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Microfluidic inertia enhanced phase partitioning for enriching nucleated cell populations in blood

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Nucleated cells in blood like white blood cells (WBCs) and other rare cells including peripheral blood stem cells (PBSCs) and circulating tumor cells (CTCs) possess significant value for patient monitoring and clinical diagnosis. Enrichment of nucleated cells from contaminating red blood cells (RBCs) using label-free techniques without the use of antibodies or centrifugation is highly desirable to ensure minimal cell loss and activation. To accomplish this, we demonstrate proof-of-concept of a new microfluidic technique that combines aqueous phase partitioning with inertial focusing to accomplish enrichment of nucleated cells in blood. This technique exploits selective affinity of RBCs to the dextran phase (DEX) to accomplish initial separation which is amplified by inertial forces that develop in high-aspect-ratio channels. In our experiments, we spiked RBC samples with representative nucleated cells, MOLT-3 cells (human, peripheral blood, T lymphoblast cell line) and MCF-7 cells (human breast cancer cell line) in a ratio of 500 : 1 (RBCs : nucleated cells) and accomplished depletion of ~96% of RBCs while retaining ~98% of nucleated cells. Higher purity can be accomplished by subjecting the enriched nucleated cell mixture to a second pass *via* the same process. The second pass further enhances RBC depletion (>99% of initial concentration) whereas nucleated cells were recovered without any further loss. This technique therefore has the potential to be utilized either alone or as a sample preparation tool in the clinical and research setting for various clinical and research applications.

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Introduction

Blood is a complex mixture containing a heterogeneous mixture of cells and platelets suspended in plasma. Red blood cells (RBCs) are anucleated and make up >99% of cells in blood. RBCs' primary function is oxygen transport and they possess limited information regarding the immune and inflammatory state of the body. Circulating nucleated cells, primarily white blood cells (WBCs) and other rare circulating cells like peripheral blood stem cells (PBSCs) and circulating tumor cells (CTCs) present significant value for both research applications and clinical analysis.

Several target applications exist for analysis of circulating nucleated cells in blood. For example: evaluation of the activation status of different WBC populations provides insights into inflammation and the immune functions of the body.[1] Neutrophils and macrophages are involved in the acute response to injury and infections whereas lymphocyte sub-populations are mediators of adaptive immunity. Understanding the temporal changes in activation of different cells can be used to study onset, progression and resolution of inflammation.[2]

Another application is the enrichment of rare cells found in circulation like CTCs or PBSCs.[3,4] These cells typically exist in numbers <1 cell mL^{-1} of blood[5] and enrichment *via* depletion of contaminating RBCs without noticeable loss of target cell populations can significantly enhance subsequent isolation/detection protocols.

Current nucleated cell enrichment/isolation techniques rely on exposure of blood to a RBC lysis buffer to accomplish lysis and depletion of contaminating RBCs.[6] The lysis buffer contains NH_4Cl and exploits the presence of the enzyme carbonic anhydrase in RBCs to achieve selective swelling and lysis. Though highly specific to RBCs, exposure of >5 min can have detrimental effects on nucleated cells such as WBCs including loss of specific sub-populations of target cells and unnecessary activation.[7,8] Further, this procedure also involves two wash steps which require centrifugation to pellet cells and remove lysed RBC debris. This is significant as ~1–5% of target cells can be lost during this procedure and it is therefore not suitable for enrichment of rare cells like PBSCs or CTCs.[9]

Another approach that is commonly used to fractionate mononuclear cell fractions is density gradient centrifugation.[10] Blood samples are layered over a medium that has an intermediate density between that of mononuclear cells

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(WBCs like lymphocytes and monocytes and other cells including CTCs and PBSCs) and RBCs and centrifuged at 300–450 g for over 30 min. Following centrifugation the mononuclear cells form a band above the density gradient and can be fractionated using a pipette. This process requires skilled personnel to perform the fractionation and requires two additional wash steps which contribute to cell loss and it is therefore not suitable for isolation of rare cells. Further, the process of centrifugation itself is a significant source of mechanical stress and activates various signalling events in target cells.[11,12]

Recently, Hur *et al.*[13] elegantly demonstrated an inertial focusing based approach to enrich nucleated cells including CTCs from blood in a label-free fashion. Inertial focusing relies on the physical properties of cells (size and deformability) to accomplish enrichment. This approach has the potential to significantly improve the throughput without the need for antibody based capture but in its current state requires significant dilution of blood samples to minimize cell–cell interactions and does not meet clinical needs in terms of throughput and efficiency. Blood samples contain $\sim 3 \times 10^9$ cells mL^{-1} and need at least a 1 : 5000 dilution to get to the optimal cell concentration of $\sim 5 \times 10^5$ cells mL^{-1} that ensures that cell–cell collisions within the channel do not interfere with inertial focusing. CTCs exist in the blood at concentrations of 1–10 cells mL^{-1}; large dilutions combined with low operating flow rates greatly increase the processing time for the isolation of CTCs. Further, the enrichment ratio defined as the ratio of $(CTCs/Blood)_{outlet}/(CTCs/Blood)_{inlet}$ attained was ~ 5.4 which indicates depletion of $\sim 83\%$ of RBCs and retention of 96% of CTCs. This technique is amenable to massively parallel adaptation for nucleated cell enrichment, where multiple devices operate in parallel but the dilution ensures that centrifugation steps are essential prior to analysis of samples which can again be a source for CTC loss.

