at 132°C and 100°C (~25 min) for UV-modified COC and PMMA devices, respectively. PEEK tubing of 175/335 µm id/od (IDEX Health &
Science, LLC) was inserted into the reservoirs and glued in place with epoxy. A constant flow programmable syringe pump (Harvard Instruments) was used to introduce fluids into the devices.

![Figure S1](image)

**Figure S1.** An autoCAD drawing of (A) the bifurcated device with the cell isolation bed containing 16 curvilinear channels spaced by 330 µm. (B) Z-configuration isolation bed containing 64 curvilinear channels spaced by 200 µm. All curvilinear channel dimensions were 25 × 80 µm (w × h).

Common to each device, the cell isolation beds consisted of curvilinear channels, which had a channel depth of 80 µm to reduce pressure drop along the channel length and the risk of channel blockage. All the channels had a nominal width of 25 µm; the width was not reduced further to accommodate passage of the largest leukocytes (*i.e.*, neutrophils).4

**Blood processing using the microfluidic device.** The microfluidic device was connected to a syringe pump with PEEK tubing via a luer lock syringe connector (Hamilton, Reno, NV). A pre-isolation rinse of the chip was performed using PBS/0.5% BSA at 4 mm/s. Fifty µL of whole blood was infused at 3.8 µL/min and 15.3 µL/min for the bifurcation and Z-configuration devices, respectively, yielding nominal linear velocities of 2 mm/s within the isolation beds. A post-isolation rinse was performed at 4 mm/s with 100-200 µL PBS/0.5% BSA to remove blood and unbounded cells. Affinity-bound cells were fixed on-chip with 4% PFA/PBS for 15 min and then rinsed with 50 µL PBS/0.5% BSA. Surface antigens of T-cells and neutrophils were stained with anti-CD3-FITC Abs and anti-CD66b-FITC Abs, respectively, in the dark for 1 h. Then, the chip was rinsed with PBS/0.5% BSA. Cells were permeabilized with 0.1% Triton-X for 5 min and nuclei stained with 1 µg/mL DAPI in PBS for 5 min followed by a rinse with 50 µL PBS/0.5% BSA at 8 µL/min. Finally, cells were identified based on staining results with the total number of isolated cells determined by scanning across the cell selection regions using a fluorescence confocal microscope.
Polymerase Chain Reaction (PCR). PCR was performed with cDNA in a total volume of 20 µL using Taq 2X Master Mix (New England Biolabs, Ipswich, MA). PCR cocktails consisted of 2 µL of primers (final concentration 500 nM), 10 µL Taq 2X Master Mix, 6 µL nuclease free water and 2 µL cDNA. PCR was carried out in a commercial thermal cycler (MJ Research Inc., Waltham, MA) with the following steps: Initial denaturation at 94°C for 2.5 min followed by 40 cycles of denaturation at 94°C for 15 s; annealing for 30 s at 63°C for GAPDH primers and 55°C for S100A9 primers; and extension at 72°C for 30 s. A final extension at 72°C for 7 min was followed by a cooling step at 4°C. All primers were obtained from Integrated DNA Technologies, Inc. The sequences (5’-3’) were GAPDH (Gene ID-2597): forward (F) – CTTTTGCGTCGCCAGCGAG; reverse (R) – TGACCTTGCCAGGGGTGCT. S100A9(Gene ID-6280) F – CTCGGCTTTGACAGAGTGCAAGAC; R – TCCCCGAGGCGCTGCTATGG. TCRB (Gene ID-6957) F – GTTTTCTTTCACTGCTGGCTTC; R – CACGGCATAAGGGTGCC; and FPR1(Gene ID-2357) F – TTTGCCTGTAACGCCAC; R – ATGCTCTATGTC TTCATGGG (www.ncbi.nih.gov).

PCR products were electrophoresed at 4.8 V/cm in 1X TBE (Tris–boric acid/EDTA, Bio-Rad Laboratories) on a 4% agarose gel with ethidium bromide (Lonza) staining. Amplicons were indexed against a DNA sizing ladder 50 – 766 bp (New England Biolabs, Ipswich, MA). Images were collected using a Logic Gel imaging system (Eastman Kodak Company).

Fluorescence staining of MOLT-3 cells and flow cytometry analysis. Prior to staining of the MOLT-3 cells for determining CD4 expression, Fc receptors were blocked with 5 µL of 1 mg/mL human IgG-Fc blocker (R&D Systems, Minneapolis, MN) and incubated at 4°C for 15 min. The following samples were prepared for analysis: (i) Unstained cells serving as an autofluorescence control; (ii) cells stained with 10 µL of 0.1 mg/mL isotype control, IgG1-FITC mAbs ( R&D Systems, Minneapolis, MN) and incubated in the dark at 4°C for 30 min; and (iii) cells stained with 10 µL of 0.1 mg/mL CD4-FITC Abs (BD Biosciences, Franklin Lakes, NJ) and incubated in the dark at 4°C for 30 min. Cells were then washed three times: One mL of cold PBS-0.5% BSA was added; the cells were centrifuged for 4 min at 350x g; supernatants were decanted and resuspended in 1 mL PBS. Propidium iodide staining (1 µg/mL) was performed on all the samples (except for the autofluorescence control) just prior to the analysis for the determination of cell viability as detected by red fluorescence. Samples were then analyzed on a
RESULTS AND DISCUSSION

Figure S2. T-cell recoveries from healthy donor blood using UV-COC_{bif} devices modified with anti-human CD4 Abs at different concentrations.

