DEFORMABILITY BASED SORTING OF RED BLOOD CELLS AND WHITE BLOOD CELLS USING MICROFLUIDIC RATCHETS

by

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Abstract

There are many situations in medicine and biology where it is desirable to sort cells in a heterogeneous sample based on their mechanical deformability, which can potentially serve as a proxy for morphology or pathology. This biophysical characteristic is particularly relevant for cells in the circulatory system, such as red blood cells and white blood cells, because deformability determines the capacity for these cells to transit through the microvasculature. Since deformability is such a fundamental characteristics of blood cells, deviations in normal cell deformability can contribute to a range of pathological conditions, such as microvascular occlusion, tissue necrosis and organ failure, observed in diseases such as malaria caused by Plasmodium falciparum. A commonly employed approach for deformability-based cell sorting is microfiltration. However, this method suffers from cell clogging at the filter microstructures, leading to reduced selectivity and device malfunction.

This dissertation presents an improved microfiltration strategy performed using the microfluidic ratchet mechanism, which relies on the deformation of individual cells through micrometer-scale tapered constrictions. Deforming single cells through such constrictions requires directionally asymmetrical forces, which enables oscillatory flow to create a ratcheting transport that depends on cell size and deformability. Simultaneously, oscillatory flow continuously agitates the cells to limit the contact time with the filter microstructure to prevent clogging and adsorption.

This work demonstrates the utility of the ratchet mechanism for cell sorting by developing a microfluidic device to sort red blood cells based on deformability. The device is used to separate Plasmodium falciparum infected red blood cells from uninfected cells. The method was shown to dramatically improve the sensitivity of malaria diagnosis performed using both microscopy and
rapid diagnostic tests by converting samples with difficult-to-detect parasitemia (<0.01%) into samples with easily detectable parasitemia (>0.1%). This work further demonstrates the utility of the microfluidic ratchet mechanism by developing a microfluidic device to isolate and sort leukocytes directly from whole blood. The method is capable of separating leukocytes from whole blood with 100% purity (i.e. no contaminant erythrocytes) and <2% leukocytes loss. Furthermore, the approach demonstrates the potential to phenotypically sort leukocytes to enrich for granulocytes and lymphocytes subpopulations.
Preface

The thesis is original and independent work by the author, Quan Guo. The work was supported by the University of British Columbia through the Four Year Fellowship program, as well as by NSERC, CIHR, Grand Challenges Canada, and Genome BC. The research requires the donation of blood from volunteers through venipuncture and finger prick. The approval was obtained from the University of British Columbia (UBC) Clinical Research Ethics Board with the certificate number H10-01243.

Prof. Hongshen Ma proposed the concept of microfluidic ratchets. The design and validation of the microfluidic ratchets principle (Chapter 4, Section 4.2) was done by myself with guidance by Prof. Ma. The design and validation of the microfluidic devices for sorting RBCs (Chapter 5, Section 5.2) and WBCs (Chapter 6, Section 6.2) were done by myself with guidance from Prof. Ma. The fabrication process (Appendix 7.3.2A.1) was initiated by myself with assistance from Sarah McFaul, Jeonghyun Lee and Aline Santoso; and the replication of the devices (Appendix 7.3.2A.1) was carried out by myself with assistance from Emel Islamzada. The instrumentation (Appendix 7.3.2A.2), i.e. the pressure board system, was initially developed by Prof. Ma and Issac Tang, with modification by Sangpil Woo and myself. The biological aspects of the research, including blood preparation (Appendix 7.3.2A.3), malaria culture (Appendix 7.3.2A.4), leukocytes immune-staining process (Appendix 7.3.2A.5) were performed by myself with assistance from Xiaoyan Deng, Kerryn Matthews, Aline Santoso and Emel Islamzada.

Chapter 2, Section 2.3 was a condensed version of the following published paper: Q. Guo, S. Park, and H. Ma, “Microfluidic micropipette aspiration for measuring the deformability of single cells,”
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As of October, 20th 2016, a version of Chapter 6, manuscript titled “Deformability based cell sorting enabling phenotypic separation of leukocytes directly from whole blood”, has been submitted for publication. I conducted all the experiment, collected all the data and co-wrote the manuscript with Simon Duffy and Prof. Ma.
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Dedication

To my Mom and Dad, and my Bunny

To my friend Norman

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Chapter 1: Introduction

1.1 Motivation

Cell sorting aims to separate subpopulations of cells from complex and heterogeneous mixtures, such as whole blood, based on a certain selection criterion. Cell sorting is often an essential step in medical diagnosis and in biological research, where it is necessary to obtain an enriched population of target cells in order to detect disease pathologies or analyze them using molecular tools.

Existing approaches to cell sorting can be categorized into labeled and label-free methods. Prominent labeled methods include fluorescence activated cell sorting (FACS) [1], [2] and magnetic activated cell sorting (MACS) [3]. FACS operates by labeling specific cell phenotypes using antibodies conjugated with fluorescent tags to target specific surface or intracellular biomarkers. The labeled cell mixtures are then processed using flow cytometry to sort target fractions into designated containers. MACS operates by labeling specific cell phenotypes using antibodies conjugated with magnetic beads. The sample is then flowed over a column with permanent magnets that capture the labeled cells using magnetic fields, while eluting unlabeled cells. The main advantage of label techniques is the high purity of target cells fraction, as long as there is an available bio-marker expressed exclusively by the specific cell type. Despite their many desirable characteristics, FACS and MACS are undesirable in many applications because the markers needed to identify a target cell fraction are insufficiently specific or are too rare to be detected, such as in the cases of circulating tumor cells [4] and regulatory T cells [5]. Additionally, these methods typically require large sample volumes, costly and potentially toxic reagents, as well as specialized expertise and instrumentation.
The development of new cell separation processes based on physical characteristics could provide selection criteria orthogonal to labeled methods. These processes also require minimal sample preparation and provide minimal stimulation of target cells. The relevant physical biomarkers include cell size, shape, cytoadherence, density, deformability, electrical polarizability, and magnetic susceptibility. So far, cells are sorted based on these physical parameters using principles such as gradient centrifugation, hydrodynamic forces, acoustic, dielectrophoretic, magnetic and optical forces, as well as microfiltration [6], [7]. Deformability is an underrated, yet essential physical parameter, particularly for circulating blood cells, such as red blood cells (RBCs), because they must traverse through the ubiquitous microvasculatures with constriction size much smaller than individual RBCs to perform gas exchange and nutrients delivery. Using deformability as a biophysical parameter can potentially not only isolate white blood cells (WBCs) from RBCs but also separate diseased RBCs, such as RBCs infected with malaria parasites, from healthy RBCs.

1.2 Research Objectives

The aim of the study is to develop a microfluidic deformability-based cell sorting technology and apply it to the following applications:

1) To sort RBCs based on deformability in order to enrich for *Plasmodium falciparum* infected RBCs (Pf-iRBCs) from uninfected RBCs (uiRBCs).

2) To isolate WBCs directly from whole blood and to sort WBCs in order to perform phenotypic separation of WBCs based on deformability.

1.3 Organization of the Thesis

The rest of the thesis is organized as follows:
Chapter 2 describes the role of cell deformability in circulating blood cells and discusses how RBC deformability changes upon infection by *Plasmodium falciparum*, one of the parasite strain responsible for malaria.

Chapter 3 reviews the principles and applications of established strategies for deformability-based cell separation, including hydrodynamic chromatography and microfiltration.

Chapter 4 presents an improved microfiltration scheme utilizing the microfluidic ratchet mechanism. Specifically, the chapter will focus on the underlying principle of the ratchet mechanism and how this mechanism permits continuous deformability-based sorting of complex samples, such as whole blood, without cell clogging or adsorption.

Chapter 5 describes the development and validation of a microfluidic device to sort RBCs infected with *Plasmodium falciparum* to improve the sensitivity of current malaria detection methods.

Chapter 6 describes the development and validation of a microfluidic device to separate WBCs from RBCs as well as to sort WBCs to enrich for different phenotypes of leukocytes, i.e., granulocytes and lymphocytes.

Chapter 7 presents the conclusion drawn from this dissertation and potential future work.
Chapter 2: Background

This chapter provides the background on deformability of circulating blood cells including RBCs and WBCs, as well as related previous work on the measurement of cell deformability using micrometer-scale constrictions.

2.1 Deformability of Red Blood Cells

2.1.1 Red Blood Cells Morphology

Red blood cells, or erythrocytes, perform the critical function of circulating throughout the body to perform gas exchange (O\textsubscript{2} and CO\textsubscript{2}) between tissues. RBCs are anuclear and have a biconcave shape with a dimple in the center. The average diameter of RBCs is 7.8 µm with a normal range from 7.2 to 8.5 µm. The thickness (or width) of RBCs range from 2.0 to 2.5 µm. The total volume of RBCs, measured as mean corpuscular volume (MCV) using hematological analyzers typical range between 80 fL to 95 fL. The variation of this parameter is the red cell distribution width (RDW), which ranges between 11.5% to 14.4% for normal individuals.

A critical feature of RBC is its mechanical flexibility, or deformability. Along their circulatory path, RBCs must transit through constrictions that are much smaller than their diameter, such as the micro-capillaries (~2.5 µm) and the inter-endothelial clefts of the spleen (0.5~1 µm) (Figure 2.1A) [8]. Consequently, normal RBCs must be able to undergo repeated and extensive deformation. The ability for RBCs to deform may be lost as a consequence of aging [9], [10], or as a result of hereditary disorders such as sickle cell disease [11], [12], spherocytosis [13], thalassemia [14], immune hemolytic anemia [15], as well as some infectious diseases like malaria [16]. The loss of RBC deformability is medically significant as rigid cells can be sequestered in tissues. RBC sequestration subsequently leads to microvascular obstruction and ultimately
contributes to tissue necrosis (Figure 2.1B). The rest of the section describes the disease of malaria. Specifically, the section focuses on the biophysical changes of host RBCs, as well as how cell sorting can help to resolve some of the challenges associated with current malaria diagnostic methods.

**Figure 2.1:** Schematic representation of RBC morphology. (A) Schematic illustration of human and mouse RBCs in proportion to the diameters of capillary and inter-endothelial splenic pore. (B) Abnormal RBCs, either from aging or caused by certain pathology, become less deformable and more susceptible to sequestration. Line v represents a threshold at which clearance from circulation is likely to occur via spleen entrapment or by other mechanism. [Reproduced from Ref. [17] with permission from American Journal of Hematology, copyright ©2013 Wiley Periodicals, Inc.]

### 2.1.2 Malaria and Biophysical Properties of *P. Falciparum* Infected RBCs

Malaria results from mosquito-borne transmission of protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* is the most prevalent and deadliest species of the parasite that causes malaria. In 2015, malaria accounted for ~400,000 deaths out of ~214 million cases
A central aspect of *P. falciparum* parasitism is the invasion of host RBCs where the parasite develops and asexually reproduces over a roughly 48-hour period. The growth and development of parasites during this intra-erythrocyte stage has been characterized as a series of morphologically distinct stages starting from *ring*, progressing to *trophozoite*, and then to the mature *schizont* stage.

Key to the pathophysiology of *falciparum* malaria is the parasite modification of the host RBCs that alters their biophysical properties during the parasites’ asexual replication. Specifically, *Pf*-iRBCs gradually lose their characteristic bi-concave shape, exhibit increased cytoadherence properties and most essentially, decreased deformability. The combined effect of these changes of host RBCs disrupts normal circulation and causes infected cells to adhere and accumulate in the microvasculature and organs [19].

Multiple factors contribute to the reduction of RBC deformability over the course of the intra-erythrocyte part of the *P. falciparum* lifecycle:

1) The malaria parasites metabolize on hemoglobin in RBCs and produce byproducts that induce oxidative stress within the host RBCs that result in membrane rigidification by cytoskeletal crosslinking and lipid peroxidation [20];

2) The parasite expresses proteins that are inserted as neoantigens on the surface membrane of the host RBCs and contribute to the host cell’s membrane rigidity [21], [22];

3) At later stages of the parasite lifecycle, the presence of solid parasites also impairs the deformation of the host RBCs [23], [24].
The changes in host RBC deformability caused by *P. falciparum* invasion has been previously studied through techniques such as micropipette aspiration [25]–[27], optical tweezer [28] and laminar shear flow assays [16], [29], [30]. Recent microfluidic studies have demonstrated mechanisms to study *P. falciparum* parasitized RBCs based on observation of capillary obstruction [31], [32], and quantification of transit time [33] and pressure [34] through constrictions. These studies suggest that deformability is a biomarker not only to distinguish healthy RBCs from *Pf*-iRBCs, but also to differentiate *Pf*-iRBCs at various intra-erythrocyte stages.