To enhance selectivity and improve throughput, we developed a new technique that combines aqueous phase partitioning with inertial focusing to enable the separation of cells and particles based on differences in surface energy and amplify the initial separation *via* inertial focusing effects.[14] This approach provides the ability to select specific cell populations which is not possible *via* inertial focusing alone. This technique enables greater than an order of magnitude higher throughput in comparison to microfluidics aqueous two phase systems (μATPS) used alone without enhancement of separation *via* inertial effects. RBCs are the biggest contaminant in the enrichment of nucleated cells. Prior μATPS studies which used polyethylene glycol (PEG) and dextran (DEX) phases alone without inertial focusing have shown that RBCs preferentially partition to the DEX phase whereas leukocytes or white blood cells (WBCs) and other nucleated cells partition preferentially to the PEG phase.[15] However, relying on μATPS alone does not provide the throughput or efficiency necessary for practical clinical utility. The addition of inertial focusing effects helps overcome the above-mentioned shortcomings. Nucleated cells (WBCs and CTCs) have been shown to be isolated without compromise in cell viability at flow rates up to several mL min^{-1}.[3,16,17]

In this manuscript, we present a new protocol for inertia enhanced phase partitioning accomplished using one phase (DEX) instead of two (DEX and PEG) as in conventional aqueous two phase systems (ATPS). This technique relies on both the migration and retardation of cells based on their differences in surface energy, which in turn is enhanced by inertial focusing. In our case we exploit the fact that RBCs preferentially partition to the DEX phase whereas WBCs and other nucleated cells do not partition into the DEX phase and remain confined to their original streamlines. By introducing samples in a thin stream at the center of a high-aspect ratio microfluidic channel and flanking the stream on both sides with DEX, we demonstrate that RBCs partition into the DEX phase following which inertial forces act to move them close to the outer walls enhancing the initial separation. The nucleated cells, on the other, hand remain confined to the center stream and can be fractionated separately. This approach is more efficient than conventional ATPS because of the use of two DEX streams as opposed to one and ensures a greater surface area for interaction of RBCs with DEX. Unlike μATPS, the throughput is at least an order of magnitude faster, which is necessary for processing clinical samples. Moreover, the enhancement of separation by enhanced inertial forces by phase partitioning creates suitable separation distances between target and non-target cells, enabling clean fractionation in comparison to recently developed inertial focusing based approaches.[13] Further, this approach is also superior to techniques that exploit inertial forces alone because the dilution of the original sample required to attain separation of target cells is 2 orders of magnitude smaller which addresses the throughput issue. Furthermore, the selectivity is also superior as nucleated cells do not prefer the DEX phase and are confined to the energetically favorable center stream. Using this new approach we demonstrate that RBC samples spiked with representative nucleated cell lines MOLT-3 and MCF-7 representative of CTCs can be sufficiently enriched to either be used directly for WBC analysis *via* techniques like flow cytometry or provide a RBC de-bulked sample for antibody-based CTC/PBSC capture and isolation.

Theoretical background

Phase partitioning

Mixtures of aqueous solutions of PEG and DEX above critical concentrations form immiscible, liquid two phase systems with a PEG-rich top phase and a DEX-rich bottom phase.[18] ATPS systems can be buffered and made isotonic and are highly useful in the segregation of components of soluble materials like cells and membrane populations based on a variety of surface properties. Even though PEG and DEX are nonionic polymers, certain ions like phosphates and sulfates partition unequally between the phases, resulting in an electrostatic potential difference between the phases. When cells with different surface energies are placed within the two

phase system, they position themselves in an energetically favorable location within either phase or at the interface of the two phases. The dominant surface energy force, in comparison to electrostatic force (order of μV), moves cells to either positively charged PEG and negatively charged DEX or to the interface between the two phases. The partition coefficient K (concentration of target population in top phase/concentration of target population in bottom) can be expressed as:

$$K = \exp[(\alpha + \gamma A)/kT]$$

where α and γ are the difference in electrostatic free energy and difference in interfacial free energy between the phases for cells/particles being partitioned, respectively. Also, A is the surface area of material, k is the Boltzmann constant and T is the absolute temperature. The exponential nature of this expression shows the relation between the partition coefficient and the properties that determine partition. When cells are placed in ATPS, the magnitude of the experienced surface energy force is proportional to the surface area of cells, surface properties such as membrane surface charge, and potential of phases. When blood cells are placed in the PEG/DEX two phase system, RBCs with lower surface energy will move into the negatively charged DEX and WBCs and other nucleated cells with higher surface energy will remain at the interface of polymers. Recent studies using microfluidics systems show that more efficient separation can be accomplished by eliminating gravitational forces and enhancing the surface to volume ratio in small scales. Exploiting microfluidic techniques makes the interface between cells and polymer large enough to perform continuous partitioning in low Reynolds number (Re) flow (Fig. 1A):

$$Re = \frac{\rho \bar{U} D_h}{\mu}$$

where ρ, $\bar{U}$, μ, are the density, average velocity and dynamic viscosity, respectively, while D_h is the hydraulic diameter, defined as:

$$D_h = \frac{2HW}{H+W}$$

where H and W are the height and width of the channel respectively.