Optimization of antibody concentration for cell isolation. To ensure Ab concentration was not a limiting factor and cell recovery results reflected the number density of the generated surface-confined carboxylic acids, we quantified cell recovery as a function of the antibody concentration in the UV-activated COC bifurcated devices (UV-COC_{bif}). The concentration of anti-CD4 Abs was varied at 0.1, 0.25, 0.5, and 1 mg/mL. Figure S2 suggested reduction in recoveries when Ab concentrations were set <0.5 mg/mL and comparable recoveries between 0.5 and 1 mg/mL. Therefore, 0.5 mg/mL was selected to be the optimal concentration for Ab immobilization for all subsequent experiments.
Figure S3. Line plots created from 20X fluorescence images for (A) PMMA and (B) COC curvilinear channels modified with 40 μM 3'-Cy3-labeled, 5'-amino modified oligonucleotides that were covalently bound to surface-confined carboxylic acids generated by UV (grey lines) or chemical activation (black lines) of the polymer surface. For controls (dotted lines colored corresponding to their treatments), fluorescently-labeled oligonucleotides were immobilized to identical surfaces without EDC coupling reagents to measure nonspecific adsorption and autofluorescence. UV activation was identical for the two polymers, but chemical activation protocols differed in terms of incubation times; a 5/1 (v/v) 2 M NaOH/IPA solution was incubated at 65°C for 30 min for PMMA devices and 12 h for COC devices. Fluorescence images of oligonucleotides immobilized to PMMA devices that were chemically treated with (C) 1/1 (v/v) 2 M NaOH/IPA for 3 min at 60°C and (D) 1/1 (v/v) 2 M NaOH/IPA for 10 min at room temperature, and (E) 5/1 (v/v) 2 M NaOH/IPA for 5 min at 60°C.

Optimizing chemical activation of PMMA and COC microchannels. We investigated the ability to activate assembled PMMA and COC microfluidic devices by filling the channels with an activating chemical solution as this could potentially produce more uniform modification throughout the channel cross-section and avoid thermal effects that would bury surface functional groups for UV-activated devices. As can be seen from Figure S3A, PMMA surfaces showed higher levels of fluorescence for the UV activated material compared to the chemically activated substrate. However, in the case of COC (Figure S3B) much higher levels of fluorescence were observed in the case of chemical versus UV activation. It has been reported that PMMA’s ester groups can be hydrolyzed to carboxy groups by incubation with 10 M NaOH at 40°C for 16 h. However, the covalent attachment of Cy3-labeled oligonucleotides containing a pendant amino group serving as a fluorescent reporter of surface-confined carboxylic acids did not indicate carboxylic acid group formation using this procedure. We hypothesized that the
addition of IPA (similar to methanol by Patel et al.⁷) would encourage interaction with the hydrophobic polymer (water contact angle = 76.4 ±1.4°) and/or solvation of the methoxide byproducts. Treatment of PMMA microfluidic channels with 1/1 (v/v) 2 M NaOH/IPA for 3 min at 60°C and 10 min at room temperature resulted in some channel roughening and deformation (Figures S3C, D). However, it was found that channel integrity was preserved up to 30 min at 65°C in 5:1 (v:v) 2 M NaOH:IPA (Figure S3E). Thus, we restricted PMMA modification using the aforementioned conditions to 30 min using 5:1 NaOH:IPA.

Flow uniformity in bifurcated and Z-configuration microfluidic devices. The uniformity of flow in the two devices, which consisted of four selection units with each unit containing an array of parallel, curvilinear isolation channels addressed by either a bifurcation or Z-configuration, were studied using fluid dynamic simulations and video microscopy. COMSOL Multiphysics 4.3a was utilized⁸ to confirm uniform flow distribution within the devices. The velocity field in Figure S4A indicated that flow was uniformly distributed within each bifurcated, cell isolation bed (Figure S4B) and also between the four beds. Furthermore, because the Z-configuration device employed the same addressing architecture for the four isolation beds as the bifurcation device, the flow was uniformly distributed between the four Z-configuration cell isolation beds, but the flow was slightly non-uniform within each bed (Figure S4B).

