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Anal Chem. Author manuscript; available in PMC 2011 January 18.

Published in final edited form as:
Anal Chem. 2005 February 1; 77(3): 933–937. doi:10.1021/ac049037i.

Biomimetic Autoseparation of Leukocytes from Whole Blood in a Microfluidic Device

Sergey S. Shevkoplyas[*,†], **Tatsuro Yoshida**[†], **Lance L. Munn**[*,‡], and **Mark W. Bitensky**[*,†]
Visual and Circulatory Biophysics Laboratory, Department of Biomedical Engineering, Boston University, 36 Cummington Street, Boston, Massachusetts 02215, and E. L. Steele Laboratory for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, 100 Blossom Street, Boston, Massachusetts 02114

Abstract

Leukocytes comprise less than 1% of all blood cells. Enrichment of their number, starting from a sample of whole blood, is the required first step of many clinical and basic research assays. We created a microfluidic device that takes advantage of the intrinsic features of blood flow in the microcirculation, such as plasma skimming and leukocyte margination, to separate leukocytes directly from whole blood. It consists of a simple network of rectangular microchannels designed to enhance lateral migration of leukocytes and their subsequent extraction from the erythrocyte-depleted region near the sidewalls. A single pass through the device produces a 34-fold enrichment of the leukocyte-to-erythrocyte ratio. It operates on microliter samples of whole blood, provides positive, continuous flow selection of leukocytes, and requires neither preliminary labeling of cells nor input of energy (except for a small pressure gradient to support the flow of blood). This effortless, efficient, and inexpensive technology can be used as a lab-on-a-chip component for initial whole blood sample preparation. Its integration into microanalytical devices that require leukocyte enrichment will enable accelerated transition of these devices into the field for point-of-care clinical testing.

The rapidly expanding field of lab-on-a-chip microanalytical devices promises inexpensive, portable, miniaturized tools that could potentially revolutionize clinical and basic research analyses by dramatically reducing sample size and handling.[1-3] An important application of this technology is the analysis of leukocytes (white blood cells) or their contents, for which these cells must first be isolated from the whole blood sample.[4-7]

Blood is a 45% suspension of cells (erythrocytes, platelets, leukocytes) in plasma. Erythrocytes (red blood cells) constitute the vast majority of all blood cells and are normally ~1000 times more abundant than leukocytes. Traditionally, several milliliters of whole blood are drawn and then centrifuged in order to separate blood cells of different density. Plasma, platelets, and white and red cells can be separated by this procedure, but it is a labor-, energy-, and time-intensive process that relies on special equipment and requires trained personnel.[8,9] Many current microanalytical devices have no integrated sample

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* To whom correspondence should be addressed. E-mail: (S.S.S.) strust@bu.edu; (L.L.M.) lance@steele.mgh.harvard.edu; (M.W.B.) mwb@bu.edu..
† Boston University.
‡ Massachusetts General Hospital and Harvard Medical School.

SUPPORTING INFORMATION AVAILABLE

A video clip in QuickTime format showing dynamics at the entry into the extraction channel. This material is available free of charge via the Internet at http://pubs.acs.org.

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preparation capabilities and continue to rely on this conventional off-the-chip technique. Currently available methods that can be used for on-the-chip enrichment of leukocytes from whole blood are often complicated, lack versatility, offer low efficiency, or require preliminary modification of cells.[3,6-8,10] Thus, development of simple and efficient methods to isolate leukocytes directly from whole blood remains an important task.

Because of its particulate nature, blood exhibits unique flow characteristics on the scale of the microcirculation.11 Erythrocytes, which are smaller and more deformable than leukocytes, tend to flow at the center of blood vessels, leaving a plasma-rich zone adjacent to the vessel wall;12 they also flow faster than leukocytes.[13] This encourages mechanical interactions (collisions) between leukocytes and erythrocytes that influence the leukocyte distribution across the vessel lumen[13,14] and among vessels of the microcirculation in vivo.[15,16] In vessels larger than ~30 μm in diameter, collisions with passing erythrocytes force leukocytes to migrate toward the vessel wall in a process known as margination[13,17,18] and help to initiate leukocyte rolling in post-capillary venules.[19-22] The tendency of erythrocytes to concentrate at the center of the blood stream also leads to plasma skimmings an uneven distribution of erythrocytes and plasma among the two daughter vessels of any nonsymmetrical bifurcation:[23] the vessel that receives less flow receives a disproportionately smaller fraction of erythrocytes and, therefore, has a lower hematocrit.