Inertial focusing in Poiseuille flow

Fluid flow in microchannels is predominantly viscous and inertial forces were thought to be extremely small and insignificant. However, recent progress has shown that these forces can be made significant and exploited for various applications through the use of appropriate channel geometries.[19] A detailed discussion of the inertial focusing principle has been previously discussed.[20] In brief, in microfluidic systems, several groups have theoretically modeled and experimentally have demonstrated the sorting and separation of particles and cells passively using inertial forces. Particle migration to equilibrium positions in a circular tube was first reported in the 1960s.[21] Particles in flow experience both viscous drag forces and inertial lift forces. Drag forces in viscous fluids act to accelerate particles in the flow direction

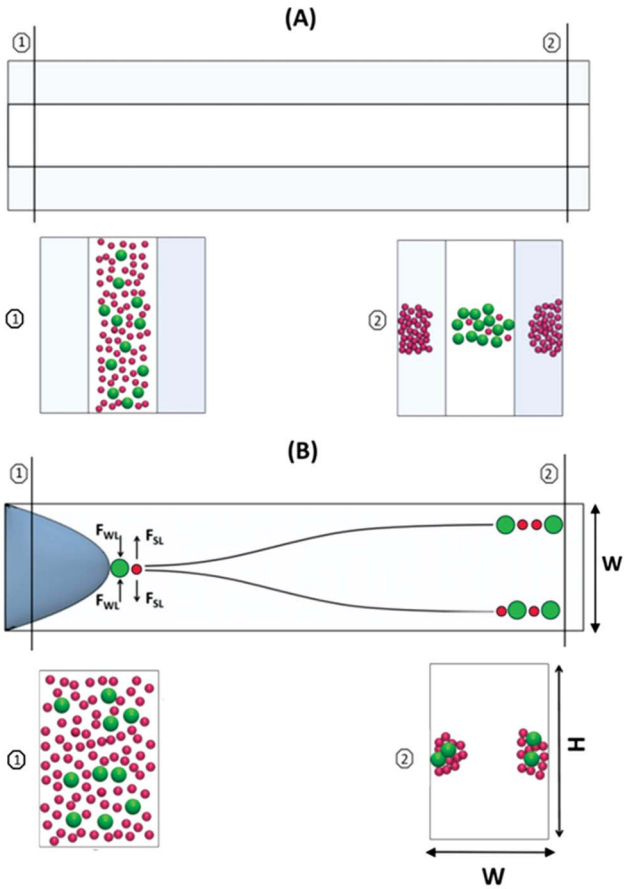

Fig. 1 (A) Separation of particles/cells in high aspect ratio channels based on phase partitioning. (B) Migration of particles/cells to lateral equilibrium position as a balance between two components of inertial lift forces, namely shear-gradient inertial lift force (F_{SL}) and wall effect inertial lift force (F_{WL}) in Poiseuille flow at the inlet and outlet of the microchannel.

and inertial forces drive particles perpendicular to the main flow direction in inviscid flow.[19] The two components of inertial lift forces, namely shear-gradient inertial lift force (F_{SL}) and wall effect inertial lift force (F_{WL}), in Poiseuille flow are responsible for the lateral migration of particles. Parabolic velocity profiles in Poiseuille flow induce a shear-gradient lift force that drives particles away from the center of microchannels, and a wall effect lift force that acts away from the wall towards the centerline in laminar flow. Several studies have shown that focusing is a function of size and randomly dispersed particles migrate at a specific lateral position in a circular channel of diameter D to a position of $0.2D$ away from the channel wall where the wall force and shear-gradient are at equilibrium.[21–24] The equilibrium positions also are dependent on the ratio of particle diameter (a_p) to channel diameter (D) and can move away from the center towards the wall with increasing Reynolds number. The net inertial lift force depends on the particle radius by a power of four ($F_L \sim a^4$) and can be calculated as a function of the particle position inside the channel.[25] Balancing Stokes drag with the shear-gradient lift force, channel length (L_I) required for focusing

particles to equilibrate at the specified flow rate can be calculated as follows:[24]

$$L_I = \frac{3\pi\mu}{2\rho U_f}\left(\frac{L_C}{a_P}\right)^3$$

where the characteristic length of a system (L_C) can be approximated to the smallest channel dimension, and U_f is an average flow velocity. When blood cells are subject to inertial focusing alone, they move to equilibrium positions closer to the outer wall (Fig. 1B).

Materials and methods

Device design and fabrication

The design of the device used was similar to that developed previously by our group[14] except for the fact that two inlets were included to allow the introduction of both samples and DEX. Devices were fabricated in polydimethylsiloxane (PDMS; Dow Corning, Midland, MI, USA) using standard soft-lithographic techniques. In brief, master molds were created by spin-coating a negative photoresist SU-8 100 (Microchem, Newton, MA, USA) on 4 in silicon wafers to obtain 95 μm height of microfluidics channels. The negative photoresist was exposed to ultraviolet (UV) light through a bright field mask of a transparency layout to create a straight high-aspect-ratio channel consisting of two inlets, a straight channel, an expanding region and five outlets (Fig. 2). The angle between two inlets was designed as 35° to produce the optimum laminar flow and prevent mixing of the blood and sample cells mixture and DEX. The width and length of the channel were fabricated to 40 μm and 4.5 cm, respectively, to ensure maximum particle sorting based on inertial lift forces. A serpentine fluidic resistor with a length of 2 cm was designed at each outlet to provide high fluidic resistance and minimize flow distortion due to the cells' transport and the irregularities of the syringe pump. The length of the expanding region was designed as 2100 μm from the end of the straight segment that increased every 3° per 150 μm to improve the efficiency of cell collection. After the unexposed photoresist was removed *via* washing with the SU-8 developer, the negative replicas were used to mold devices out of PDMS mixed with a cross-linking agent in a ratio of 10 : 1 and baked in a 100 °C oven for 1 h. Molded PDMS devices were removed from the silicon wafer, trimmed using a scalpel and access holes were cored out using syringe needles (Luer Hub 20G × 1/2 in, Smallparts, USA). The channels were formed by irreversibly sealing the PDMS with standard glass slides (Fisher Scientific., USA) following treatment with O_2 plasma in a Plasma Asher (Nordson March Instruments, Amherst, OH, USA). Inlet and outlet tubing (Tygon, Smallparts, USA) was press fitted into the cored holes prior to use.

