SINGLE-STEP UNTRAHIGH ENRICHMENT OF LEUKOCYTES FROM WHOLE BLOOD ENABLED BY CELL ROLLING ON BIOMIMETIC ADHESIVE SURFACES

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ABSTRACT

Isolation of leukocytes such as neutrophils from whole blood is an important pre-analytical step for many routine laboratory procedures including diagnosis of bacterial infections, accessing chemo-readiness, HLA typing, and other genomic analysis. The standard procedure for isolating leukocyte populations based on density gradient is time and labor intensive and requires large sample volumes. Using asymmetric patterns of the P-selectin – a physiological ligand involved in cell trafficking, we demonstrate single step isolation of neutrophils from whole blood. Cells exhibiting non-specific adhesion are not drawn out of the flowing stream, enabling a staggering leukocyte enrichment ratio of 500,000 which is four orders of magnitude higher than other continuous-flow chip based methods. The isolated population of leukocytes is enriched in neutrophils exhibiting high purity (92%) and recovery (~70%).

KEYWORDS: Cell separation, Leukocyte rolling, Selectin

INTRODUCTION

Separation and analysis of cells based on surface markers plays an important role in biological research and clinical diagnosis. Existing microscale cell sorting techniques are either non-specific to antigen type, require expensive accessory equipment for their operation, or capture cells on adhesive surfaces and necessitate special elution procedures for recovering the cells. We have recently demonstrated that transient receptor-ligand interactions that result in cell rolling [1] on a surface under fluid flow can be used to control the flow of cells on a receptor-patterned substrate [2], which paves the way for a new technique for label-free cell separation. By incorporating these asymmetric P-selectin patterns inside microfluidic channels we demonstrated separation of HL60 cells (expressing PSGL-1 which mediates interaction with P-selectin) from K562 cells (does not interact with P-selectin) [3].

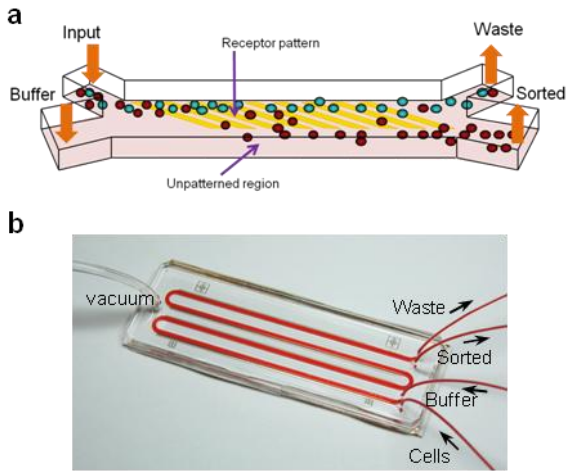

Figure 1. (a) Schematic of the cell separation device. Cells are introduced from one side in a buffer flow. Rolling of cells on patterned receptors causes them to separate laterally from where they can be collected in different channels.(b) The microfluidic device used in this study.

In this paper we focus on isolation of neutrophils, which form the first line of defense against bacterial infection and an important component of the innate immune system. Neutrophil separation is routinely performed in clinical labs for detecting infection and inflammation, accessing chemo-readiness and for genomic analysis. The conventional density gradient method, which is the gold standard for neutrophil separation, requires ~2-3 h and consumes milliliters of samples, and is not easily amenable to point-of-care applications, e.g. assessment of neonatal sepsis in developing countries. Since neutrophils exhibit avid rolling on P-selectin compared to other leukocytes, we hypothesized that asymmetric P-selectin patterns could be used for sorting neutrophils. In this work, using our separation device [3] we demonstrate high purity separation of neutrophils (>92%) from whole blood in a single step. The neutrophils were found to be viable and functionally unaltered after separation.

EXPERIMENTAL

A detailed description of the fabrication process in given in our earlier work [3]. Briefly, lithographically patterned gold-coated glass slides were cleaned and immersed in 1% PEG-trimethoxysilane (Gelest) solution overnight to passivated the glass, followed by treatment with 1 mM dithio-bis-succinimidyl propionate for 2 h to functionalize the gold. Then the substrates were incubated with 15 μg/mL P-selectin for 1 h and stored in 1% BSA solution until used in the experiments.

978-0-9798064-5-2/μTAS 2012/$20©12CBMS-0001

The flow cell was molded in PDMS using standard lithography techniques, and consisted of a serpentine channel of rectangular cross-section (100 µm x 1 mm) with total length of 20 cm (Figure 1b). The device was attached to the substrate after alignment using vacuum, creating a reversible bond.