2.1.3 Malaria Diagnostic Approaches

2.1.3.1 Microscopy-based Giemsa Stain

Over the past decades, little has changed for malaria diagnosis. Malaria is still diagnosed using microscopic examination of blood smears stained with Giemsa dye [35], which can be prepared as either thin or thick blood film. Thin blood film is methanol fixed and stained with Giemsa to emphasize the parasite inclusions within host RBCs. Observed under the microscopy, normal RBCs cytoplasm is pinkish but may become darker and have purple rim. Ring-stage parasite is clearly visible within the RBC and has a light brown dot (nucleus of parasite) less than 1 μm within blue cytoplasm less than half of the erythrocyte. Sometimes, multiple rings can be found. As the parasites mature, the parasite cytoplasm becomes darker and the brown hemozoin pigment (metabolic byproduct of parasite) is clearly visible. At schizont stage, the brown dot fills more than half of the erythrocyte. To determine the concentration of parasites, the term parasitemia is used, which is defined as the ratio of number of parasitized RBCs over the total number of RBCs in percentage. Due to the fixed monolayer of RBCs, the morphological identification of the parasite to the species level and the intra-erythrocyte development stages is easier with great specificity.
RBCs prepared using thick film are stained without being fixed and the cells are lysed during staining, leaving only the parasite bodies, white blood cells and platelets on the smear. To determine the parasite concentration in blood using the thick film method, the term parasite density is used instead of parasitemia, which is defined as the number of parasites per µL of blood. Specifically, the number of parasites is counted until 200 WBC have been seen and then multiplying the parasites counted by 40 will give the parasite count per µL of blood, with the assumption that there are 8000 WBCs per µL of blood. Because thick blood film concentrates the layers of whole blood on the small surface of a microscopy slide, it greatly enhances the sensitivity over thin film approach. Although thick film method is much better than the thin film for the detection of low levels of parasitemia, it cannot identify the parasite species and intra-erythrocyte stages. Thick film method also requires more expertise in finding the parasites among WBCs and platelets.

Despite the high specificity of Giemsa stain microscopy, it is a rather technical and laborious procedure, which requires the examination of at least 100 microscopic fields before a negative result can be reported. The diagnosis becomes even more challenging with asymptomatic patients containing only a few parasites (<10 parasites) per µL of peripheral blood [36]–[38]. Generally, Giemsa microscopy has a sensitivity limit of 100 parasites per µL or 0.002% parasitemia for thick smear and 0.02% parasitemia for thin smear. Automated malaria diagnosis using digital imaging process software has been attempted to replace manual counting and parasite identification [39], [40]. However, the specificity and sensitivity of the automated diagnosis also has a direct correlation with the parasites density.
2.1.3.2 Rapid Diagnostic Tests

Rapid diagnostic tests (RDTs) [41, 42] represent simple, quick (~20 minutes), accurate and cost-effective diagnostic tests for determining the presence of malaria parasite. RDTs are based on the principle of detecting specific antigen in blood using the method of immunochromatography, whereby a colored detecting antibody binds to lysed parasites antigen, carried by capillary action on a nitrocellulose strip and arrested by a secondary capture antibody. The presence of the antigen is indicated by a color change on the strip and doesn’t require skilled interpretation. The most common malarial antigen is *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2), which contributes to more than 90% of the malaria RDTs on the market. It can detect parasite density as low as <10 parasites per µL. However, the use of *Pf*HRP2 suffers from antigen persistence in the blood stream even following the elimination of the parasites. This limit of *Pf*HRP2 antigen RDTs not only causes false positive but also makes it unsuitable for monitoring malarial drug efficacy following treatment. Another malaria antigen commonly used in malaria diagnostics is plasmodial lactate dehydrogenase (pLDH). The detection method based on pLDH is not as sensitive as those based on *Pf*HRP2 but pLDH based method doesn’t suffer from antigen persistence issue. Similar to gold standard microscopy, the performance of RDTs is directly related to parasitemia with more errors, reduced sensitivity and specificity at parasite density less than 200 parasites per µL. Unlike microscopy, RDT is not able to determine the stages of the iRBCs or to quantify the parasite density in patients’ blood. Both information is valuable when monitoring the drug efficacy or designing patient specific drug treatment plan.
2.1.3.3 State-of-the-art Malaria Diagnostic Techniques

A significant challenge to the control and eventual eradication of malaria is that this disease is largely endemic to resource-poor regions, where advanced laboratory facilities may not be available. Therefore, sensitive molecular diagnostic method such as polymerase chain reaction (PCR)-based techniques cannot be used. Despite recent effort to integrate sample processing, malaria DNA extraction, amplification and detection on a single small micro-chip, which dramatically reduces the processing time and improves the motility of the device, the technique is still years away from being implemented in low-resource setting [43].

Malaria parasites can also be detected using fluorescent dyes that have an affinity for the nucleic acid in the parasite nucleus and observed under fluorescence microscopy or analyzed using flow cytometry [44]. And recently, the process has been miniaturized on a microarray chip system for accurate, sensitive and rapid diagnosis of malaria in the field-setting [45]. In 2014, a research team from the Singapore-MIT comes up with an alternative approach for early diagnosis of Pf-iRBCs by detecting the hemozoin crystallites using magnetic resonance relaxometry (MRR), a technique similar to magnetic resonance imaging (MRI) [46]. Hemozoin is a metabolic by-product of malaria parasites known to be present in all stages and all human-infecting strains of malaria. The technique is inexpensive, and able to detect parasite density as low as 10 parasites per µL blood, equivalent to 0.0002% parasitemia, using a new index known as transverse relaxation rate.

Despite the recent advances in malaria diagnosis, microscopy-based Giemsa smear technique still remains the gold standard due to its ability to determine parasitemia or parasite density, parasite intra-erythrocyte stage as well as parasite strain. These are critical metrics for clinical prognosis, monitoring of drug resistance, hospitalization criteria and assessing severity of infection. None of
the abovementioned techniques, apart from PCR-based molecular assays, are able to provide a full spectrum of malaria diagnostic information. The potential for human error of microscopy-based technique, in particularly at low parasitemia level, can be overcome through a step of enrichment of iRBCs. Specifically, deformability-based sorting of iRBCs can provide an enriched population of Pf-iRBCs and improve the sensitivity of both microscopy- and RDTs-based techniques. In particular, enrichment of early ring stage parasites is of more significance since iRBCs with mature parasites tend to either adhere to the endothelium blood vessel or to be sequestered by microvasculature of spleen or other organs due to their increase in cyto-adherence and rigidity [47].

2.2 Deformability of White Blood Cells

White blood cells (WBCs), or leukocytes, are the immune cells that protect the body from infectious disease and foreign substances. Leukocytes are spherical and differ in shape from biconcaved erythrocytes, but they are rather similar in diameter (~8 µm) [48]. While circulating in the blood stream, leukocytes are significantly less deformable than erythrocytes [34], [49] and are known to cause microvasculature obstruction, such as in the capillaries of lungs [50], [51]. Deformability is not only a discriminating characteristic between erythrocyte and leukocyte, it can also potentially serve as a proxy for morphology, determining leukocytes phenotypes. Leukocytes can be divided into granulocytes and agranulocytes. Granulocytes include neutrophils, eosinophils and basophils and are identified through their multi-lobed nucleus and numerous cytoplasmic granules. Agranulocytes, or mononuclear leukocytes, including lymphocytes and monocytes, contain a large spherical nucleus. While the average size (diameter) of these subpopulations of circulating leukocytes differs, from the smallest lymphocytes (average diameter 6.2 µm), intermediate neutrophil (7.0 µm) and eosinophils (7.3 µm), to the largest monocytes (7.5 µm), the
size distribution overlaps significantly [48], making it difficult to discriminate these cells based solely on size [52].

These morphological difference, i.e. granularity and size, can be a potentially contributing factor to their ability to deform through micro-constrictions. Previously, the deformability of three major subpopulations of human leukocytes, including lymphocyte, neutrophil and monocyte, has been investigated using cell poker [51], micropipette aspiration [53], [54] and its microfluidic derivative (discussed in Section 2.3) [55]. The results indicate that monocytes are the most rigid cells, followed by lymphocytes and neutrophils. Furthermore, the ability of these leukocyte subsets to traverse through constrictions has been studied using polycarbonate membrane pore filters (5 µm diameter) to mimic the retention of these leukocytes in pulmonary capillaries. The results revealed that monocytes to be retained by the filter to the greatest extend, followed by neutrophils and lymphocytes [51]. These facts suggest the potential to use cell deformability as a surrogate for morphology not only to separate leukocyte from erythrocytes but also to phenotypically sort leukocytes.

2.3 Measurement of Cell Deformability

In order to establish cell deformability as a physical biomarker for cell morphology or pathology, it is important to develop a robust method to measure cell deformability. Previously during my master thesis (2009-2012), I applied the principle of traditional micropipette aspiration and developed the microfluidic version of this prominent technique, as shown in Figure 2.2A.
Figure 2.2: Microfluidic micropipette aspiration of individual cells.
(A) Schematic illustration of an individual cell passing through a 3D micron-scale tapered constriction; (B) Deformation of a single cell through funnel constriction at the instability with leading and trailing radii edge (R_a and R_b).

Specifically, the deformability of an individual cell was investigated by pressurizing the cell through micron-scale constrictions. The microstructure was designed such that the entrance of the funnel is larger than the diameter of the cells, while the exit is smaller. The funnel openings were sized to create a temporary seal with each cell as it passes through; and therefore the force applied across the funnel infers the forces required to squeeze individual cells through the constriction. To derive an intrinsic property of cells from the force measurement, an individual cell was modelled using a simple liquid drop model [53], which considers a cell as a liquid drop with a constant cortical tension, T_c. The threshold pressure required to squeeze an individual cell through the constriction depends on the rigidity of the deformed cells and cell deformation geometry, including the radii of the leading and trailing cell surfaces, which are in turn determined by the geometrical constraint provided by the funnel (Figure 2.2B). The relationship can be modelled using the Young-Laplace law:

$$\Delta P = T_c \left( \frac{1}{R_a} - \frac{1}{R_b} \right)$$  \hspace{1cm} (2.1)
In this model, \( R_a \) and \( R_b \) are the leading and trailing radii and \( T_c \) is the intrinsic cortical tension (pN·µm\(^{-1}\)), representing the deformability of the test cell. Using this mechanism, the cortical tension values of various cell types are measured, including hematological cells, such as erythrocytes, lymphocytes and granulocytes [55] and malaria \( Pf \)-iRBCs at different intra-erythrocytic stages [34] (0~16 hours: ring stage; 16~24 hours: early trophozoite; 24~40 hours: late trophozoite; 40~48 hours: schizont). Figure 2.3 illustrates the deformability of these cell types making up a spectrum. Each cell is accompanied by images taken from microscopy Giemsa or Wright stain.

**Figure 2.3:** Deformability spectrum of circulating cells including erythrocytes (RBCs), malaria *Plasmodium falciparum* infected RBCs (\( Pf \)-iRBCs), leukocytes (neutrophils, lymphocytes). The unit for the scale is dimensionless. It is a number defined as \( T_c/T_{C-RBC} \) (\( T_c \) represents average cortical tension of cells and \( T_{C-RBC} \) is the average cortical tension value of RBCs).

The normalized cortical tension is defined as the ratio of average cortical tension of certain cell types or disease states divided by the average cortical tension of healthy RBCs (\( T_c/T_{C-RBC} \)). As a result, the normalized cortical tension of RBCs is 1. As shown in Figure 2.3, the rigidity of parasitized RBCs increases progressively as the parasite matures [34]. Furthermore, leukocytes are
significantly more rigid (30X to 60X) than erythrocytes. Lymphocytes and neutrophils are the two abundant subtypes of leukocytes in circulation but they differ markedly in their cell deformability.

**Table 2.1**: Cortical tension value of *Pf*-iRBCs at different intra-erythrocyte development stages as well as two leukocyte phenotypes (neutrophil and lymphocyte)

<table>
<thead>
<tr>
<th>Cell types or disease states</th>
<th>Cortical tension (pN/µm)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy RBCs</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Ring <em>Pf</em>-iRBCs</td>
<td>4.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Early trophozoite <em>Pf</em>-iRBCs</td>
<td>8.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Late trophozoite <em>Pf</em>-iRBCs</td>
<td>21.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Schizont <em>Pf</em>-iRBCs</td>
<td>Average: 486 Range: 51 - 1095</td>
<td>N/A</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>37.0</td>
<td>6.8</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>74.7</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 2.1 shows the cortical tension values of healthy RBCs, ring stage *Pf*-iRBCs, early-trophozoite, late-trophozoite and schizont *Pf*-iRBCs, as well as neutrophil and lymphocyte. The standard deviation data is not shown in the scale (Figure 2.3) but in the table. The cortical tension of healthy RBCs is rather consistent with <10% deviation, probably contributed by the size variation, corresponding to the 11-15% RDW for healthy people. The cortical tension value of *Pf*-iRBCs at progressive development stages shows an increase in variation. At schizont stage, the cortical tension values vary significantly from 51 to 1095 pN/µm.

In conclusion, the clear distinction between the biomechanical properties of these cells suggests that deformability-based sorting could potentially offer an effective means to sort *Pf*-iRBCs from
uiRBCs based on intra-erythrocyte stages, to separate erythrocytes from leukocytes, and to isolate specific phenotypes of leukocytes from whole blood.
Chapter 3: Literature Review

This chapter reviews existing approaches for deformability-based cell sorting, which include approaches based on hydrodynamic chromatography and microfiltration.

3.1 Hydrodynamic Chromatography

Hydrodynamic chromatography aims to alter the flow path of certain cells based on their distinct deformability created through hydrodynamic effects when the cell sample is flowed through a fluidic microchannel. These approaches include deterministic lateral displacement (DLD) and cell margination.