Blood samples

Whole blood samples were withdrawn from healthy volunteers using a finger stick protocol approved by the University of Louisville Institutional Regulatory Board (IRB) and mixed with 1% sodium heparin to prevent coagulation. Samples were kept on ice until required for experiments. Samples were used within an hour of withdrawal.

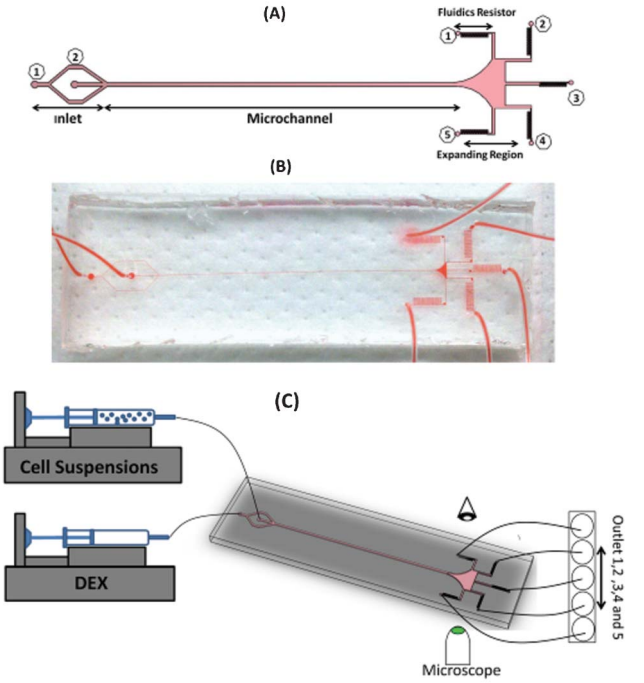

Fig. 2 (A) Schematic and (B) actual view of microfluidic device (the microchannel is filled with a red dye for visualization). The microdevice consists of 2 inlets, a straight segment microchannel (L_I), an expanding region, fluidics resistors and 5 outlets. (C) Illustration of set-up used for inertia enhanced phase partitioning experiments.

Nucleated cell culture

MOLT-3 cells and MCF7 cells were purchased from American Type Culture Collection (ATCC) and were cultured in the growth media suggested by ATCC. MOLT-3 cells were cultured in suspension in HyClone (RPMI-1640) medium (1 ×) (Thermo scientific, USA) supplemented with 10% fetal bovine serum (FBS) together with 1% penicillin–streptomycin–glutamine (Mediatech Inc. Cellgro, USA). MCF-7 cells were cultured in low-glucose Dulbecco's Modification of Eagle's Medium (DMEM) (Thermo scientific, USA) together with 10% FBS (Thermo scientific, USA), 1% penicillin–streptomycin (Mediatech, Inc. cellgro, USA) and 1% glutamine 2 mM (Invitrogen Corporation, Cibco, USA). All cultures were incubated at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. Prior to experiments cultured cells were extracted and stained using a nuclear acid staining dye SYTO 13 (Invitrogen, Molecular Probes, USA) to distinguish nucleated cells from blood cells and then spiked into blood samples at a pre-determined concentration.

Cell sample preparation

For each experiment, a 60 μL sample of whole blood was withdrawn from a healthy volunteer and diluted in 5 mL of 1 × phosphate buffered saline (PBS), a dilution of 1 : 80 resulting in a final RBC concentration of 2.6 × 10^7. Finally, MOLT-3 and MCF-7 cultured cells were spiked into the blood sample such

that the ratio of nucleated cells to blood was $\sim$1 : 500. This sample was loaded into a 10 mL syringe and connected to the inlet of the device. Though these cell concentrations are not indicative of the concentrations of CTCs in blood, these concentrations were chosen due to reliability issues with conventional cell counting systems like the hemocytometer (>62 cells) and flow cytometer (>10 000 cells).

Phase partitioning

For phase partitioning, a two-phase polymer system was prepared using 4% (w/w) PEG (8000, Fisher Scientific, USA) and 5% (w/w) DEX (BP1580-100, Fisher Scientific, USA).[14] PEG and DEX were dissolved into 1 × PBS and the pH was adjusted to 7.4. The mixture was transferred to a 50 mL conical tube and vigorously shaken to achieve complete dissolution and allowed to settle overnight at room temperature. Following settling, the PEG and DEX phases were carefully extracted from the top and bottom of the tube, respectively, by using separate 10 mL pipettes. Only the prepared (charged) DEX solution (viscosity about 0.016 Pa s) was used for the experiments.

Set-up for inertial focusing and phase partitioning experiments of RBCs and nucleated cells

RBC-nucleated cell mixtures were made prior to the experiments. Blood isolated from the body has $\sim 3 \times 10^9$ cells mL^{-1} and is not suitable for inertial focusing or phase partitioning without dilution. However, prior efforts have accomplished significant dilutions (2×10^5 RBCs mL^{-1}), which diminishes the relevance of the experiments. Therefore our dilutions were in the range of 2.6×10^7 RBCs mL^{-1} which is $\sim$2 orders of magnitude greater than used in previous studies. Concentrations of MOLT-3 cells and MCF-7 cells were in the range of $\sim 5.2 \times 10^4$ and 5.4×10^4, respectively. Diluted blood samples were mixed with SYTO labelled nucleated cell samples and loaded into a 10 mL syringe. The experimental set-up is show in Fig. 2(C) where injection of samples and DEX into the channel was accomplished using a syringe pump (Harvard Apparatus, PHD 2000 or Harvard Apparatus, 11 plus). To avoid sedimentation of cell suspensions each syringe was rotated every 3 min. Flow through the device was observed in real time under an inverted microscope (ECLIPSE, TE2000-U, Nikon, Tokyo, Japan) equipped with a 12 bit QIMAGING camera (RTIGA-2000R, CANADA). High-speed videos and images of cell positioning within the channel were recorded and analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA, USA) at both inlets and outlets. Cells collected at each outlet were counted *via* standard hemocytometry using a fluorescence microscope to distinguish SYTO labelled nucleated cells from unlabeled RBCs and expressed as average number of cells at each outlet $\pm$ standard deviation (SD). All experiments were performed in triplicate (n = 3).