Blood was collected in citrate vacutainers from consenting donors following approved IRB protocols and used within 3 h from the time of collection. Whole blood was diluted (1:1) with buffer (DPBS) containing 30µM of Ca^{++} and 200U/ml of polymxinB (to prevent endotoxin activation) and infused parallel to a buffer stream (of the same composition) at a total wall shear stress of 0.5 dyn/cm^2. The sorted and waste streams were collected in separate vials. An antibody cocktail (CD235a, CD45, CD66, CD14) along with multicolor flowcytometry was used to determine cell lineages in the collected samples.

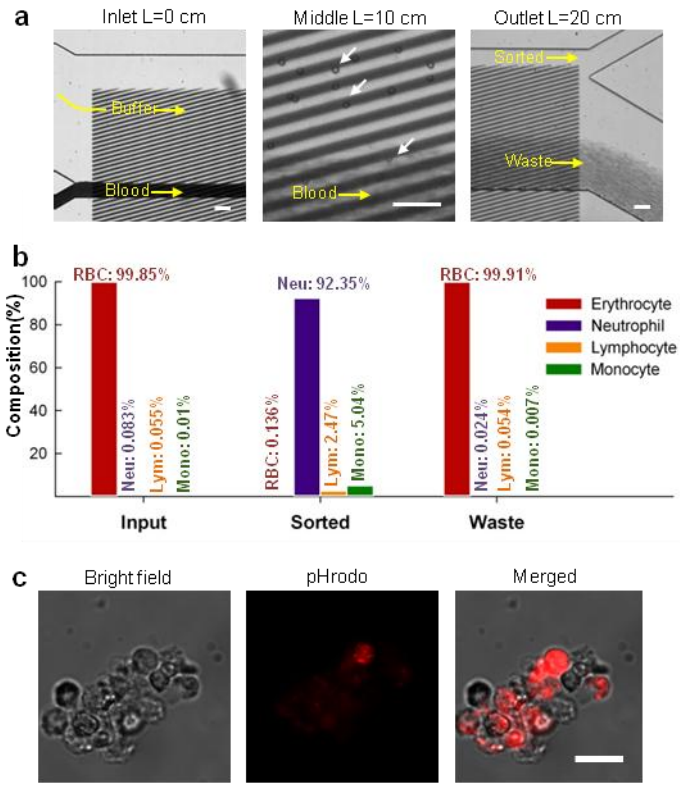

Figure 2. (a) Images of the different segment of the device in operation. Neutrophils could be seen (arrows) rolling out of the blood stream in the middle panel. Scale bar: 100 µm. (b) Composition of the input, sorted and the waste fractions after sorting of whole blood as analyzed via flow cytometry. (c) The sorted neutrophils successfully phagocytosed E.coli particles (tagged with pH sensitive dye pHrodo). Scale bar is 20 µm.

RESULTS AND DISCUSSION

When a stream of anticoagulated blood was introduced parallel to a buffer stream in 1:9 ratio at a wall shear stress of 0.5 dyn/cm^2, we observed that neutrophils specifically interacted with the P-selectin patterns, followed the edges of the patterns (Figure 2a), and were displaced laterally in a device length-dependent manner. In contrast, the red blood cells showed a passive spreading across the width of the channel, possibly due to cell-cell hydrodynamic interactions. The composition of the samples were found using a pan leukocyte marker (CD45) and granulocyte specific marker (CD66). We found that collecting 25% of the flow from the purified end resulted in a neutrophil purity of 92% (Figure 2b) with very little red blood cell contamination (<0.13%). The recovery of the neutrophil as caclulated from the purity values was ~70%. Tryphan blue dye exclusion assay confirmed that the viability of the sorted neutrophils were more than 97%. We also performed phagocytosis assays on the sorted neutrophils to establish functional capabilities. Heat-killed E. coli cells tagged with pHrodo® (invitorgen) were added to the sorted neutrophils or whole blood in excess (>1000 bacteria per neutrophil). pHrodo® is a pH sesitive dye (fluorescess only at low pH) can be used to simultaneously assess phagocytosis and sucessful acidification of the phagosome. We found that almost all of the sorted neutrophils (>98%) demonstrated successful phagocytosis at levels similar to the neutrophils in whole blood (Figure 2c).

CONCLUSION

In this paper we demonstrated high-efficiency, high-purity separation of neutrophils from whole blood by rolling on surfaces patterned with P-selectin in a continuous flow. To our knowledge, this method is the first report of a continuous separation method for neutrophils with purities comparable to existing antibody based methods. This technology can be easily extended to other weak adhesive ligands and should be useful as a general separation method in lab-on-a-chip and point-of-care devices.

ACKNOWLEDGEMENTS

The authors acknowledge the funding from the Deshpande Center for Technological Innovation at MIT (R.K. and J.M.K.), NIH grants HL-095722 and HL-097172 (J.M.K.) and NSF CAREER award 0952493 through the Chemical and Biological Separations program (R.K.). The devices were fabricated at the Microsystems Technology Laboratory (MIT).

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