3.1.1 Deterministic Lateral Displacement

Deterministic lateral displacement (DLD) [56] is a microfluidic technique originally developed for separating particles based only on their sizes. This technique has also recently demonstrated its potential to sort biological cells based on deformability [57]–[59]. The DLD device comprises a periodic array of micrometer-scale obstacles. As shown in Figure 3.1A, the principle of DLD can be explained by considering streamlines taken by infinitesimally small fluid particles in a microfluidic device where Reynolds number $<< 1$, and thus fluid flow is entirely laminar. In this scenario, the streamlines are entirely independent of each other and do not cross or merge. Therefore, infinitesimally small fluid particles following one streamline will not move into another. Particles of finite size, however, can be shifted onto a different streamline because of size exclusion, which occurs when the size of the particle exceeds the available space between the streamline and the edge of a boundary. In this case, the particle will be shifted onto a different streamline, where the amount of shift depends on particle size, and is therefore the basis of size-based separation using this mechanism.
Figure 3.1: Principle of deterministic lateral displacement. (A) Conceptual design and principle of the deterministic lateral displacement separation. (B) Images showing GTA-treated RBCs interacting with the pillar at certain shear stresses; untreated RBCs can deform and fit within one streamline, which results in no lateral displacement; GTA-degraded RBCs retain the discoid shape and shift into another streamline, causing lateral displacement of these cells way from the original flow direction. [Figure 3.1B reproduced from ref. [58] with permission copyright ©2014 Holms et al.]

DLD can also be extended to sort cells based on deformability by taking advantage of the fact that fluid flow near an obstacle could apply significant shear stress to compress nearby cells in transit (Figure 3.1B). The velocity difference across the cell, ranging from zero velocity at the boundary to the velocity at the opposing side of the cell, results in a shear stress that deforms the cell and modifies its shape. This can potentially influence the separation angle of the RBCs trajectory through the DLD [60]. Experimentally, this phenomenon has been validated by Holmes et al. [58]. Specifically, they demonstrated the successful separation of untreated RBCs from 0.01% glutaraldehyde (GTA)-treated RBCs based on deformability (Figure 3.1B). GTA is a fixative agent that induces cross-linking and stabilization of RBC membrane proteins to reduce deformability in a concentration dependent manner, while the RBC shape and size remain unchanged. Figure 3.1B
clearly shows that GTA-treated RBCs retain their discoid shape and do not deform, compared with untreated RBCs which deformed significantly under the flow shear stress.

In summary, DLD is a hydrodynamic method for manipulating and separating particles and cells in the laminar flow regime using pillar arrays to alter the flow paths of certain cells. The technique performs extremely well for size-based particle separation. Deformability-based cell sorting is possible in principle, but existing studies have been limited to chemically degraded RBCs and have not been extended to medically relevant samples.

### 3.1.2 Cell Margination

Another form of deformability-based cell separation using hydrodynamic chromatography is cell margination, which relies on the non-inertial lift force experienced by deformable cells near the wall of a microchannel. In a microchannel without any obstacles, rigid spherical particles typically do not cross streamlines in the laminar flow regime (Re<<1) where viscous effect dominates inertial effect. However, softer cells can deform and reorient themselves under shear flow stress; and are able to experience the non-inertial lift force that can help them to cross streamlines. Originally discovered by Goldsmith and Mason in 1961 [61], this force arises from shear stress experienced by deformable cells near the boundary of a channel, such as RBCs, which produces a characteristic tank-treading motion. Tank treading motion refers to the rotation of the RBC membrane, in a similar motion to a treadmill, while cell is stretched axially. Consequently, RBCs remain a constant inclination with respect to the flow streamline which causes the non-inertial lift. As a result of this hydrodynamic lift, RBCs are pushed perpendicularly across streamlines away from the wall. Olla et al. [62] provided a quantitative theoretical calculation of the non-inertial lift force on objects experiencing tank tread motion, which predicted that,
1) Spherical objects experienced no such lift forces at Re<<1 and;

2) the lift force relied on the ability of the objects to deform to the ellipsoid shape.

These theoretical prediction was later proved to be in good agreement with the experimental results for vesicles [63] as well as RBCs and platelets [64]. Specifically, Geislinger et al. [64] investigated the potential to separate RBCs, blood platelets and solid microspheres from each other using the non-inertial hydrodynamic lift at various flow rates and fluid viscosities. A version of the cell margination effect observed for blood flow in capillaries is known as the leukocyte margination [65]. In this scenario, the highly deformable RBCs are pushed towards the center of the blood vessel, while the less deformable and spherical leukocytes experience less lift force and are marginalized near the capillary walls. The phenomenon typically occurs at high hematocrit level as the accumulated RBCs contribute to leukocyte margination by colliding and pushing the non-RBCs away from the centerline towards the side walls.

Figure 3.2: Image and Schematic illustration of the principle of margination. (A) Biomimetic margination design of the bifurcated microfluidic channel for the concentration of leukocytes, a direct implementation of Fåhræus-Lindqvist effect. (B) Conceptual design for the separation of ring stage Pf-iRBCs using non-inertial lift forces. [Figure 3.2A reproduced from ref. [66] with permission Copyright ©2005, American Chemical Society; Figure 3.3B Reproduced from ref. [67] with permission Copyright ©2014 Geislinger et al.]
The margination effect has been used in microfluidic devices to isolate subpopulation of cells based on deformability. For example, leukocyte margination was directly implemented using a biomimetic bifurcation design to isolate leukocytes from whole blood [66]. As shown in Figure 3.2A, this particular device was able to enrich leukocyte concentration over RBCs by 34-fold [66]. Hou et al. [68] presented another realization of the cell margination microfluidic device for deformability-based separation of RBCs infected with *Plasmodium falciparum*. As described in Section 2.1.2, RBCs infected with *P. falciparum* experience a dramatic reduction in their deformability as the parasite grows inside. As a result, deformable uiRBCs experienced greater lift forces, which concentrated in the channel center while sterically displacing malaria iRBCs (late stages) towards the wall. After flowing through a long separation channel (~3 cm), iRBCs were enriched and collected through both the top and bottom side-channel outlets. The group was able to capture iRBCs with a yield ~75% for ring stages and >90% for late stages with 2-fold enrichment of parasitemia. A significant shortcoming of the approach is the low sample (iRBCs) purity due to the requirement of high hematocrits (~40%) input sample and high flow rate (0.2-5 µL·min⁻¹). More recently (2014), Geislinger et al. demonstrated the separation of malaria iRBCs [67] using the hydrodynamic lift force but only at low flow rate and sample hematocrit. The schematic illustration of the concept is shown in Figure 3.2B. The cells injected were focused to be near the lower wall using the sheath flow (not shown in the figure) before they traversed through the separation channel (20 mm long) where they experienced the non-inertial lift effect. The bifurcation between Outlet 1 and 2 occurs at a distance 50 µm above the lower wall with outlet 2 connected at an angle 49° with Outlet 1 (Figure 3.2B). The group was able to achieve higher
enrichment (4.3-fold) than Hou et al. (2-fold) at the expense of low throughput (~12,000 cells per hour).

In summary, cell margination enables deformability-based cell separation using the non-inertial lift force to selectively transport deformable cells towards the center of a microfluidic channel, while rigid cells are marginalized towards the channel walls. This mechanism has been adapted to separate leukocytes from RBCs [66], as well as malaria iRBCs from normal RBCs [67]. A key challenge in current studies is the need for high-density cell samples, which dramatically limits enrichment of target cells over background cells, and the poor selectivity of target cells over the contaminant RBCs. Consequently, the cell margination mechanism is perhaps best utilized as an initial pre-processing step, before refining target cell selection through secondary and tertiary selection steps.

3.2 Microfiltration

Microfiltration is the process of flowing a cell sample through an array of microscale constrictions in order to capture target cells based on their size and deformability. Microfiltration techniques have been used extensively for biophysical cell separation, such as for the separation of leukocytes from whole blood. The strategies for deformability based cell separation using microfiltration can be classified into three basic filter geometries (pore, weir, and pillar) and two filter schemes (dead-end and cross-flow filtration).

3.2.1 Filter Geometry

Figure 3.3 illustrates the three basic filter geometries. The pore filter microstructure consists of a membrane perforated with a 2D array of holes. The diameter of the hole defines the critical cut-off dimension for capturing target cells of certain size and deformability. The weir filter
microstructure consists of microchannels containing a sudden decrease in the channel cross section, which creates a barrier in the flow path to trap larger and more rigid cells while allowing the passage of smaller and softer cells. The size of the gap between the top of the obstruction barrier and the ceiling of the fluidic channel defines the critical cut-off dimension. The pillar filter microstructure consists of an array of micro-posts spaced appropriately to form constrictions to capture target cells. The narrowest distance, or gap, between each pair of pillars defines the critical cut-off dimension.

**Figure 3.3:** Three basic filter geometries including (A) membrane pore; (B) weir; (C) pillar. [Figure 3.3B and C reproduced from ref. [7] with permission Copyright ©2015 by Annual Reviews]

### 3.2.2 Filtration Schemes

There are two types of filtration schemes: dead-end and cross-flow filtration, as shown in Figure 3.4. In dead-end filtration, cells are simply infused into the filter microstructure using a pressure-driven flow. The process is extremely simple to perform, but a key challenge is cell clogging, which can reduce the selectivity of the process and limit the ability to extract the segregated cells for subsequent analysis. Specifically, as target cells are captured in the filter, they close off available constrictions, which increases the hydrodynamic resistance of the filters. If the sample infusion pressure is kept constant, the sample flow rate will be reduced as a result. Eventually, the
sample flow comes to a complete stop, hence the name dead-end. In some cases, the increased hydrodynamic resistance is compensated by increasing the filtration pressure. However, since clogging is an unpredictable process, raising the filtration pressure greatly increases the potential to rupture the cells. Consequently, this approach necessitates fixing the cells to preserve their integrity, which greatly limit the available molecular analyses that could be applied to the captured cells [69], [70]. A related issue to clogging is fouling, which occurs because captured cells remain in contact with the filter microstructure for a significant period of time. The prolonged contact between the cell and the filter microstructure promotes the adsorption, which significantly limits the ability to extract the captured cells after separation.

**Figure 3.4:** Two common microfiltration schemes including (A) dead-end filtration and (B) cross-flow filtration.

To overcome clogging and enable continuous cell sorting, improved filtration technique using cross-flow has been developed. Figure 3.4B is a typical cross-flow filtration scheme, in which a flow tangential to the filter surfaces is applied to remove the captured cells. This process is known as cross-flow or tangential-flow filtration. The scheme diverts most of the flow and cells stuck at the filters away and hence the filters are less prone to clogging. Since the cross-flow scheme aims to allow the less deformable cells to be stopped in a suspended state instead of being trapped in
the filter permanently, this filtration process could be performed continuously. Recent literature provides examples of using cross-flow filtration to separate leukocytes from whole blood in several cases, which is summarized in Table 3.1.

Although cross-flow strategy can significantly reduce cell clogging and adsorption, the selectivity of the approach is limited because the cross-flow action disrupts the filtration process. Previous studies on the separation of leukocytes from RBCs have found that when the cross-flow is too strong, RBCs were not given sufficient opportunity to come in contact with the filters before they were carried away by the cross-flow to leukocytes outlet. Consequently, the purity of the separated leukocytes was typically quite low in existing examples of cross-flow microfiltration device (refer to Table 3.1). When the cross-flow is set to be slow to avoid the contamination of RBCs, it typically results in poor declogging, which can result in low leukocyte recovery [71].

3.2.3 Performance of Existing Microfiltration

3.2.3.1 Definition of Performance Metrics

Performance metrics for evaluating cell sorting techniques include recovery rate or capture efficiency of the target cells, purity of the target cells, enrichment of the relative fraction of target cells, depletion rate of background cell, as well as the overall sample handling throughput. Recovery rate (Equation 3.1) is defined as the fraction of the captured target cells relative to the total number of target cells in the original sample. Depletion rate of background cells (Equation 3.2) is the fraction of background contaminate cells depleted by the device. Purity of the output sample (Equation 3.3) is the fraction of target cells relative to the total captured cells. Purity is a critical parameter, indicating the contamination of unwanted cells. Enrichment is the enhancement of the purity of target cells at the input of the separation system relative to the purity of the target
cells at the output. Depletion rate of background cells, purity, and enrichment are closely related parameters. Studies in this field typically report only one of these three parameters. Furthermore, for leukocyte and malaria Pf-iRBCs separation from whole blood, purity (Table 3.1, Table 5.1 and Table 6.2) is often not reported due to the abundance of the background contaminant RBCs.

Volumetric flow rate is typically reported when defining throughput. Alternatively, the number of cells sorted per unit time is used to indicate throughput. Viability or functionality of the captured target cells needs to be maintained since the separated cells at their original states are preferred for subsequent molecular assays or for clinical therapeutic transfusion.

\[
\text{Recovery Rate or Separation Efficiency} = \frac{\text{Target Cells}_{\text{output}}}{\text{Target Cells}_{\text{input}}} \quad (3.1)
\]
\[
\text{Depletion Rate of Background Cells} = 1 - \frac{\text{Background Cells}_{\text{output}}}{\text{Background Cells}_{\text{input}}} \quad (3.2)
\]
\[
\text{Purity} = \left(\frac{\text{Target Cells}}{\text{Target Cells} + \text{Background Cells}}\right)_{\text{output}} \quad (3.3)
\]
\[
\text{Enrichment} = \frac{\text{Purity}_{\text{output}}}{\text{Purity}_{\text{input}}} \quad (3.4)
\]

### 3.2.3.2 Performance of Existing Microfiltration Techniques for Leukocyte Sorting

Table 3.1 summarizes some of microfiltration techniques developed so far for the separation of leukocytes from whole blood, as an exemplary application. Specifically, the table identifies the filter types and filtration schemes employed by each paper and summarizes their performance metrics, including recovery rate, purity or enrichment and throughput (defined in Section 3.2.3.1). Here, the parameter of capacity is also included to indicate the ability of the device to process high density sample. The purpose of introducing this parameter along with the throughput further
highlights the issue of cell adsorption and clogging issue, commonplace to traditional microfiltration techniques, particularly for dead-end filtration.