Previously, we used silicon micromachining and poly(dimethylsiloxane) (PDMS) replica molding techniques[24-27] to fabricate microchannel networks with dimensions and topology similar to those of the actual microcirculation.24 Using whole human blood, we were able to reproduce and document a number of scale-specific microcirculatory phenomena in such networks.

Here, we describe the operation principle and demonstrate the efficiency of a simple microfluidic device that takes advantage of the natural flow properties of blood (leukocyte margination and plasma skimming) to separate leukocytes from whole blood. It consists of a network of rectangular microchannels of specific geometry and connectivity and requires only a small pressure gradient to produce a 34-fold enrichment of the leukocyte-to-erythrocyte ratio in a single pass. The unique biomimetic design of this microchannel device allows, in fact, autoseparation of leukocytes from the rest of the blood. This novel leukocyte enrichment technique could be used to design custom lab-on-a-chip components for integrated sample preparation in microanalytical devices that require leukocytes, their DNA, or RNA.[3,6,7]

EXPERIMENTAL SECTION

Microfabrication

Fabrication of microchannel networks and the experimental setup were previously described in detail.[24,25] Briefly, a silicon wafer containing a negative image of the microchannel device was created at the CNF (Cornell Nanofabrication Facility, Cornell University, Ithaca, NY) using electron beam lithography (EBMF-10.5/CS, Cambridge Instruments) and reactive ion etching (Bosch process, Unaxis SLR 770 ICP Deep Silicon Etcher, Unaxis USA Inc., St. Petersburg, FL) techniques. This master wafer was then used as a negative mold to cast replicas of the device in PDMS (RTV 615 A/B; G. E. Silicones, Waterford, NY). Each of these cast replicas was then trimmed to size and affixed onto a predrilled, PDMS-coated glass slide (Micro Slides; VWR Scientific, West Chester, PA) to form the microchannel device. Before assembly, all contact surfaces were exposed to air plasma (Plasma Cleaner/Sterilizer, Harrick Scientific Corp., Ossining, NY). This treatment generates silanol (Si–OH) groups on exposed PDMS surfaces, which provides permanent binding between the cast microchannels and the PDMS-coated glass slide; it also makes the microchannel wall

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surfaces hydrophilic.[27] Assembled microdevices were flushed with 1% (w/v) aqueous solution of mPEG-silane (Shearwater Polymers, Inc., Huntsville, AL) to prevent cell adhesion and then washed with GASP buffer (1% BSA, 9 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mM NaCl, 5.5 mM glucose, pH 7.4, osmolarity 290 mmol/kg).

Setup

The microchannel device assembly was mounted in a custom holder on a calibrated motorized microscope stage (BX-51, Olympus America Inc, Melville, NY). A water column was used to provide the operational pressure gradient of 5−40 cmH_2O across the microchannel flow system. Sequences of images were acquired with a CMOS digital camera (Silicon Video 2112, Epix Inc., Buffalo Grove, IL) using a wide band-pass blue-violet filter (407 ± 52 nm, Edmund Industrial Optics, Barrington, NJ) to improve contrast. Images were recorded and then analyzed off-line using custom software (designed using Matlab 6.5, The Math Works Inc, Natick, MA) to determine white cell distributions and the leukocyte-to-erythrocyte ratios in the extraction channel. All microchannels of the device had the same depth of 10.3 μm. Blood samples were introduced into a feeding reservoir incised in the PDMS replica to provide access to the channels. Blood exited the device through a 2-mm hole drilled through the glass slide and then drained into a waste collection reservoir. In the configuration described here, 15−70 μL of blood (depending on the size of the feeding reservoir) is required for operation.

Samples

Whole human blood was collected by venipuncture from healthy consenting volunteers into Vacutaner tubes containing EDTA (10 mL, 17.55 mg of (K_3)EDTA, BD, Franklin Lakes, NJ) similar to the standard clinical procedure. Initial red and white cell concentrations in whole blood were determined in duplicate using a Sysmex K-1000 automated cell counter (Sysmex Corp. of America, Long Grove, IL). Blood samples were used within 4 h of collection.