Results and discussion

Characterization of inertial focusing using individual cell populations

To confirm the ability to achieve inertial focusing of deformable cells within fabricated microchannels and determine optimal flow rates for focusing, RBCs and two types of nucleated cells (MOLT-3 and MCF-7) were introduced into the fabricated channels individually and the optimal flow rates for efficient inertial focusing were determined. Cell sample flow rates (Q) were varied between 5–100 μL min^{-1} (Re = 1–25 in straight channel) and introduced *via* inlet 2 whereas inlet 1 was closed. For RBCs, at a flow rate of 5 μL min^{-1} focusing the cells at two lateral equilibrium positions was achieved with $\geqslant$70% extracted from two lateral outlets (1 and 5). This can be explained as a consequence of extremely high RBC concentration in whole blood ($>1 \times 10^9$ cells mL^{-1}) which caused some RBCs to remain at the centre of the channel and exit through outlet 3. However, increasing the flow rate in increments of 5 μL min^{-1} caused the percentage of RBCs exiting from outlets 1 and 5 to be increased and at flow rates more than 20 μL min^{-1} >90% of RBC was recovered at these two outlets. In general, we found that the larger the flow rate (larger Re number), the greater the focusing of cells at two lateral equilibrium positions. MOLT-3 and MCF-7 cells on the other hand are larger and more deformable than RBCs. Therefore, to accomplish focusing at lateral equilibrium positions requires larger forces in comparison to RBCs. For flow rates between 5 and 30 μL min^{-1}, there was no inertial focusing and cells were recovered from all five outlets. However, increasing the flow rate to >30 μL min^{-1} caused both cell types to focus along the two lateral equilibrium positions and enabled isolation at the lateral outlets. At flow rates $\geqslant$30 μL min^{-1}, more than 90% of nucleated cells were collected from outlets 1 and 5. These results confirm effects seen by Hur *et al.*[13] suggesting that RBCs focus close to the outer wall, whereas the more deformable nucleated cells require larger forces to attain focusing and their equilibrium positions are further away from the outer wall than RBCs.

Optimization of flow rates for phase partitioning

The overall goal of inertia enhanced phase partitioning for nucleated cell enrichment is to introduce the sample in a narrow stream flanked on both sides by a partitioning phase (DEX) and selectively separate cells that have an affinity to the DEX phase (RBCs) from cells that do not have an affinity to the DEX phase (nucleated cells). Inertial forces that develop will enhance initial separation and move the two cell types further away from each other to enable clear fractionation. Therefore, the sample stream needs to be flanked on both sides by a wider DEX stream. However the DEX solution has a higher viscosity than cell sample solutions; therefore, for all experiments, the ratio of flow rates necessary to ensure equal distribution within the micro-channel was determined to be $\sim$1 : 4 (DEX : cell sample). For this ratio, the widths of both DEX streams and the sample stream were at $\sim$1/3 of the channel width ($\sim$13.3 μm).

Characterization of phase partitioning

To obtain conditions for high efficiency inertial focusing and determine if surface energy based interactions dictate the direction of cell migration in two phase systems, RBCs and CTCs (MOLT-3 and MCF-7) were introduced individually at the centre of the microfluidic channel from inlet 2 with the DEX phase flanking the sample on both sides introduced *via* inlet

1. Results confirm that RBCs have a strong affinity to the DEX phase. Although inertial focusing moves RBCs to a lateral position as described previously, surface energy based phase partitioning into the DEX phase enhanced the inertial focusing and confinement of >94% RBCs at the outer walls and isolation *via* outlets 1 and 5 was accomplished at flow rates of <20 μL min^{-1}. In contrast to RBCs, both MOLT-3 cells and MCF-7 cells did not show an affinity to the DEX phase and preferentially remained within the centre stream resisting inertial forces that push outward towards the walls and approximately 100% of cells were recovered from middle outlets 2, 3 and 4. Furthermore, increasing the flow rate of samples to more than 20 μL min^{-1} enhanced the inertial forces causing nucleated cells to begin penetration of the DEX phase. Therefore, at flow rates between 5 and 20 μL min^{-1} the inertial focusing effects are offset by surface energy based partitioning of nucleated cells, forcing them to stay confined within the inlet sample stream without moving towards the walls. These conditions are similar to those previously used for MCF-7 enrichment using inertial focusing alone[13] and were used for the subsequent sorting of cell mixtures.

Enrichment of MOLT-3 cells from blood samples

To demonstrate the ability of our protocol to accomplish enrichment of nucleated cells from blood, the premixed RBC–nucleated cell mixtures were introduced into the channel at concentrations of 2.6 × 10^7 and ~5.5 × 10^4 cell mL^{-1} which results in ~1 : 500 (nucleated cell : RBC) ratio. The mixture was introduced into the channel at 15 μL min^{-1} *via* inlet 2 and DEX introduced *via* inlet 1 at a flow rate of 4 μL min^{-1} such that the DEX streams flanked the sample on both sides. Based on the characterization by studies described previously, this flow rate was found to be the most efficient to enrich nucleated cells. RBCs have a strong affinity to the DEX phase and partition into the DEX phase whereas the nucleated cells do not have an affinity to the DEX phase and remain confined within the center stream. Further, inertial forces act on RBCs to move them to equilibrium positions close to the outer wall thereby enhancing the initial separation. The same inertial forces act on the nucleated cells as well.