In general, for dead-end filtration, the device can either only process limited amount of blood (up to 1 mL) or requires diluted whole blood. Techniques utilizing cross-flow schemes can perform continuous separation but also requires diluted whole blood in many cases. The performances of cross-flow microfiltration for leukocyte separation from RBCs are generally better than those of dead-end microfiltration. Specifically, Ji et al. [72] compared the performances of cross-flow and dead-end microfiltration with the same cut-off size (~3.5 µm) for isolating leukocytes from human whole blood samples. The results confirm that cross-flow filtration schemes with pillar filter geometry has better performances in terms of leukocytes recovery rate, purity and blood handling capacity than those of dead-end filtrations using either pillar, weir or pore.

In summary, microfiltration is a simple and economical strategy to separate biological cells based on deformability despite issues associated with cell clogging and adsorption. Although microfiltration using a cross-flow scheme can reduce the likelihood of clogging and adsorption, the cross-flow cannot solve the clogging issue completely without disrupting the filtration process. The potential underlying issue for the sub-optimal selectivity of cross-flow schemes arises from the fact that the cross-flow is not applied directly against the filtration but at a 90° angle to filtration flow. This issue suggests the idea of developing a declogging flow in the direction against the filtration flow to achieve more efficient declogging effect. This strategy will be discussed in detail in Chapter 4.
Table 3.1: Performances specifications of recent research in leukocytes separation from whole blood using microfiltration

<table>
<thead>
<tr>
<th>Filtration Strategies and Filter Types*</th>
<th>Leukocyte Recovery rate</th>
<th>Leukocytes Purity</th>
<th>RBC depletion rate</th>
<th>Throughput</th>
<th>Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead end; Pillar [73]</td>
<td>18-25%</td>
<td>n.d.</td>
<td>84-89%</td>
<td>15-50 µL min(^{-1})</td>
<td>Diluted whole blood (1:2 in PBS)</td>
</tr>
<tr>
<td>Dead end; weir [74]</td>
<td>71%</td>
<td>210-fold enrichment</td>
<td>n.d.</td>
<td>4000 cells s(^{-1})</td>
<td>Diluted whole blood (1:10 in PBS)</td>
</tr>
<tr>
<td>Dead end; weir [75]</td>
<td>60%</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3-15 µL hour(^{-1})</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Dead end; pore [76]</td>
<td>&gt;90%</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Only process 1.5 µL whole blood</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Dead end; pore [72]</td>
<td>72-85%</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Only process 200 µL whole blood</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Dead end; weir [72]</td>
<td>~70%</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Only process &lt;50 µL whole blood</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Dead end; pillar [72]</td>
<td>70-95%</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Only process 300 µL whole blood</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Cross flow; weir [77]</td>
<td>98%</td>
<td>72%</td>
<td>n.d.</td>
<td>3.6 µL hour(^{-1})</td>
<td>Diluted whole blood</td>
</tr>
<tr>
<td>Cross flow; pore [71]</td>
<td>27.4 ± 4.9%</td>
<td>93.5 ± 0.5%</td>
<td>n.d.</td>
<td>~17 µL min(^{-1})</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Cross flow; pillar [78]</td>
<td>90%</td>
<td>n.d.</td>
<td>90%</td>
<td>10 µL min(^{-1})</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Cross flow; weir [78]</td>
<td>20%</td>
<td>n.d.</td>
<td>40%</td>
<td>10 µL min(^{-1})</td>
<td>Diluted whole blood (1:100 in PBS)</td>
</tr>
<tr>
<td>Cross flow; pillar [72]</td>
<td>70-95%</td>
<td>n.d.</td>
<td>n.d.</td>
<td>20 µL min(^{-1})</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Cross flow; pillar [79]</td>
<td>97%</td>
<td>n.d.</td>
<td>50%</td>
<td>5 µL min(^{-1})</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. Data not available
Chapter 4: Principle and Design of Microfluidic Ratchets

4.1 Introduction

Section 3.2 previously described the microfiltration systems and discussed how this approach suffers a dramatic loss of cell selectivity due to clogging of the microstructures and subsequent fouling of the device. The improved cross-flow filtration scheme is not effective at declogging the cells from the filters as the cross-flow is applied orthogonally to the filtration direction. A potential remedy is to periodically introduce a reverse-flow in the opposite direction to the filtration flow to release clogged cells. However, with a simple microfiltration structure, the effectiveness of this approach is limited, because the reversibility of inertia-less, low Reynolds number flow in microchannels will undo the initial separation.

In this chapter, a novel ratchet mechanism is briefly introduced to solve the reversibility issue. The ratchet mechanism was developed during my master thesis (2009-2011). The underlying principle of the microfluidic ratchet microfiltration scheme relies on the asymmetrical deformation of individual cells through tapered funnel shape constrictions. Specifically, deforming cells along one direction would require less pressure than along the other direction. As a result, oscillatory flow of an appropriate magnitude coupled with this physical asymmetry, allow some cells to transport through the tapered constriction unidirectionally in a ratcheting manner, while other cells are blocked by the constriction and released on the subsequent flow reversal. Under precisely controlled pressure, this transport process can enable deformability-based cell sorting based on a cell’s capacity to deform through micrometer-scale size constrictions. The oscillatory flow, consisting of filtration flow and reversal flow, plays a critical role of minimizing contact between cells and the funnel constrictions to prevent cell clogging and adsorption.
The rest of this chapter is divided into two sections. Section 4.2 discusses the underlying principles of the microfluidic ratchet cell sorting mechanism. Section 0 describes the conceptual design of a microfluidic ratchet cell sorter.

4.2 Microfluidic Ratchet

4.2.1 Deterministic Microfluidic Ratchet

To enable asymmetric transport of individual cells in the low Reynolds number flow regime, microstructures are designed as a 2D tapered constriction, where the opening at the entrance side is larger than the typical cell diameter, while the opening at the exit side is smaller than the typical cell diameter (Figure 4.1A-B). Key geometrical parameters of this microstructure include:

1) the pore size $W_0$, defined as minimum width of the funnel;

2) the thickness of the 2-D funnel microstructure ($H_0$) and

3) the shape of the funnel taper.

The tapered constrictions are sized such as a cell is laterally constrained ($W_0 < \Phi_{\text{cell}}$) and must deform in order to transit, but is vertically unconstrained ($H_0 > \Phi_{\text{cell}}$) to provide stress relief. $\Phi_{\text{cell}}$ is defined as the diameter of a spherical or discoid shape cell. Under these conditions, the deformation force required to transit cells through such a constriction can be modelled using Young Laplace’s Law (Equation 2.1) and liquid drop model described in Section 2.3.
Figure 4.1: Microfluidic ratchet enabling asymmetric deformation of individual cells. (A-B) Tapered funnel constrictions enable unidirectional flow of cells (ratchet behavior) under oscillation excitation consisting of upward filtration flow and downward reverse flow; (C-D) conceptual design of the ratchet cell sorting. [Figure 4.1 C and D reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]

From this model, the pressure ($\Delta P$) required to deform a cell through the funnel constriction is a function of membrane cortical tension ($T_C$), as well as the leading and trailing radii ($R_a$ and $R_b$) of the cells. Because of their confinement in an asymmetric funnel-shape taper, the cell requires a different deformation pressure to transit through the taper along the direction of the taper versus against the direction of the taper [81]. Specifically, when a cell deforms along the direction of the taper (Figure 4.1A), both the leading and trailing radii are constrained, which reduces the difference between $R_a$ and $R_b$, resulting in smaller transiting pressure. Conversely, when a cell deforms against the direction of the taper (Figure 4.1B), only the leading edge ($R_a$) is constrained while the trailing edge ($R_b$) is unconstrained, which results in larger differences between $R_a$ and $R_b$, leading to larger transiting pressure.

During my master thesis, the existence of the pressure asymmetry has been experimentally confirmed by deforming L1210 mouse lymphoma cells ($\Phi_{cell}$: 8-12 µm) through funnel
constrictions with a range of pore sizes (6–11 µm) and shapes (θ = 0°, 5°, and 10°) [81]. The microfluidic device (shown in Figure 2.2) was specifically designed to measure the deformation pressure required to transit L1210 cells both along the direction of the funnel taper (forward pressure, Figure 4.1A) and against the direction of the funnel taper (backward pressure, Figure 4.1B). Furthermore, the pressure asymmetry, defined as the ratio of backward to forward pressure, increased as the taper angle (θ) decreased from 10° to 5°, but disappeared as the angle was reduced to 0°. These results agreed with the theoretical predictions obtained using the Young-Laplace Law (Equation 2.1). The existence of the pressure asymmetry forms the basis of the microfluidic ratchet to allow ratchet transport of individual cells using oscillatory flows.

4.2.2 Deformability-dependent Unidirectional Transport

To confirm that oscillatory flow through tapered constrictions can indeed transport single cells in a ratcheting manner, a separate microfluidic device containing 37 funnel constrictions of 6 µm pore size arranged in series at a pitch of 60 µm, was designed to track the movement of single cells through the funnel constrictions series (Figure 4.2) [81]. A negative control experiment was performed by placing a L1210 mouse lymphoma cell under unbiased oscillatory flow in a microfluidic channel without microstructures (region in Figure 4.2A①). Unbiased oscillatory flow refers to the flow swinging upward and downward at the same speed and duration. Since the fluid flow is pressure-driven, the unbiased oscillation is achieved using pressure pulsation of the same magnitude and duration from both upward and downward directions. Expectedly, these cells showed no net motion confirming the reversibility of low Reynolds number flow in the microfluidic channel. When the same cell was then placed within a series of funnels, the same unbiased oscillatory flow caused the cell to ratchet along the direction of the funnel taper.
Specifically, the cell was able to transit through the funnel constriction in the forward phase of the oscillation flow, but was prevented from returning to its starting point during the backward phase of the oscillation flow. Furthermore, cell ratcheting was found to only occur when the pressure exceeds the threshold deformation pressure (Figure 4.2B) for the cell to transit along the direction of taper within certain oscillatory frequency range (Figure 4.2C), confirming the transport process is selective based on cell deformability. The phenomenon is named microfluidic ratchet as it allows one-way motion of individual cells, analogous to ratchet-pawl configuration. The ratchet mechanism underpins the fundamental principle of the microfluidic deformability-based cell sorting.

Figure 4.2: Microfluidic ratchet enabling one-way flow of individual cells. (A) Images of the individual cell (mouse lymphoma cell, $\Phi_{\text{cell}}=10.5$ $\mu$m) ratcheting through a series of funnels of the same size ($W_0=6$ $\mu$m) under a certain oscillatory pressure ($350$ Pa) and frequency ($0.5$ Hz). (B) The displacement of a single mouse lymphoma cell in the funnel chain with $0.5$ Hz oscillation at various pressure amplitudes. The no-funnel curve is a control experiment which tracks the cell motion in Figure 4.2A(1). (C) Frequency dependence of ratchet motion with an
oscillation pressure amplitude of 150 Pa. [Reproduced from Ref. [81] with permission, copyright ©2011 American Physical Society]

4.3 Cell Sorting using Microfluidic Ratchet

Based on the microfluidic ratchet mechanism discussed in Section 4.2, deformability-based cell sorting can be achieved under oscillatory flows through asymmetric funnel microstructures. While deformable cells ratchet through the funnels, rigid cells are blocked by the constrictions and subsequently released under the reverse flow. To process a large number of cells, a microfluidic device is designed containing a 2D array of micrometer-scale funnel constrictions as shown in Figure 4.3. Specifically, the pore size remains the same at each row of the funnels, but gradually decreases from bottom to top rows. Cells are infused from the bottom-left corner of the sorting region and transported through the array by vertical oscillatory flows and constant horizontal cross-flow.

Figure 4.3: Principle of deformability-based sorting using microfluidic ratchet. Conceptual design of the ratchet cell sorting. [Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]
As shown in Figure 4.3, the upward phase of the oscillatory flow works to discriminate the cells based on deformability, the downward phase of the oscillatory flow works to release the cells to prevent clogging and fouling, and the simultaneous cross-flow works to extract the sorted cells. These three flows combine to propel the cells in a zig-zag diagonal path through the constriction array. As cells reach a limiting pore size that prevents their transit, they proceed horizontally between funnel rows towards the outlet reservoirs. Since cells with different size and deformability will be blocked by different pore sizes, the cell population are sorted based on the ability to deform in this manner.

The microfluidic ratchet cell sorting process presents several advantages over traditional microfiltration (Section 3.2). Firstly, during the ratchet sorting process, within the funnel microstructures, the cells do not experience significant deformation until nearing their blocking funnel row. As a result, the integrity and viability of the cells are maintained throughout the sorting process. Secondly, because this oscillatory flow ensures that cells are not permanently trapped within the funnel microstructure, the hydrodynamic resistance of the filter remains constant. As a result, all incoming cells experience a constant filtration force. Furthermore, the oscillatory flow ensures that cells come into contact with the filtration microstructures only momentarily, which prevents cell adsorption and enables processing of high cell concentration samples, such as whole blood which typical have a hematocrit (volume of cells over total volume of the sample) of 40-45%. Finally, the multiple funnel array design can sort a cell sample into multiple outlets, which provide significantly greater ability to analyze heterogeneous samples compared to binary cell separation using traditional microfiltration.
Chapter 5: Deformability-based Sorting of RBCs using Microfluidic Ratchets

5.1 Introduction

5.1.1 Motivation

Previously in Section 2.1, the disease of malaria has been reviewed in detail including the biology of malaria parasitism, specifically the factors contributing to the loss of deformability of malaria iRBCs, and the current malaria detection approaches, such as gold standard microscopy and RDTs. The significances of deformability-based separation of Pf-iRBCs from uiRBCs can be summarized as follows,

1) In clinics, there exists a need to sort patient blood sample to enrich for iRBCs from the abundant uiRBCs in order to improve the sensitivity of the current diagnostic tests.