The flow distribution simulations were compared to empirical observations acquired by filling the devices with red dye solution.⁹ Time lapse images were extracted from the videos (Figure S5). As shown in the images depicted in Figures S5A-D, the dye front was nearly linear across the four bifurcation beds indicating uniform flow between and within the bifurcation beds, which agreed with the simulation results (Figures S4A and S4B). Flow was uniform between the Z-configuration isolation beds, but showed a parabolic profile within each bed (Figure S4B). As shown in the images depicted in Figures S5E-G, dye filled more rapidly in the outer channels than the central channels, similar to that depicted in Figure S4B. Similar flow profiles were observed for filling devices with blood. These data agreed with previous results using a similar type of device, but consisting of a single cell isolation bed.⁹
Figure S4. (A) COMSOL simulation showing the velocity field of buffer throughout the bifurcated cell isolation device. (B) Linear velocities normalized to an average of 2 mm/s acquired by (filled bars) 14 groups of the parallel channels in the Z-configuration device based on the results depicted in Figure S5E-H and (solid line) compared to theoretical predictions via numerical simulation (solid black line). Dashed line indicates the distribution of linear flow velocities through all four beds of the simulated bifurcation device shown in Figure S4A.

Figure S5. Time-lapse images of dye filling the (A-D) bifurcated device and (E-H) Z-configuration device. Images A-D and E-H were taken at 0, 15, 30, and 60 s, respectively.

Determination of CD4 expression of MOLT-3 cells by flow cytometry. Expression of CD4 antigen on MOLT-3 cells was measured after subtracting isotype control-FITC and CD4-FITC double positive events as shown in the histograms in Figure S6. This method indicated that only 26% of MOLT-3 cells were CD4+, which is in agreement with data reported by Greenberg et al.\textsuperscript{10} The low fluorescence signal observed for CD4+ MOLT-3 cells in flow cytometry (Figure S6) indicated these cells have much lower CD4 expression than T-cells (46,000-202,000 CD4 molecules/cell; mean = 100,000 molecules/cell).\textsuperscript{11-13}
Recovery of leukocyte sub-populations. Initial recovery tests were performed with MOLT-3 cells (average cell diameter 9.3 ±2.4 µm) using isolation beds modified with anti-CD4. However, we observed very poor recovery of these cells; 0.36 ±0.3% for UV-COC$_{bif}$, 0.13 ±0.1% for CH-COC$_{bif}$ and 0.52 ±0.1% for CH-COC$_{Z}$. After correcting for the CD4-MOLT-3 expression from the flow cytometry analysis (see Figure S6), the recoveries were 1.4 ±0.3 %, 0.5 ±0.1% and 2.0 ±0.1%, for the UV-COC$_{bif}$, CH-COC$_{bif}$ and CH-COC$_{Z}$ devices, respectively. These efficiencies were lower than that observed for the human T-cells (~10%), but exhibited similar patterns between the activation modality and device design; chemically activated COC$_{Z}$ devices isolated the highest absolute number of cells.

The two primary factors contributing to the low recovery of MOLT-3 cells were likely the small cell size relative to the channel width and the low CD4 expression levels for these cells. Because the 9.3 ±2.4 µm diameter MOLT-3 cells were isolated in 25 µm wide channels, we would expect low recovery as there is a low probability of cell/surface-confined Ab interactions. Reduction in channel width to slightly larger than the MOLT-3 cell diameter should improve their recovery. However, the use of much narrower channels may be problematic for blood processing due to the risk of device failure arising from clogging because of the presence of large leukocytes (~15 µm). If the Ag expression level is low, shear forces in the channel may also remove isolated cells from the selection bed due to the small adhesion force and thus, produce
lower recovery. These considerations were also true for human T-cell isolation from whole blood; in spite of their smaller size (~7 µm average diameter) compared to MOLT-3 cells, these cells showed higher recovery most likely because of the higher expression of CD4 producing better cell/surface adhesion forces.

**Raw data for recovery of leukocyte subsets.** We processed 50 µL of whole blood using both the Z- and bifurcation configurations selecting T-cells using anti-CD4 antibodies and neutrophils with anti-CD66b antibodies. The results for this investigation are shown in Table S1 along with the standard deviations. See main text for a discussion of this data.

**Table S1.** Enumeration values for isolated T-cells and neutrophils from whole blood using COC and PMMA microfluidic devices with different geometries and activation protocols.

| Cell type     | Chip Design | Modification-substrate | Cell count (n= 4) Mean ± SD | Negative control | Purity |
|---------------|-------------|------------------------|----------------------------|------------------|--------|
| **T-Cells**   | Bifurcated  | UV-PMMA                | 325 ±85                   | ---              | ---    |
|               |             | UV-COC                 | 798 ±168                  | 19 ±5            | 97 ±1  |
|               |             | CH-PMMA                | 7 ±3 (8)                  | ---              | ---    |
|               |             | CH-COC                 | 703 ±386                  | 16 ±3            | 92 ±5  |
| **Neutrophils** | **Z-configuration** | UV-COC                 | 918 ±91                   | ---              | 94 ±3  |
| **Neutrophils** | **Z-configuration** | UV-COC                 | 1096 ±537                 | ---              | ---    |
|               |             | CH-COC                 | 2949 ±901                 |                  | 97 ±2  |
| **T-cells**   | Z-configuration | UV-COC                 | 596 ±154                  | 7 ±1             |        |
|               |             | CH-COC                 | 2565 ±1194                | 30 ±9            | 98 ±1  |
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