However, the inertial forces are insufficient to overcome the surface energy based repulsion from the DEX phase causing confinement within the centre stream. After passing through the channel, the samples were collected at each of the 5 outlets. Results show that ~94% of RBCs were isolated *via* outlets 1 and 5 whereas ~98% of the MOLT-3 cells introduced at the inlet were fractionated at the center outlets 2, 3 and 4 (Fig. 3). More specifically, 50% of MOLT-3 cells were collected at the outlet 3 (center) and 48% of MOLT-3 cells were evenly distributed between outlets 2 and 4.

Enrichment of MCF-7 cells from blood samples

An identical experiment was performed with blood samples spiked with MCF-7 cells instead of MOLT-3 cells. Results were similar with ~94% of RBCs depleted *via* outlets 1 and 5 and ~97% of the MCF-7 cells were recovered at outlets 2, 3 and 4 with 76% of MCF-7 cells isolated *via* outlet 3 and the remaining 21% of the MCF-7 cells were distributed between outlets 2 and 4 (Fig. 4).

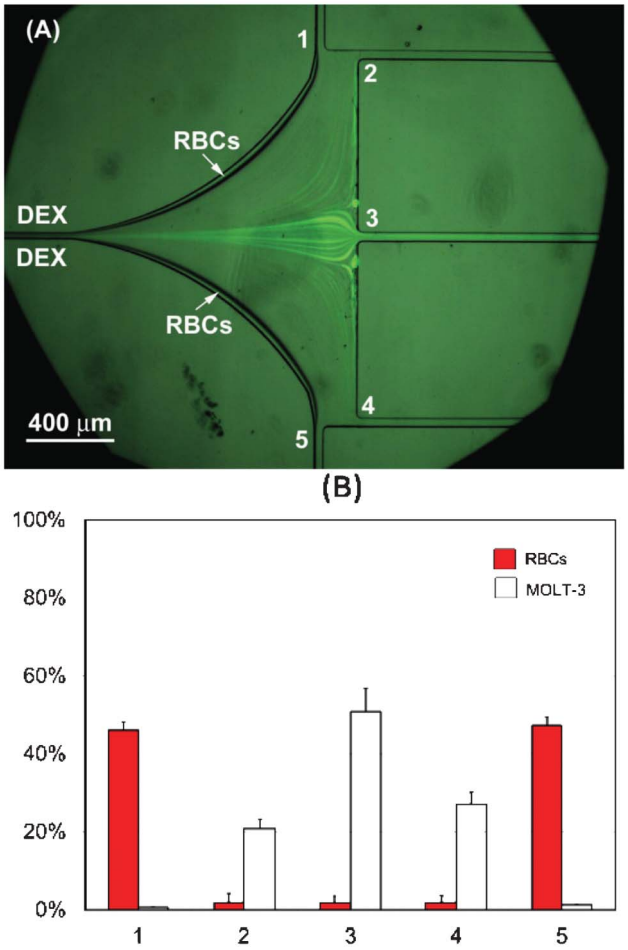

Fig. 3 MOLT-3 cell enrichment. (A) Microscope image of MOLT-3 (green fluorescent) cell separation from RBCs (dark, unlabelled cells) *via* different outlets due to inertia enhanced phase partitioning. (B) Summary of percentage of cells collected at each outlet.

Comparison of MOLT-3 cell enrichment in the absence of phase partitioning

To determine if the separation of nucleated cells from blood samples at the concentrations used in prior experiments for the separation of MOLT-3 and MCF-7 cells was possible using inertial focusing alone (using the method described by Hur *et al.*[13]), and to establish the importance of using phase partitioning (DEX) for enrichment of nucleated cells form blood samples with high concentrations of RBCs, we performed experiments where premixed RBC–nucleated cell (MOLT-3) mixtures were introduced into the channel at concentrations of 2.6 × 10^7 and ~5.5 × 10^4 cell mL^{-1} (1 : 500 (nucleated cell : RBC) ratio) without the DEX phase at *Re* values of 25–50. At the best *Re* values for maximum depletion/focusing of RBCs, results clearly show that at high concentrations of RBCs the separation is poor with high RBC contamination (20% in outlets 2,3 and 4) and the random distribution of MOLT-3 cells in all outlets is due to cell–cell interactions (Fig. 5).