2) In research, it is essential to sort Pf-iRBCs based on their intra-erythrocyte stages to further understand the molecular process associated with the maturation of the malaria parasites, which aids in finding novel targets for anti-malarial drug compounds discovery and testing.

The current challenges for malaria detection include:

1) Patient blood parasitemia can be as low as a few parasites per μL whole blood (<0.0002% parasitemia), which is significantly below the detection limit of Giemsa microscopy.

2) Early stage Pf-iRBC is typically what’s found in clinical blood samples [19], [82] because late stage iRBCs are prone to cyto-adhesion in blood vessel and sequestration in capillaries. Early stage Pf-iRBCs appear as a faint ring shape structure inside the iRBCs in Giemsa stained blood smear under microscopy, and are easy to miss by inexperienced technicians.
These two factors require the ability of the separation technique to be able to distinguish the subtle differences in deformability between early ring stage iRBCs and uiRBCs as well as the ability to enrich from ultra-low parasitemia sample to a level of parasitemia easily and confidently detected by Giemsa microscopy and RDTs.

5.1.2 Literature Review

The existing biophysical methods to separate malaria iRBCs from uiRBCs have been summarized in Table 5.1. The performance metrics for cell sorting have been defined in Section 3.2.3.1. In the case of malaria sorting, the parameter purity is often replaced with the term parasitemia. And the enrichment factor is related to the parasitemia by equation 5.1. The throughput is usually expressed as number of cells per unit of time (minute or hour).

\[
Enrichment\ Factor = \frac{\text{Parasitemia}_{\text{output}}}{\text{Parasitemia}_{\text{input}}} \quad (5.1)
\]

The most commonly used benchtop approach is density gradient centrifugation [83] based on the fact that iRBCs at later intra-erythrocyte stages are less dense than early stage iRBCs and uiRBCs. However, the method suffers from limited selectivity and can only separate late stages intra-erythrocyte iRBCs. As a result, gradient centrifugation is used only in malaria research to process large volumes of cultured malaria samples of high parasitemia to isolate late stage iRBCs.

Alternatively, RBCs have been biophysically separated based on size, deformability, permeability and cyto-adherence, using margination (discussed previously in Section 3.1.2) [67], [68], dielectrophoresis [84], and surface-enhanced cyto-adherence [85]. As shown in Table 5.1, these methods are effective when isolating Pf-iRBCs at late (trophozoite and schizont) stages of infection. However, they are not effective when target cells are distinguished by subtle differences,
such as ring stage *Pf*-iRBCs. *Pf*-iRBCs can also be isolated using magnetic attraction of biocrystallized hemozoin. This approach is also effective for isolating late stage *Pf*-iRBCs [86] and recently has been shown to have some effect on early stage *Pf*-iRBCs using microfluidics, though only at high parasitemia [87]. None of the existing approaches, however, provide an effective method to sort RBCs based on deformability and to enrich for early stage *Pf*-iRBCs at clinically relevant concentrations (<0.01%).

**Table 5.1:** Performances specifications of recent label-free methods in malaria iRBCs separation from uiRBCs

<table>
<thead>
<tr>
<th>Biophysical Parameters/Method</th>
<th>Malaria Plasmodium species</th>
<th>Performance Metrics</th>
<th>Intra-erythrocytic stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density / Density Gradient Centrifuge [83]</td>
<td><em>falciparum</em></td>
<td>&gt;5%</td>
<td>n.d.</td>
</tr>
<tr>
<td>Permeability / Dielectrophoresis on chip [84]</td>
<td><em>falciparum</em></td>
<td>0.01-5%</td>
<td>50-200X</td>
</tr>
<tr>
<td>Magnetic property / on-chip magnet [87]</td>
<td><em>falciparum</em></td>
<td>~10%</td>
<td>10X</td>
</tr>
<tr>
<td>Size and Deformability /margination [67]</td>
<td><em>falciparum</em></td>
<td>1.6%</td>
<td>4.3X</td>
</tr>
<tr>
<td>Size and Deformability /margination [68]</td>
<td><em>falciparum</em></td>
<td>0.25%</td>
<td>2X</td>
</tr>
<tr>
<td>Surface adhesion /on-chip immobilization [85]</td>
<td><em>gallinaceum</em> (avian)</td>
<td>3.2-20.1%</td>
<td>5-23X</td>
</tr>
</tbody>
</table>

n.d.: Data not available

Using the microfluidic ratchet cell sorting strategy discussed in Chapter 4, the ability to separate *Pf*-iRBCs at different intra-erythrocyte stages, as well as to enrich for ring stage *Pf*-iRBCs at...
clinically relevant concentrations will be demonstrated. These advances aim to dramatically improve the sensitivity of malaria diagnosis. The rest of the chapter will be organized as follows:

Section 5.2 describes the microfluidic ratchet design, hydrodynamic resistance model and operation of the devices. Section 5.3 validates the ability of the device to sort RBCs based on deformability using RBCs treated with glutaraldehyde at various concentrations as a model to mimic the loss of deformability of malaria iRBCs at various parasite intra-erythrocyte stages. Subsequently, the ability of the device to sort Pf-iRBCs based on their intra-erythrocyte stages is investigated. Section 5.4 demonstrates the ability of the ratchet sorting to improve the sensitivity of malaria diagnostic approaches, including microscopy and RDTs. Section 5.5 discusses the limitations of the current study. The chapter concludes with a summary and discussion.

5.2 Microfluidic Ratchet Device for RBCs Sorting

5.2.1 Microfluidic Device Overview

The microfluidic ratchet device developed to sort RBCs is shown in Figure 5.1. In the figure, the device was infused with different food color dyes to highlight the geometric design. The central part of the device is the sorting region containing a 2D array of funnel constrictions. The funnel array is connected by a left cross flow inlet (CFI) and sample inlet (SI), to nine outlet collectors (O1-9) on the rightmost edge of the device. Oscillation inlet 1 and 2 (Osc1 and Osc2) line the top and bottom of the sorting matrix. These flows combine to propel the sample stream to form a characteristic diagonal trajectory through the funnel array.
Figure 5.1: Images of the microfluidic ratchet device for RBC sorting operation.
[Figure 5.1, Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]

5.2.2 Microfluidic Device Design Details

5.2.2.1 Sorting Region of the Microfluidic Ratchets for RBCs Sorting

To sort RBCs, rectangular sorting region consists of 35 rows and 630 columns of funnel constrictions, occupying an overall area of 1250 µm by 6350 µm (Figure 5.2A). The geometry of individual funnel shape is shown in Figure 5.2D. Specifically, there are three critical geometrical parameters that determine the function of the funnel filters to sort cells based on deformability. They are pore size, funnel thickness and funnel shape. These parameters have been introduced in Section 4.2.1, where the design of individual funnel for measuring single cell deformability was discussed. Here, the design of these funnel design parameters for cell sorting is discussed.
Table 5.2: Pore size arrangement

<table>
<thead>
<tr>
<th>Pore Sizes (µm)</th>
<th>1.5</th>
<th>1.75</th>
<th>2</th>
<th>2.25</th>
<th>2.5</th>
<th>3</th>
<th>3.5</th>
<th>6</th>
<th>7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Rows</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Outlet Number</td>
<td>O1</td>
<td>O2</td>
<td>O3</td>
<td>O4</td>
<td>O5</td>
<td>O6</td>
<td>O7</td>
<td>O8</td>
<td>O9</td>
</tr>
</tbody>
</table>

1) **Pore size**: The pore size ($W_0$) is kept constant along each row, and decreases every four rows from bottom to the top of the funnel matrix. In total, the 35 rows of constrictions correspond to 9 different pore sizes (Table 5.2). Each outlet number corresponds to a blocking pore size for segregating a subpopulation of cells. For example, O3 collects the fraction of RBCs which are blocked by the 2 µm pore but can traverse through 2.25 µm pore. O1 collects the most deformable fraction of RBCs while O9 collects the least deformable fraction. Together, the funnel arrays sort the input sample cells into 9 fractions based on deformability. The design of the pore size for RBC sorting is limited by fabrication capability. The minimum gap width that can be fabricated using the current photolithography process (see Appendix 7.3.2A.1 Photolithography) is 1.5 µm with a resolution of 0.25 µm. The design of four repeating rows of identify funnel pore was intended to decreases the likelihood of the funnel pillars defects leading to the accidental passage of RBCs through the funnel pore that otherwise would block the cells.

2) **Funnel thickness (or height) of the sorting region**: The thickness of the funnel determines the orientation of the RBCs as they deform through the funnel constriction. Previously, devices with funnel thickness 3-6 µm have been fabricated to sort RBCs. When the microchannel is too thin (3 µm), it greatly increases the surface interactions between the RBC and the
microstructures, RBCs adsorbed and clogged the sorting region within 10 minutes of operation. Sorting region thickness of 6 µm will enable RBCs to traverse sideway through the funnel. This situation causes minimal deformation of individual RBCs, and decreases the sensitivity of the deformability-based RBC sorting. Through trials and errors, the thickness of the sorting region is designed to be 4.5 µm (Figure 5.2B), which is sufficient to constrain RBCs in a planar configuration while still allowing them to be transported freely by fluid flow.

3) **Funnel taper shape:** The shape of the funnel is critical for the ratchet effect described in Section 4.2.1. Previously, McFaul et al. [88] determined through simulation that parabolic shape funnel yielded greater directional asymmetry in deformation pressure. Specifically, parabolic shaped funnel created greater differences between forward (Figure 4.1A) and backward pressure (Figure 4.1B) with less overall compression of each cell, compared with straight funnel shape. Curved structure also provides a smoother transition of cells through the constriction, which is less likely to damage the cell. As shown in Figure 5.2D, the current version of the funnel filter is a parabolic shape with the Equation 5.2.

\[
y = \pm \left( kx^2 + \frac{W_0}{2} \right), \quad k = 5000 \text{ m}^{-1} \tag{5.2}
\]
**Figure 5.2:** Design detail of the microfluidic ratchets for RBCs sorting.  
(A) Image of the microfluidic device sorting a population of RBCs.  
(B) Cross-sectional region of a-a’ showing the thickness of the sorting region (funnels) and inlet/outlet channels.  
(C) RBCs transit the funnel matrix until reaching the blocking pore sizes.  
(D) The parabolic funnel shape design.  

[Figure 5.2 A and C, Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]
5.2.2.2 Supporting Components of the Microfluidic Ratchets for RBCs Sorting

The supporting components to the sorting region include the Osc1, Osc2, CFI and SI as well as the outlets. They are responsible for controlling the flow rate and direction of the cell sample through the sorting region. The height of these peripheral channels is ~10 µm, compared to the 4.5 µm for the sorting region (Figure 5.2B). The carrier fluids through the channels are phosphate buffer saline (PBS) with 0.2% (v/v) Pluronic™ F-127 to keep cells viable during the sorting process and to minimize the non-specific binding of the cells to the silicone microstructures. Figure 5.3 illustrates the detail design of the supporting microchannels.

1) **Tree microchannel network:** As shown in Figure 5.3B-D, the inlets (CFI, Osc1 and Osc2) are connected to the sorting region through the “tree network” design. Specifically, a single inlet channel is split into a series of multiple branches of channels. The tree network design aims to diminish the effects of parabolic velocity profile associated with a single channel at low Reynolds number flow (Figure 5.3B). The design ensures a uniform flow rate across the sorting region both vertically and horizontally. Therefore, all incoming RBCs experience approximately the same flow rates regardless of their positions in the sorting region. This property is critical for the vertical oscillatory flow in that parabolic flow profile across the sorting region will lead to higher filtration pressure near the middle of the matrix as compared to the sides of the matrix. For the Osc1 and Osc2, a single inlet channel was eventually split into 512 nozzles connecting to the sorting region (Figure 5.3C and D). The cross-flow inlet channel was split into 32 separate channels (Figure 5.3B). The sample inlet was split into 4 sample introduction channels (Figure 5.3C). Together the 36 channels (CFI and SI) lead to the 36 gaps between each adjacent rows of funnels in the sorting region. The four channel sample
inlets design (Figure 5.3C) increases the cross-sectional area of the cell sample introduction and therefore can decrease the likelihood of cell-debris clogging issue at the sorting region inlet, causing premature clogging or termination of the sorting process.

2) **Half-circle barriers:** As shown in Figure 5.3D, a single row of dome-shape structure was incorporated in between the oscillation tree network and the sorting region. The structure has a minimum gap size of 1.5 µm, intended to prevent cells from entering the oscillation channels. This design was proved to be not necessary because the angle of the RBCs stream can be precisely controlled by the pressure applied to each inlet and no cells can escape into the Osc1 or Osc2.