RESULTS AND DISCUSSION

The microseparation device measures ~0.7 × 7 mm and consists of a simple network of microchannels schematically shown in Figure 1. Flow of blood through the device is driven by a small pressure gradient (~40 cmH_2O). The device operates as follows:

Whole blood enters the device through a 70-μm-wide supply channel. Initially, leukocytes are distributed uniformly across the channel (Figure 1, 1). As smaller, more flexible erythrocytes seek the faster flow region in the center of the channel lumen,[28] they collide with leukocytes forcing them to migrate toward the sidewalls.[18] Therefore, before the first bifurcation is reached, the leukocyte distribution across the channel changes considerably, with most of the leukocytes traveling near the channel sidewalls (Figure 1, 3).

This phenomenon of leukocyte margination in the supply channel appears more dramatic than that observed in real blood vessels. The depth of all channels comprising the device (10.3 μm) approximates the average leukocyte diameter (10−12 μm), such that out-of-the-plane motion of leukocytes is effectively restricted. Due to this pseudo-2D geometry, practically all erythrocyte–leukocyte collisions result in lateral migration of the leukocytes toward the nearest sidewall. In contrast, margination in real blood vessels is less efficient because migration of leukocytes is not constrained to any preferential direction. Therefore, when leukocytes marginate in vivo, they approach the vessel wall via a random walk (that reflects collisions with erythrocytes from multiple angles) and arrive at points scattered around the vessel perimeter.[18]

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The concentration of leukocytes in the blood immediately after entering the supply channel (2100/μL, Figure 1, 1) is less than half that in the original whole blood sample (4300/μL). This is because erythrocytes and plasma flow more easily than leukocytes from the sample reservoir, effectively lowering leukocyte concentration. This phenomenon likely reflects specific design features of the current sample introduction system.

As leukocytes marginate to the sidewalls, they begin to flow slower than the rest of the blood, because of the shape of the velocity profile in the channel.[29-31] This results in gradual accumulation of leukocytes traveling adjacent to the sidewalls and thus leads to the increase of overall leukocyte concentration along the supply channel (Figure 1, 1−3). A flux balance shows that the leukocyte concentration (C) ratio between any two points along the channel is determined by the average leukocyte velocities (v_i): $C_2/C_1 = v_1/v_2$. In the case shown in Figure 1, the velocity ratio is ~2; i.e., the concentration of leukocytes increases ~2-fold along the supply channel, reaching 4500/μL (Figure 1, 3). Note that this value is almost equal to that in the original sample. Thus, the gradual accumulation of leukocytes along the supply channel compensates the 2-fold reduction of leukocyte concentration caused by dynamic dilution at the entry into the channel.

After passing through the first bifurcation, leukocytes continue to travel near the outside wall of each daughter channel. These two channels have equivalent asymmetric leukocyte distribution profiles, and either can be used for further processing. The asymmetry in leukocyte distribution in segment 4 causes the majority of the leukocytes to enter segment 6 of the second bifurcation, with very few entering segment 5 (data not shown). This further increases the leukocyte concentration in segment 6 by ~2-fold. The asymmetry persists here, with most leukocytes remaining near the right-hand sidewall.

The main part of the leukocyte enrichment device consists of a 135° bend 6 with a small extraction channel 8. The bend alters the velocity profile[32] causing erythrocytes to move quickly around the inside, bypassing leukocytes that travel more slowly in the plasma-rich region near the sidewall. As the erythrocytes pass the slower leukocytes, they trap them near the wall and propel them into the extraction channel (Figure 1, asterisk; movie S-1, Supporting Information). Approximately 67% of all leukocytes from the segment 6 enter the extraction channel 8; the rest continue along the right-hand sidewall of the adjacent, larger channel 7 with a characteristic asymmetric distribution (Figure 1). Compared to the initial whole blood sample, the leukocyte concentration in the extraction channel 8 is increased ~10-fold, achieving 42 300/μL.

Both leukocyte margination and plasma skimming are principally important for the enrichment function of the device: accumulation of leukocytes near the sidewall in the plasma-rich region causes ~2/3 of them to enter the extraction channel; at the same time, plasma skimming reduces the erythrocyte concentration in the extraction channel to less than 1/3 of its initial value. Note that “plasma skimming” in this context differs somewhat from its conventional definition due to the high concentration of leukocytes in the plasma rich region. The combined effect of these phenomena translates into an increase in the leukocyte-to-erythrocyte ratio from 1:1100 in the original blood sample to 1:32 in the extraction channel (Figure 1, 8)—a 34-fold enrichment.