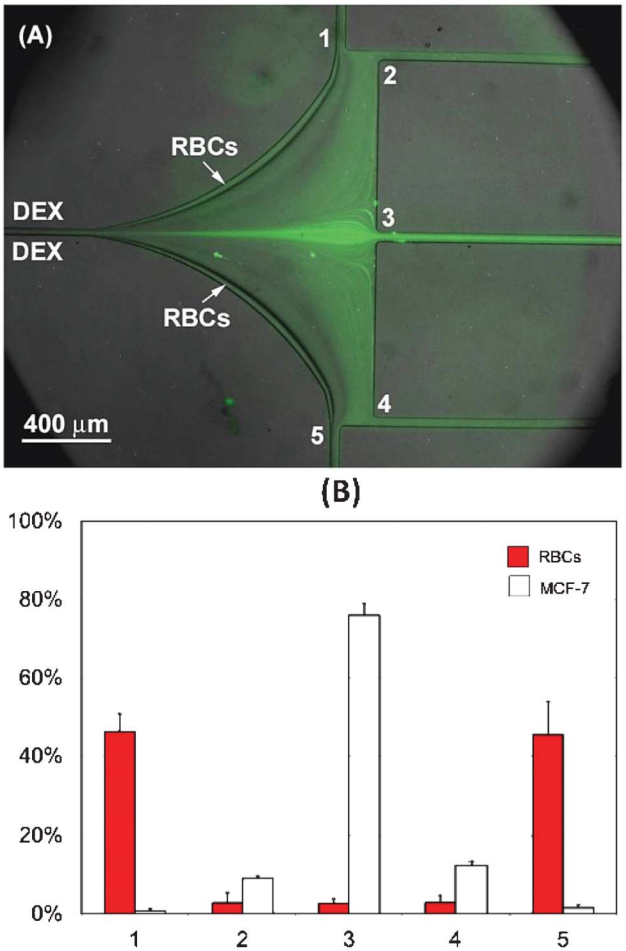

Fig. 4 MCF-7 cell enrichment. (A) Microscope image of MCF-7 (green fluorescent) cell separation from RBCs (dark, unlabelled cells) *via* different outlets due to inertia enhanced phase partitioning. (B) Summary of percentage of cells collected at each outlet.

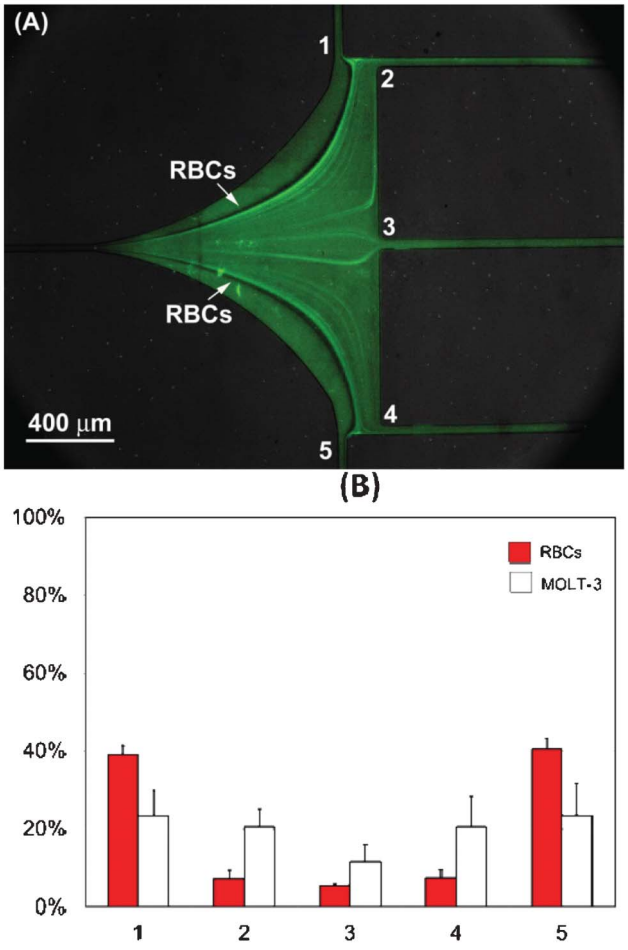

Fig. 5 MOLT-3 cell enrichment. (A) Microscope image of MOLT-3 (green fluorescent) cell separation from RBCs (dark, unlabelled cells) *via* different outlets due inertial focusing alone in the absence of phase partitioning at similar RBC and nucleated cell concentrations. (B) Summary of percentage of cells collected at each outlet.

Therefore, we conclude that the affinity of RBCs to the DEX phase and repulsion of nucleated cells away from the DEX phase enables enrichment of nucleated cells in blood at concentrations previously not possible when using inertial forces alone to accomplish enrichment.

Further enhancement of RBC depletion *via* a second pass

Despite removal of 94% of RBCs *via* the first pass through the device using inertia enhanced phase partitioning, a sizable number of RBCs still remain. The first pass accomplishes the enrichment of nucleated cells from 1 : 500 (nucleated cell : RBC) to 1 : 30. To determine if a second pass of MOLT-3 enriched samples (sample collected *via* outlets 2, 3 and 4) can be further enriched, we pooled samples from outlets 2, 3 and 4 and introduced them into the device for a second time. The second separation is highly efficient and ensures depletion of >99% of initial RBCs without the loss of any additional nucleated cells (Fig. 6). Further, ~80% of MOLT-3 cells were now isolated *via* outlet 3. The second pass therefore results in a 1 : 2 (nucleated cell : RBC) ratio which is sufficient for analysis *via* techniques such as flow cytometry. However, it should be noted that contamination with WBCs that exist at a concentration of $\sim 5 \times 10^6$ cell mL^{-1} in whole blood and $\sim 6 \times 10^4$ cell mL^{-1} in our diluted samples still remains as they elute with the nucleated cells.

Significance of results and potential applications

Microfluidics based approaches provide attractive alternatives for cell sorting in comparison to conventional macroscale protocols. The primary advantage of microfluidics is the ability to address cells at the single cell level thereby minimizing mechanical or chemical stress necessary for separations. More recently microfluidic approaches have been used to accomplish antibody-free or label-free isolation of cells thereby minimizing cell activation due to the antibody binding event. Approaches that have been successful thus far have exploited differences in size,[13] shape,[26] osmotic resistance[17] and deformability.[13] However, these techniques have been limited in their ability to accomplish high purity, high efficiency separations. This can be attributed to the overlap in physical properties and poor resolution of microfluidic devices to