3) **Outlet:** Figure 5.3E shows the design of the outlets. Each outlet collects RBCs blocked by the four repeating rows (three for O1) of funnel filters as discussed in 5.2.2.1.
Figure 5.3: Design detail of the supporting channels to the sorting region. 
(A) RBCs follow a diagonal trajectory in response to the sample inlet (SI) flow, cross flow inlet (CFI) flow and biased oscillation flows (Osc1 and Osc2). (B) Blow-up image of the outlet design, indicating that four rows of repeating pore sizes lead to one outlet. Tree channel design for (C) cross-flow inlet channels as well as for (D) oscillation channels. (E) the dome-shape barriers designed to prevent the cells from escaping into the oscillation channels. [Figure 5.3 A, Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]
5.2.3 Hydrodynamic Model

The motion of cells through the funnel array is controlled from microchannels that feed fluid from the top, bottom, and left sides of the array as shown in Figure 5.1. The relative magnitude of these flows controls the path followed by cells in the array, while the oscillation fluid flows, particularly the upward oscillation, controls the deformation forces applied to each cell. To optimize the magnitude and oscillation period of these flows, the system was initially modelled using a finite element package (COMSOL) to determine the geometries for the microchannels. After fabricating the devices, the flow rates are fine-tuned by adjusting the pressure applied to all the inlets (CFI, SI, Osc1 and Osc2) empirically, discussed later in Section 5.2.4.

Laminar fluid flow can be modelled using standard methods of linear electrical circuit analysis. Specifically, fluid flow in a microfluidic channel is linearly proportional to the pressure drop across the length of the channel. For incompressible fluid, the volume is conserved and therefore, pressure and the flow rate can be determined from,

\[ \Delta P = R_H \times Q, \]  

where \( \Delta P \) is the pressure difference (Pa) across the fluidic channel, \( Q \) is the volumetric flow rate (m\(^3\)·s\(^{-1}\)) and \( R_H \) is the hydrodynamic resistance (Pa·s·m\(^3\)). The design of the microfluidic device is guided by the hydrodynamic resistance model based on the following design objectives:

1) The fluid flow within the sorting region is determined by the pressure applied to the supporting microchannels including CFI, SI and Osc1\&2; and influenced by the presence of funnel filling cells, which can change the hydrodynamic resistance of the sorting region. To minimize the effect from cells occupying the filters, it is imperative to design the supporting microchannels
to have dominant resistance in order to desensitize the variation of the flow due to the presence of the cells adsorbed in the funnels. The dominant resistance can be achieved by adding serpentine shape microchannels.

2) The pressure required to squeeze an individual RBCs through micron-scale constrictions has been determined previously to be on the order of a few pascals [34], [49]. To achieve such minute pressure range, the pressure applied at the oscillatory channels (on the order of hundreds of pascals) needs to be attenuated to the order of a few pascals range.

Figure 5.4: Hydrodynamic resistance model of the microfluidic ratchet cell sorting device. (A) Image of the microfluidic device and (B) its equivalent hydrodynamic circuit model analysis. Electric circuit equivalent to the (C) horizontal flow network and (D) vertical oscillation network. [Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]
Figure 5.4B illustrates the equivalent hydrodynamic circuit for the microfluidic device (Figure 5.4A), which has components including cross flow network ($R_{CF}$), sample inlet network ($R_{SI}$), oscillation network ($R_{OSC}$) and outlet networks ($R_{O}$). Fluid flow in the sorting region can be considered as a superposition of the horizontal constant flow circuit (Figure 5.4C) and vertical oscillation flow circuit (Figure 5.4D). In the vertical circuit, the hydrodynamic resistance of the sorting region, $R_{V\_SORT}$, is the sum of resistance for individual funnel constrictions,

$$R_{V\_SORT} = \sum_{i=1}^{n_{row}} r_i, \quad (5.4)$$

where $n_{row}$ and $n_{column}$ are the number of funnel rows and columns in the matrix, $r_i$ is the resistance of the individual funnel and the value of each $r_i$ is determined using finite element simulation (COMSOL multiphysics, full list of the values shown in Table 5.3). In the horizontal circuit, the hydrodynamic resistance of the sorting matrix, $R_{H\_SORT}$, can be determined from the resistance of the spacing between each funnel row ($r_{spacing}$) using

$$R_{H\_SORT} = \frac{r_{spacing}}{n_{spacing}}, \quad (5.5)$$

where $n_{spacing}$ is the number of horizontal spacings in the sorting matrix.

To realize the objectives outlined at the beginning of this section, the supporting microchannels ($R_{O}$, $R_{OSC}$, $R_{CF}$ and $R_{SI}$) are designed to present a dominant hydrodynamic resistance (>50X) over that of the funnel matrix, allowing precise control of fluid flow using pressure-driven flow from the inlets (full listing of the hydrodynamic resistance values are in Table 5.4). This heavy-peripheral-light-center hydrodynamic design provides a constant flow rate in the funnel matrix, whose resistance may vary with the number of cells in the funnel matrix, and thereby ensures that
each cell experiences a nearly constant deformation pressure. The design further serves to dramatically reduce the pressure applied at the inlets to derive an attenuated version for each cell. Specifically, the pressures ranging from 14 to 20 kPa applied at the oscillation inlets are reduced to less than 10 Pa at each funnel constriction, which was previously determined to be appropriate to distinguish normal RBCs and ring-stage iRBC through similarly sized microfluidic constrictions [34].

**Table 5.3:** Summary of hydrodynamic resistance of ratchet funnel

<table>
<thead>
<tr>
<th>Pore size (µm)</th>
<th>1.5</th>
<th>1.75</th>
<th>2</th>
<th>2.25</th>
<th>2.5</th>
<th>3</th>
<th>3.5</th>
<th>6</th>
<th>7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic resistance ($\times 10^{14} \text{ Pa} \cdot \text{s} \cdot \text{m}^{-3}$)</td>
<td>64.6</td>
<td>46.3</td>
<td>34.9</td>
<td>27.5</td>
<td>22.5</td>
<td>16.1</td>
<td>14.3</td>
<td>12.4</td>
<td>5.07</td>
</tr>
</tbody>
</table>

**Table 5.4:** Summary of hydrodynamic resistance of sorting device components

<table>
<thead>
<tr>
<th>Components</th>
<th>$R_{\text{CFI}}$</th>
<th>$R_{\text{SI}}$</th>
<th>$R_{\text{OSC}}$</th>
<th>$R_{\text{O}}$</th>
<th>Sorting region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic resistance ($\times 10^{14} \text{ Pa} \cdot \text{s} \cdot \text{m}^{-3}$)</td>
<td>150</td>
<td>120</td>
<td>100</td>
<td>1200</td>
<td>1.65 ($R_{\text{V,SORT}}$); 20 ($R_{\text{H,SORT}}$)</td>
</tr>
</tbody>
</table>

5.2.4 **Microfluidic Cell Sorting Operation**

Operation of the microfluidic device involves initially introducing the cell sample under predetermined pressure settings for the SI, CFI and Osc1&2 as well as oscillation frequency for Osc1 and Osc2. These parameters were empirically determined to produce a characteristic diagonal stream across the rectangular sorting region. The filtration pressure (Osc2) is set by observing the angle of the samples diagonal stream. If the filtration pressure (Osc2) is too low, the cells do not have sufficient time to test each row of funnel and will exit the sorting region prematurely. If the
filtration pressure is too high, RBCs will exit the sorting region through the top oscillation channel. This also increases the likelihood of rupturing Pf-iRBCs and releasing the parasites while being squeezed through their limiting funnel opening, causing cell adsorption and potential clogging of the system. The acceptable range of filtration pressure was determined to be 14 kPa to 20 kPa, which confines the cell sample within the sorting region while allowing RBCs, particularly Pf-iRBCs to reach their limiting funnel opening and to be collected in the corresponding outlets. The oscillation was set to bias upwards (Osc2) to filter the cells for 4 seconds and downwards (Osc2) to declog the cells for 1 second. To minimalize the interruption of reverse flow for declogging, it was determined that 1 second of downward oscillation at 14 kPa was sufficient to release any cells trapped in the funnel constrictions. Upward oscillation longer than 4 seconds may increase the likelihood of cells being adsorbed in the funnels, which may lead to the eventual clogging of the funnel matrix. The pressure for the SI determines the throughput of the sorting process as a smaller SI pressure will limit the number of cells infused into the sorting region. The CFI pressure creates a constant cross-flow and plays a critical role in forming the characteristic diagonal trajectory. A high CFI pressure will force the cells to traverse through the sorting matrix prematurely and a low CFI pressure will cause the sample infused from SI to travel into the CFI networks during the upwards oscillation. Accordingly, the pressure values for both CFI and SI are set between 5 to 6 kPa.

5.3 Device Validation

5.3.1 Sorting Chemically Degraded RBCs Based on Deformability

To establish the ability of the microfluidic ratchet sorting device to sort RBCs based on deformability, RBCs are rigidified artificially by exposing them to small concentrations of
glutaraldehyde (GTA). GTA is a fixative agent that induces cross-linking and stabilization of RBC membrane proteins and reduces deformability in a concentration dependent manner. Blood preparation procedure was described in detail in Appendix 7.3.2A.3. The effect of GTA on RBCs deformability has been verified quantitatively using the microfluidic micropipette aspiration assay described in Section 2.3. Specifically, I have measured the deformability (as defined by their cortical tension) of individual RBCs exposed to 0%, 0.005%, 0.01%, 0.015% and 0.025% (v/v) GTA concentration, as shown in Figure 5.5A. The results indicate that:

1) There is a progressive increase in rigidity of GTA-treated RBCs at concentrations from 0.000% to 0.015%. GTA concentration greater than 0.025% will make RBCs entirely rigid and unable to be deformed through the constriction at the maximum available pressure;

2) The effect of GTA on the deformability of RBCs population is non-uniform, indicated by the error bar.
Figure 5.5: Validation of deformability-based cell sorting using microfluidic ratchets. (A) Comparison of deformability of RBCs rigidified by GTA at progressively increasing concentrations. (B) Percentage distributions across the outlets of the RBCs treated with various concentrations of GTA for RBCs from three different donors. (C) Micrographs of 0.000% and 0.050% GTA treated RBCs in the funnel constrictions. [Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]

Subsequently, RBC samples exposed to 0.000% up to 0.050% GTA were sorted using the microfluidic ratchets. The sorting process distributed these cells into the outlets in a manner consistent with their decreased deformability. Specifically, RBCs exposed to 0.000% and 0.010% GTA were distributed in O1-3, while increasing the GTA concentration to 0.015%, 0.025%, and 0.050% resulted in progressive rightward shifts in their distributions (Figure 5.5B). RBCs exposed to 0.050% GTA retained their discoid shape, which prevented them from transiting through 6 µm
pores (Figure 5.5C). This sorting experiment was repeated three times using blood from three different donors. The resulting RBC distributions were consistent and demonstrated the repeatability of the deformability-based sorting process.

5.3.2 Sorting \(Pf\)-iRBCs Based on the Intra-Erythrocyte Stages

After the validation of deformability-based RBCs sorting using GTA model, \(Pf\)-iRBCs at different intra-erythrocyte stages of parasite development were then sorted using the microfluidic ratchets. The deformability of various stages of \(Pf\)-iRBCs has been measured using microfluidic micropipette aspiration, described in Section 2.3 [34]. The result is summarized in Figure 5.6A, which indicates a progressive decrease in deformability as the parasite matures within host RBCs.

To obtain \textit{falciparum} malaria parasites at various intra-erythrocyte stages individually, the malaria cultures (Appendix 7.3.2A.4, Synchronization) were synchronized to be approximately at the same stages of parasite maturation. This can be validated using the Giemsa stain thin blood smear method (Figure 5.6B, Appendix 7.3.2A.4, Giemsa Staining). For the sorting experiment, aliquots from a \textit{P. falciparum} culture at 4, 16, 28, 36, 44-hour post-synchronization (Figure 5.6B) were sorted and then counted at each outlet. Each sample was maintained at 5% hematocrit. Prior to processing, each sample is stained using Hoechst DNA stain (Appendix 7.3.2A.4, Hoechst Fluorescence Staining) to facilitate enumeration of the \(Pf\)-iRBCs after sorting. After the sorting process was finished, the iRBCs were counted through manual screening of each outlet under the fluorescence microscopy. UiRBCs incubated in the identical environment as the parasite culture were used as a control. These cells were primarily distributed in O1-4. At the 4 and 16-hour time points, \(Pf\)-iRBCs were predominantly at ring stage (Figure 5.6B, 4-hour image) and had a distribution centered around O3. At the 28, 36, and 44-hour time points, the \(Pf\)-iRBCs were
predominantly *trophozoite* and *schizont* stage, and had a distribution centered around O4 and O5. In general, the *Pf*-iRBC distribution exhibited a monotonic rightward shift that directly correlated with incubation time after synchronization (Figure 5.6C), which can be better visualized as a cumulative distribution (Figure 5.6D). The normal distribution curves were fitted to each sample distribution results to better illustrate the shift in distributions. The ability to distinguish *Pf*-iRBCs at different stages of development could likely be improved by further optimization of the constriction matrix geometries (pore size $W_0$ and funnel thickness $H_0$), as well as the filtration pressure. Nonetheless, these results are consistent with our previous efforts to measure the deformability of *Pf*-iRBCs at different stages of intra-erythrocyte development (Figure 5.6A).
Figure 5.6: Deformability-based sorting of Pf-iRBCs at different intra-erythrocyte stages. 