Any practical microanalytical device for point-of-care use must incorporate a robust and convenient way to prepare a sample directly on the chip to minimize the required raw sample size and potential user-induced errors.[1-3] Most current prototypes, however, concentrate more on the analysis and quantification steps, rather than integrated sample preparation procedures, either because sample preparation is often the most difficult[33] or least exciting[7] part of the assay.[10] Thus, even though such devices generally require only

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submicroliter sample volumes for analysis, off-the-chip sample preparation via conventional centrifugation may require several milliliters of blood.[8,9]

Currently available approaches to on-the-chip sample preparation are based on filtration[4,8,33] or dielectrophoresis (DEP)[34] or require preliminary cell modification steps such as fluorescent labeling[35,36] or tagging with magnetic beads.[37,38] The current state of the art of these and other sample preparation methods is the focus of recent reviews.[3,6,7,10]

The new leukocyte enrichment method described here has many important advantages over these technologies. It is simple, has no active (moving or otherwise) elements, requires only a small hydrostatic pressure gradient to function, and can be easily manufactured using conventional microfabrication. Further, it operates on (anticoagulated) whole blood, actually benefiting from physiological levels of hematocrit—the same factor that confounds other sample preparation techniques. It provides positive selection of leukocytes, which are not trapped in a specific area of the chip (in contrast to both filtration33 and DEP34) but continue to flow and can be easily transported elsewhere on the chip for further purification or directly to analytical unit(s) for examination. Importantly, leukocyte handling (and thus potential leukocyte activation) is minimized and no cell preprocessing is required, as opposed to fluorescence or magnetically activated cell separation procedures.[35-38]

The separation is efficient, producing a greater than 30-fold increase in the leukocyte-to-erythrocyte ratio in a single pass. This is higher than that of a single-spin centrifugation “buffy coat” preparation of whole human blood (~10−20-fold enrichment) and is sufficient for many applications such as PCR, which requires at least a 10-fold enrichment.[8]

Future work is needed to improve purity and provide better characterization of this new leukocyte enrichment technique. For example, modification of the input reservoir may eliminate the initial dynamic reduction of leukocyte concentration caused by the impeded traffic of leukocytes during their entry into the supply channel (Figure 1, 1). In addition, separation units can be arranged in series to further purify the extraction channel effluent (Figure 1, 8). However, the low hematocrit (~1/3 of initial sample) and high leukocyte concentration (~10 times higher than in initial sample) characteristic of the effluent may diminish the efficiency of secondary separation. Decreased frequency of erythrocyte–leukocyte collisions and increased number of leukocyte–leukocyte interactions could hinder leucocyte margination and reduce separation efficiency. Another possible approach aimed at higher separation efficiency would involve coating the supply channel walls with adhesion molecules, such as P-selectin, which mediate leukocyte rolling in vivo. This slow “adhesive” rolling of leukocytes would facilitate their accumulation along the supply channel and may improve overall enrichment.

CONCLUSIONS

An obvious advantage of applying a biomimetic approach to the design of microfluidic lab-on-a-chip components is that the technical solutions that we borrow from nature have been already rigorously tested, optimized, and approved by evolution. Examples of successful application of this approach include check valves[39] and adhesion-based cell separation.[40] In this paper, we described a new, simple method for obtaining highly enriched leukocytes from a small sample of whole blood, the design of which has been inspired by natural microcirculatory phenomena. This technique could be used as an initial sample preparation step for the miniature analytical assays that require leukocytes or their genetic material. By providing convenient and efficient sample preparation in situ, this technology should facilitate implementation and commercialization of future lab-on-a-chip devices.

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Supplementary Material

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Acknowledgments

This work was performed in part at the Cornell Nanofabrication Facility (a member of the National Nanofabrication Users Network), which is supported by the National Science Foundation, its users, Cornell University, and Industrial Affiliates. We thank R.K. Jain for useful comments on the manuscript. This work was supported by a Predoctoral Fellowship Award from the American Heart Association (S.S.S.) and an NHLBI grant (L.L.M).

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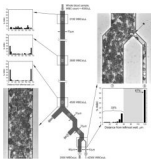

Figure 1.
Microseparation device. The geometry of the device is illustrated in the center; snapshots and plots of leukocyte (WBC) distributions are shown at the sides of the figure. Blood enters the supply channel from the sample reservoir (not shown). Flow is from top to bottom in all segments. All channels in the device are drained into a low-pressure reservoir (not shown). The circled numbers correspond to points referenced in the text. Length of the supply channel (points 1−3) is ~5.5 mm; distance between the first and the third bifurcation is 0.48 mm. Flow rates in segments 7 and 8 were approximately 120 and 16 pL/s, respectively.

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