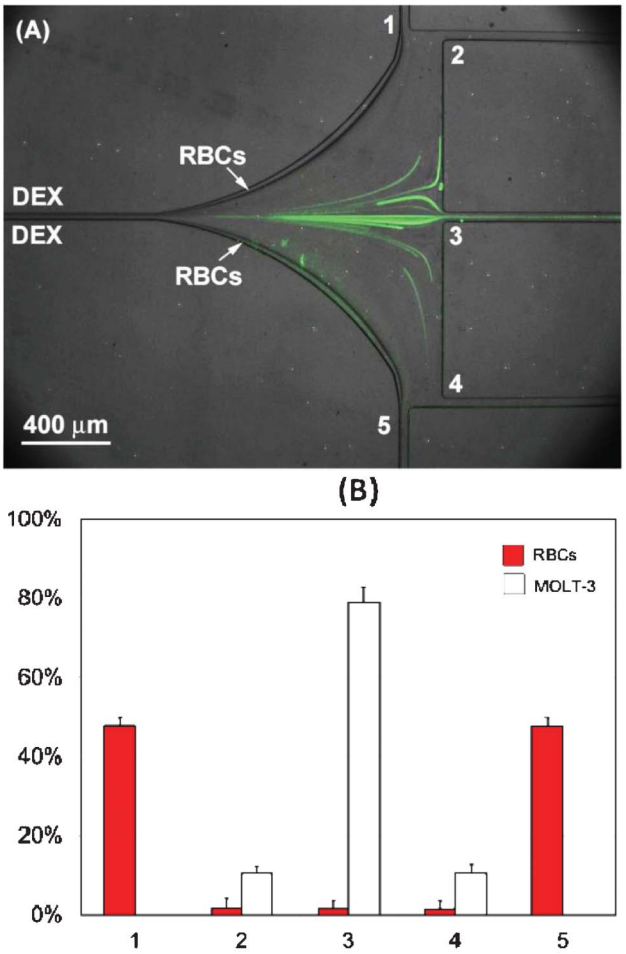

Fig. 6 MOLT-3 cell enrichment: 2nd pass. (A) Microscope image of MOLT-3 (green fluorescent) cell separation from RBCs (dark, unlabelled cells) *via* different outlets due to inertia enhanced phase partitioning. (B) Summary of percentage of cells collected at each outlet.

fractionate cells at the outlet. One property that has not been extensively exploited to separate cells using microfluidic approaches is phase partitioning. The primary reason is the poor fractionation efficiency at the outlets. Unlike size based sorting, phase partitioning allows selection of cells similar in size (small differences in size) but with differences in surface energy. However, without improvement in fractionation efficiency this technique is not desirable for cell sorting. To overcome this limitation, we combine phase partitioning with inertial focusing to ensure initial separation is based on phase partitioning and then the initial separation is enhanced by inertial forces that move the initially separated cells farther away from each other. This ensures selection of cells based on differences in surface energy and clean fractionation as cells are moved >10 μm from each other.

We therefore demonstrate a new technique that exploits the selectivity of phase partitioning in combination with inertial focusing effects to accomplish the enrichment of nucleated cells in blood. This technique provides a label-, chemical- and centrifugation-free approach for enrichment of nucleated cells from blood. Cells isolated using this technique can be used directly for analysis using techniques like flow cytometry or can be used for sample preparation and seamlessly interfaced with antibody based capture platforms. This approach is a significant improvement over techniques that employ either inertial forces or phase partitioning alone in several ways. First, the flow rates are significantly (~1 order of magnitude) larger than the flow rates used for phase partitioning alone. In terms of the dilution of blood samples, our technique is ~2 orders of magnitude greater than studies that use inertial forces alone. Following the 1st pass our results are superior to those achieved using inertial microfluidics alone. In our experiments the RBC:MCF-7 ratio was 500 : 1 and our RBC concentration was 2.6×10^7 cells mL^{-1}. We depleted 94% of RBCs and retained 98% MCF-7 cells. In comparison the inertial focusing efforts by other groups used RBC : MCF-7 ratio of 100 : 1 and the RBC concentration was 2×10^5 cells mL^{-1} (2 orders of magnitude dilution) and 83% depletion of RBCs and 96% retention of MCF-7 cells were obtained. Therefore our 1st pass is superior to inertial focusing alone. The 2nd pass further enhances RBC depletion without loss of MCF-7 cells. Further, due to the differences in densities and viscosities of DEX and samples, the volume of fluid collected at the outlets is not equal. Nearly 80% of the fluid is isolated at the outlets 1 and 5 whereas only 20% of the fluid exists *via* outlets 2, 3 and 4. This ensures that cell samples do not need additional centrifugation steps to accomplish sample concentration. The throughput of this device can be further enhanced by the operation of multiple separation channels in parallel to process large amounts of blood. For reliable detection of rare cells like CTCs, this device can be used as a standalone platform to obtain enrichment of CTCs in blood for subsequent detection/diagnosis using techniques like flow cytometry. The major advantage is the ability to directly label and analyze samples using a flow cytometer as >95% of RBCs are depleted after the first pass and more than 99% of the RBCs are depleted after the second pass with this protocol. Alternatively, this device can be used as a sample preparation tool to enrich CTCs prior to introduction into immuno-affinity capture devices like those developed by Nagrath *et al.*[3] and Stott *et al.*[16] Depletion of >94% of RBCs provides a significantly cleaner sample and a higher probability for CTC interaction with the capture of antibodies in a smaller device for more efficient separation. The use of phase partitioning in conjunction with inertial focusing effects therefore allows processing of blood cells at ~2 orders of magnitude higher concentrations and ensures the retention of >98% of CTCs and has the potential to be used as a valuable sample preparation tool in both research and clinical settings. A similar case can be made for PBSC isolation.

Conclusions

In summary we developed a new technique to enrich nucleated cell populations in blood. We demonstrate proof-of-concept of the ability of this device to accomplish depletion of >94% of RBCs while preserving ~98% of nucleated cells. A second pass ensures further depletion of RBCs (>99%) without any further

loss of nucleated cells. This technique therefore has great potential for use either directly or as a sample preparation tool for CTC detection and isolation in clinical and research settings.

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