(A) The deformability of freshly-drawn RBCs unexposed to parasite culture, exposed but uiRBCs from a P. falciparum culture, as well as Pf-iRBCs at the ring, early trophozoite, late trophozoite and schizont stages (from Guo et al. 2012 [34]). (B) Micrographs of Giemsa stained uiRBC and Pf-iRBCs at 4 to 44-hours post-synchronization. Percentages of iRBCs at ring (R), trophozoite (T) and schizont (S) stages at each time point are shown within the images, validated by Giemsa stain microscopy. (C) Normalized distribution of uiRBCs and Pf-iRBCs at 4-44 hours after ring-stage synchronization. (D) Result in C shown as cumulative distribution. [Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]
5.4 Device Application

5.4.1 Improve the Sensitivity of Microscopy-based Malaria Diagnosis

The potential to use deformability-based cell sorting to enrich for Pf-iRBCs to improve the sensitivity of malaria diagnosis performed using microscopy is studied. To model clinical samples, Pf-iRBCs synchronized at the ring-stage with approximately 5% parasitemia were doped into uiRBCs to create the desired parasitemia. Initially, samples at a moderately low parasitemia (0.01-0.1%) were sorted in order to determine the outlets distribution of Pf-iRBCs. The hematocrit of the samples was maintained at constant 5%, regardless of the starting parasitemia. Prior to processing, each sample is stained using Hoechst DNA stain to facilitate enumeration of the Pf-iRBCs after sorting. Pf-iRBCs were significantly enriched in O4-7 and depleted in O1-3 (Figure 5.7A). In the latter case, samples from O1-3 were pooled together in order to provide statistically meaningful measures of parasitemia.
Figure 5.7: Deformability-based sorting of RBCs improves the sensitivity of malaria diagnosis performed using microscopy.

(A) The distribution of Pf-iRBCs in low-parasitemia samples after sorting by the microfluidic ratchet. Pf-iRBCs selectively accumulate in O4-7 and can achieve an increasingly greater magnitude of enrichment for samples with a low starting parasitemia (indicated by dotted line).

(B) Enrichment of Pf-iRBC from 11 samples with parasitemia ranging from 0.04% to 0.0004%. The output sample was pooled from O4-7. The initial and enriched parasitemia is shown relative to the detection limit for thin and thick film microscopy.

(C) The resulting enrichment factors from the 11 samples.

(D) Dot plot showing the correlation between the initial parasitemia and the enriched parasitemia. [Reproduced from Ref. [80] with permission, copyright ©2012 The Royal Society of Chemistry]

To investigate the enrichment of ring-stage Pf-iRBCs at even lower parasitemia, a series of samples with parasitemia ranging from 0.0004% to 0.04% were prepared and sorted. Fractionated samples collected from O4-7 were pooled together to measure the resulting enriched parasitemia.
The performance metrics used to evaluate the microfluidic ratchets are output parasitemia and enrichment factor is defined as the ratio of the enriched parasitemia and the starting parasitemia. In parasitology, in lieu of purity, parasitemia is used to indicate the concentration of parasite density in blood, defined as the ratio of parasitized RBCs number over total number of RBCs. Enrichment factor has been defined in Section 5.1.2, equation 5.1. For the microfluidic ratchets, the enrichment is defined as

\[ \text{Enrichment Factor} = \frac{\text{Parasitemia}_{0-7}}{\text{Parasitemia}_{\text{input}}} \]  

(5.6)

As shown in Figure 5.7B, samples with starting parasitemia orders of magnitude lower than the detection limits of thin-film and thick-film microscopy were enriched to a detectable range (1%-3% parasitemia), equivalent to enrichment factors of 100 to 2500X (Figure 5.7C). Interestingly, the magnitude of enrichment shows some dependence on the initial parasitemia of the sample, where the sorting process produced a greater enrichment for samples with lower initial parasitemia (Figure 5.7D). This result likely arises from a bystander effect, where metabolic by-products released from Pf-iRBCs, such as hemin, rigidifies uiRBCs in the sample to effectively reduce the selectivity of deformability-based sorting [89]. Regardless of this effect, however, our results show that deformability-based ratchet sorting is able to dramatically lower the detection limit of malaria diagnosis performed using microscopy.

**5.4.2 Improve the Sensitivity of RDTs**

Finally, the potential to use deformability-based cell sorting to improve the sensitivity of malaria detection performed using rapid diagnostic tests (RDTs) was investigated. RDTs have been reviewed in Section 2.1.3.2 as an alternative diagnostic approach. RDTs strips based on
plasmodium lactate dehydrogenase (pLDH) were selected because of their low false positive rate [90]. Firstly, RDTs sensitivity was evaluated over a range of parasitemia as shown in Figure 5.8A. The positive result is indicated by a color appearing band to the right of the quality control band. The experiment established their detection limit of pLDH RDTs to be 0.004% parasitemia (Figure 5.8B). Subsequently, ring-stage Pf-iRBC samples at 0.003% and 0.0006% parasitemia were prepared, as well as a positive control at 0.1% parasitemia. As shown in Figure 5.8C, the RDT was not able to detect the infection without enrichment at 0.003% and 0.0006% parasitemia (indicated by non-appearing positive band), whereas the enriched output pooled from O4-7 of the microfluidic device were detected positive (indicated by visible positive band). In these cases, the optical density of the detection band for the enriched samples were similar to 0.02% or 0.01% parasitemia respectively (Figure 5.8C). These results confirm that microfluidic enrichment could dramatically increase the sensitivity of RDTs for *falciparum* malaria.
5.5 Limitations

The limitations of the current study for deformability-based RBCs sorting are discussed as follows:

1) There is a significant overlapping of the distribution of various stages of parasite development as shown in Figure 5.6. Firstly, this could be contributed by the heterogeneity of the deformability within the iRBCs population of the same intra-erythrocyte stages, especially at the later stages,
which is evident in the deformability data shown in Figure 5.6A. Secondly, it is difficult to achieve a tight synchronization. As shown in Figure 5.6B, at hour-4, there is approximately 10% of the iRBCs not at expected ring stages, determined from manual inspection of the thin-smear images. The ability to distinguish Pf-iRBCs at different stages of development could likely be improved by further optimization of the geometries of the constriction matrix.

2) To model clinical relevant Pf-iRBCs, cultured P. falciparum was used. The model doesn’t represent the authenticity of the patient blood due to the absence of the leukocytes in the culture. Leukocytes are spherical and more rigid than erythrocytes, and therefore, can easily be retained in the microchannel designed for RBCs sorting, leading to device clogging or even failure. A potential remedy to the limitation is to add a step of leukocyte depletion prior to the sorting enrichment.

3) The throughput of the microfluidic device is currently limited to ~ 1 million cells per hour. This is largely due to the difficulty in processing iRBCs at high hematocrits (>10% hematocrit). Despite the extreme deformability of RBCs, the iRBCs suffer from increased cytoadherence and have the potential to foul the device, causing the experiment to fail. The issue is currently addressed by:

- adding Pluronic™ F-127 to at a concentration of 0.2% (v/v), which serves as a lubricating agent to prevent non-specific binding of cell debris to the funnel filters, demoting the adhesion of iRBCs to filter regions;

- preparing the incoming iRBCs sample at <5% hematocrit.

The limiting throughput problem can also be potentially solved by paralleling a number of devices to sort samples at the same time.
5.6 Discussion and Conclusion

Reduced RBC deformability is central to the pathology of *falciparum* malaria. Consequently, deformability-based sorting represents a fundamental approach that could be used to enrich pathological cells to improve diagnostic sensitivity or to fractionate these cells for further study. However, deformability-based sorting of RBCs has not been previously achieved because of the extreme softness of these cells, which requires exquisite control of the deformation force applied to each cell in order to alter its flow path.

Here, a novel method to sort RBCs based on deformability was presented. The method relies on ratchet transport created by deforming single cells through tapered constrictions using oscillatory flow, enabling continuous and perpetual fractionation of the input cell sample. The filtration microstructures remain unobstructed during the sorting process, which ensures that all cells experience a consistent filtration force. Additionally, the oscillatory flow prevents the adsorption of *Pf*-iRBCs to the filtration microstructure, enabling the extraction of target cells after separation. After the deformability-based mechanism of the ratchet sorting process was validated using GTA model, the ability of the microfluidic approach to separate *Pf*-iRBCs based on the intra-erythrocyte stages was demonstrated. Furthermore, this method can enrich ring-stage *Pf*-iRBCs by >100X, therefore dramatically improving the detection limits of malaria diagnosis performed using microscopy and rapid diagnostic tests (RDTs).

Despite the limitations discussed in Section 5.5, deformability-based sorting could overcome a key challenge associated with the detection of malaria infection at low parasitemia containing mostly early stage iRBCs. While existing high-sensitivity malaria detection methods involve PCR-based analyses that require specialized laboratory infrastructure, the ability to biophysically enrich for
infected RBCs by 100X (and potentially up to 2500X) could effectively lower the limit of detection for malaria diagnosis performed using conventional microscopy and RDT methods [91]. Furthermore, microfluidic enrichment could be used to develop simple diagnosis platforms based on automated microscopy [39], [40], where existing methods are currently limited by error rate.
Chapter 6: Deformability-based Sorting of WBCs using Microfluidic Ratchets

6.1 Introduction

Leukocytes, or white blood cells (WBCs), can provide access to a veritable treasure-trove of information on the health status of each individual [92]. For example, leukocytes can serve as indicators of pathogen infection, especially for leukocyte-specific pathogens such as the human immunodeficiency virus (HIV). Hematological assays performed on leukocytes typically require initial depletion of erythrocytes, as well as isolation of specific phenotypes from whole blood.

Key challenges in such sample preparation steps include:

1) Complete depletion of erythrocytes is necessary since the hemoglobin within RBCs can lead to chemical interference and deterioration of PCR-based test performances [93];

2) Rapid isolation of leukocytes, such as short-lived neutrophils, is required;

3) Activation of leukocytes is preferably avoided. Activated leukocytes do not represent the initial status of the immune system, which can limit the utility of the information acquired from these leukocytes [94].

RBC depletion is currently achieved in clinics or in research settings via selective bulk lysis of RBCs or differential centrifugation. Both of these macro-scale methods are labor intensive and they fail to efficiently isolate all leukocyte subpopulations. Besides, these approaches require a large volume of blood (a few mL), which makes it difficult to process small volume samples, such as blood collected through finger prick. Importantly, they also stress the cells for extended periods of processing time with undesirable chemicals (hypotonic solution or density gradient), leading to leukocytes activation, which can corrupt the information obtained from these cells.
Leukocyte phenotypic separation and analysis is currently achieved through labelling the specific surface markers on leukocytes subsets with fluorescence probes or magnetic beads using FACS or MACS. The monoclonal antibodies label specific to cells of interest is costly and requires expensive equipment for subsequent separation. Furthermore, the lengthy blood sample labelling procedure and toxic chemical reagents potentially exposes the cells to undesirable environment, resulting in the non-specific activation of leukocytes. Alternatively, phenotypic separation has also been achieved through patterning immobilized antibodies binding to specific antigens on leukocyte surfaces, a process known as panning [95]. However, the technique typically requires a debulking step to eliminate erythrocytes, such as RBCs lysis.

Recently, a number of microfluidic methods have been developed to process small volume of blood either to deplete RBCs for leukocytes or to achieve phenotypic separation of leukocytes from whole blood. For example, a benchtop RBCs lysis assay has been applied on microfluidic chips [96] to automatically lyse RBC via deionized water, which shows dramatic reduction of enriched leukocytes activation over traditional macro-scale RBCs lysis. Alternatively, biophysical methods using microfluidics have been developed based on intrinsic physical properties, including cell size, deformability, electrical and magnetic properties, to achieve label-free cell sorting. These microfluidic techniques include hydrodynamic chromatography (discussed in Section 3.1), dielectrophoresis [97], magnetophoresis [98]–[100] and micro-filtration (discussed in Section 3.2 and Table 3.1). So far, DLD has the ability to separate leukocytes from whole blood and sort leukocytes into different phenotypes such as granulocytes, lymphocytes and monocytes, although the selectivity is rather poor due to the overlapping size distribution among these leukocytes subtypes [48], [52]. Margination has also been directly implemented using microfluidic
bifurcations design to deplete RBCs but these methods exhibit variable performance [66], [101], [102]. Dielectrophoresis (DEP) and magnetophoresis are active approaches as they use additional force fields generated with electrodes or magnets integrated on small fluidic chips. In DEP, leukocytes can be distinguished from erythrocytes based on their differences in dielectric properties, contributed by their size, nuclear and membrane morphology [103]. In magnetophoresis, leukocytes can be discriminated from deoxygenated erythrocytes based on their differences in magnetic properties. Both DEP and magnetophoresis methods have poor selectivity of leukocytes over erythrocytes and require additional apparatus to generate electrical and magnetic fields.

This chapter describes the utilization of the microfluidic ratchets to separate leukocytes from whole blood as well as to sort leukocytes based on phenotypes. The rest of the chapter will be organized as follows. Section 6.2 will introduce the overall design, model and operation of the microfluidic devices for leukocyte sorting. Section 6.3 will validate the ability of the device to deplete RBCs to achieve pure suspensions of leukocytes. Section 6.4 will demonstrate the ability of the ratchet sorting device to sort lymphocytes, granulocytes and monocytes directly from whole blood. The chapter concludes with a discussion and conclusion in Section 6.5.

6.2 Microfluidic Ratchet Device for Leukocyte Sorting

The schematic illustration of the leukocyte sorting process is shown in Figure 6.1A. The design of the microfluidic device for sorting leukocytes is similar to the design for sorting RBCs (described in Section 5.2.2). The key design parameters (pore sizes and funnel thickness) are designed through three iterations of the trial and errors. The changes in sorting region geometry throughout the three iterations of design are summarized in Table 6.1.
Table 6.1: Design changes of the three iterations of leukocyte sorting device

<table>
<thead>
<tr>
<th>Iteration</th>
<th>Pore size arrangement</th>
<th>Funnel thickness (µm)</th>
<th>Sorting region size (µm × µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iteration 1</td>
<td>1.5, 1.75, 2, 2.25, 2.5, 3, 3.5, 6, 7.5</td>
<td>8</td>
<td>1250 × 6350</td>
</tr>
<tr>
<td>Iteration 2</td>
<td>1.5, 1.75, 2, 2.25, 2.5, 3, 3.5, 6, 7.5</td>
<td>11</td>
<td>1250 × 6350</td>
</tr>
<tr>
<td>Iteration 3</td>
<td>2, 2.5, 3, 3.5, 4, 4.5, 5.5, 6.5, 8</td>
<td>11–15</td>
<td>1875 × 9525</td>
</tr>
</tbody>
</table>

The first iteration of leukocyte sorting device utilized the same RBCs device configuration except the funnel thickness was modified to be 8 µm, slightly bigger than the average size of the leukocytes (lymphocytes ~6.2 µm, neutrophils ~7.0 µm and monocytes ~7.5 µm [48]). It was found that the microfluidic device can only sort dilute whole blood (1:5 in PBS) for less than 10 minutes before the leukocytes started to clog the entrance. The sorting region thickness was later increased to be ~11 µm which shows a significant improvement in the capacity of the device to process whole blood. In this iteration of trial, it was found that 3 µm is a critical cut-off pore size for the separation of erythrocytes and leukocytes, which is similar to 3.5 µm pores used by other microfiltration systems with pillar, weir, and membrane filters [72].

For the third iteration, the pore sizes (W₀) for the sorting region were designed to be 2, 2.5, 3, 3.5, 4, 4.5, 5.5, 6.5 and 8 µm with the expectation that RBCs were exclusively collected in O₁-3. O₄-9 is intended to not only isolate leukocytes from RBCs but also to distinguish the subpopulations of leukocytes. This is the first iteration of design to explore the effect of pore sizes on the selectivity of various leukocyte phenotypes. The results will provide guidelines for upcoming iterations of
pore size design in order to improve the selectivity of the microfluidic ratchets for leukocyte phenotypic separation based on deformability. The funnel thickness ($H_0$) is designed to be 11-15 µm based on the iteration 2 result.

**Figure 6.1:** Mechanism and design of microfluidic ratchet cell sorting device. 
(A) Schematic illustration of the ratchet cell sorting region consisting of an array of 2D funnel microstructures with smaller and smaller opening from bottom to top rows. Through sample inlet (SI), whole blood cells are introduced and form a diagonal streak under the combined flows of cross-flow inlet (CFI) and biased oscillatory flows (Osc1 for filtration and Osc2 for de-clogging); smaller and softer RBCs travel all the way up while bigger and more rigid leukocytes are blocked by certain funnel openings midway through the sorting region and subsequently separated from the RBCs. (B) Image of the overall microfluidic device infused with different food color dyes illustrating components of the microfluidic device including sample inlet (SI), cross-flow inlet (CFI), sorting region and nine outlets (O1-O9).

The design of the microfluidic device (iteration 3) infused with color dyes is demonstrated in Figure 6.1B. Sorting leukocytes from whole blood is performed using the funnel matrix consisting of 35 rows by 630 columns of funnel constrictions, same as the RBC sorting device. However, the sorting region size is enlarged to $1875 \times 9525$ µm in order to improve the throughput. The design of the supporting microchannels, i.e., CFI, SI, Osc1 and Osc2, and O1-9, is modelled using hydrodynamic resistance analysis and follows the same design principles as that of the RBCs sorting device, discussed in Section 5.2.2. As a result, the pressures ranging from 14 to 20 kPa
applied at the oscillation inlets are reduced to approximately 5 to 30 Pa at funnel constrictions, which was on the same order of pressure magnitude for individual leukocyte and erythrocytes deformation through funnels determined previously [34], [55].

6.3 Device Validation – RBC Depletion

To test the microfluidic device and evaluate the separation of leukocytes from whole blood, blood samples were pre-stained with Hoechst 33342 DNA stain (see material and methods in Appendix A.5, DNA Staining of Leukocytes) and infused into the sorting region (Figure 6.2A). As shown in Figure 6.2B-D, the sample formed the characteristic diagonal trajectory. Individual leukocytes (blue) that have reached their limiting funnel constrictions were found to transit horizontally as expected (Figure 6.2E). As expected, RBCs were collected in fractions O1-3 (Figure 6.2F; 2, 2.5 and 3 μm pores) while leukocytes were preferentially segregated among O4-9 (Figure 6.2G; 3.5, 4, 4.5, 5.5, 6.5 and 8 μm pores). At the end of the sorting, the downward oscillation is increased from 14 kPa to 17 kPa to release the clogged cells at the funnels.
Figure 6.2: Microfluidic ratchets to separate leukocytes directly from whole blood. (A) Image of the sorting region of microfluidic ratchets infused with food color dyes illustrating the diagonal trajectory of the sample inlet flow; (B-E) images of the leukocytes immunostained with Hoechst 33342 being separated from the whole blood which traverses diagonally through the funnel matrix; (F-G) images of the outlets showing the separate reservoirs RBCs and leukocytes are collected into.

To evaluate how funnel thickness and oscillation pressure affect the leukocytes’ distribution across the outlets (defined as leukocyte deformability profile), experiments were performed using devices with funnel thicknesses of 11 and 15 µm, and oscillation pressures between 14-20 kPa. This operating range was established using previous experiments of acceptable operating conditions (see Section 5.2.4). As shown in Figure 6.3A-C, higher filtration pressures increased the frequency for cells to transit narrower openings, represented by a leftward shift in the leukocyte deformability profiles for three different donors’ blood samples. Altering the funnel thickness from 11 to 15 µm provides a higher ceiling to facilitate cell deformation, which requires smaller pressure to deform
leukocytes through the pores, leading to a leftward shift in the leukocytes deformability profiles in cases of donors 1 and 3, and a broadening of the leukocyte distribution in donor 2 (Figure 6.3D-F).

![Figure 6.3](image)

**Figure 6.3**: Leukocytes deformability profiles as a function of filtration pressure (Osc2) and funnel thickness.  
(A-C) Leukocyte distribution profiles of three different donors at various filtration pressures with fixed funnel thickness of 11 µm. (D-F) Leukocyte distribution profiles of three donors at two different funnel thickness with a fixed filtration pressure of 17 kPa.

The purity and leukocyte recovery rate or separation efficiency were investigated as the parameters of oscillation pressure and funnel thickness. It was observed that RBCs were exclusively collected in O1-3 in all scenarios except when the filtration pressure was decreased to 14 kPa, in which case, a small fraction of RBCs escaped into O4, leading to decreased recovery rate of pure leukocyte population (Figure 6.4A). The thickness of the device does not influence the recovery rate of
leukocytes separation from RBCs (Figure 6.4B). Recovery rate or separation efficiency has been defined in equation 3.1. Specifically, for leukocytes sorting, it is defined as follows. Here, it was assumed that no cells were lost in the sorting region or Osc1&Osc2.

For filtration pressure = 17 and 20 kPa,

\[
Separation \ Efficiency = \frac{Leukocyte_{O4-9}}{Leukocyte_{O1-9}}
\]  

(6.1)

For filtration pressure = 14 kPa,

\[
Separation \ Efficiency = \frac{Leukocyte_{O5-9}}{Leukocyte_{O1-9}}
\]  

(6.2)

As shown in Figure 6.4, optimal leukocyte separation could be achieved with a funnel height of 11 µm and filtration pressure of 17 kPa, which provides a leukocyte recovery rate of 98-99% and generates pure leukocyte suspensions in O4-9. Interestingly, when investigating the repeatability of microfluidic device by testing blood from one donor at three different time points, it was observed that the leukocyte deformability profiles displayed two different distribution states (Figure 6.4C), where they primarily accumulated either in O6 or O8. A similar variation was also observed among donors (Figure 6.3). While the cause of this variation merits further investigation, this phenomenon may simply reflect the capriciousness and dynamic nature of the human immune system [104], which may be reflected in the changes in the neutrophil population presented in the next section.
6.4 Device Validation – Phenotypic Sorting of Leukocytes

6.4.1 Sorting Whole Blood for Granulocytes and Lymphocytes

To investigate whether deformability-based sorting could resolve granulocyte and lymphocyte subpopulations, donor leukocytes were pre-stained with a lymphocyte-specific (T-cells and B-cells) cocktail of monoclonal anti-CD3 and anti-CD19 antibodies conjugated with Alexa Fluor 488 as well as granulocyte-specific (neutrophils, eosinophils and basophils) anti-CD66b antibodies conjugated Alexa Fluor 647. The leukocytes immunostaining procedure was introduced in details in Appendix A.5, Leukocyte Immunophenotyping. Following microfluidic ratchet enrichment, leukocytes were distributed in O4-9. Lymphocytes were preferentially distributed around O6 (retained between 4.5 to 5.5 µm pore size, Figure 6.5A-C), where such cells can be obtained at 62-68% purity (Figure 6.5D-F). Granulocytes were distributed around O8 or O9 (retained between 6.5 to 8 µm pore size, Figure 6.5A-C), where such cells can be obtained at 88%-95% purity in these outlets (Figure 6.5D-F).

Figure 6.4: Performance of deformability-based separation of leukocytes from whole blood as a function of (A) filtration pressure and (B) funnel thickness. (C) Intra-individual differences of leukocyte distribution.
Figure 6.5: Phenotypic sorting of whole blood cells for lymphocytes and granulocytes. (A-C) Distributions of lymphocytes and granulocytes for three different donors blood; (D-F) composition of lymphocytes and granulocytes in each outlet indicating the relative abundance of both phenotypes; (G-H) images of the immunostained lymphocytes (green) in O6 and granulocytes (red) in O8 from Donor 1.

The intra-individual variability of the leukocyte deformability profiles was established through four independent experiments on blood from a single donor, while the inter-individual variability was assessed using experiments on blood from three different donors. Both intra- and inter-individual deformability profiles for lymphocytes were remarkably consistent, with ~60% retention in O6 (Figure 6.6A-B). In contrast, granulocyte distribution varied significantly, both
intra- and inter-individually (Figure 6.6C-D). This result is not surprising as granulocytes are dominated by neutrophils, which represent more than 90% of these cells, and neutrophils are known to adopt different biophysical characteristics upon activation by chemical [50] or mechanical stresses [105]. Furthermore, since granulocytes represent 60-70% of all leukocytes, the variation in deformability in these cells may explain the variability of intra-individual leukocyte deformability profiles in Figure 6.4C.

![Deformability profiles of lymphocytes and granulocytes.](image)

Figure 6.6: Deformability profiles of lymphocytes and granulocytes.  
(A-B) Deformability profiles of lymphocytes of three donors.  
(C-D) Deformability profiles of granulocytes of three donors. Each inter-individual distribution profile data point is a mean of triplicate experiments.

### 6.4.2 Sorting Whole Blood for Monocytes

The distributions of monocytes were evaluated to determine whether they could be separated from lymphocytes and granulocytes based on deformability. Monocytes make up 2%-8% of the total
Leukocyte population. They average 7.5 µm in diameter [48], making them larger than either lymphocytes (6.2 µm) or granulocytes (7.0 -7.3 µm). Monocytes were sorted from whole blood after pre-staining with anti-CD14 conjugated with Alexa Fluor 488 and retained primarily in O8-9, by the 6.5 µm constrictions (Figure 6.7). This result suggests that these cells could be efficiently enriched from lymphocytes but not from granulocytes. The purity of monocytes in O8 ranges 4.5-20%, relative to granulocytes, which represents only a modest increase from the standard frequency of these cells in peripheral blood leukocyte population. However, the nearly exclusive accumulation of monocytes within these outlets suggests that future iterations of this device could resolve monocytes from granulocytes by optimization of the pore size (in the range between 6.5 µm and 8 µm) and funnel thickness.

![Figure 6.7: Phenotypic sorting of whole blood cells for monocytes.](image)

(A-C) Distribution of monocytes sorted from whole blood from donor 1 for three trials with inset graphs demonstrating the composition of leukocytes subsets in O8 and O9.

### 6.5 Discussion and Conclusion

The microfluidic ratchet mechanism employed in this study overcomes some key limitations of both conventional microfiltration and microfluidic biophysical cell sorting systems to enable leukocyte separation from whole blood with 100% purity, as well as phenotypic sorting of leukocytes to enrich for granulocytes and lymphocytes. Microfiltration methods developed
previously using pillars, weir and membrane pores have all shown a tendency to clog, which increases hydrodynamic resistance, reduces selectivity and necessitates increased filtration pressure or periodic washing of the filter region. While secondary tangential flow partly alleviates device clogging, the oscillating flow used in microfluidic ratchet sorting enables clog-free and nearly perpetual processing of high-density whole blood samples. In fact, the selectivity of previous microfiltration systems is often poor or unreported (Error! Reference source not found.). In comparison, the microfluidic ratchet consistently generates pure leukocyte isolates from whole blood with only 1-2% loss. The microfluidic ratchet also achieves superior enrichment performance relative to other microfluidic approaches (Table 6.2), including magnetophoresis, as well as other size-based sorting strategies, such as dielectrophoresis, DLD and leukocyte margination. Because the ratchet mechanism sorts cells by combined size and deformability, this capability permits sorting of leukocyte subpopulations despite the fact that they overlap significantly in size [48]. A critical limitation of the current microfluidic ratchet device is its relatively low sample throughput (~5 μL whole blood per hour). However, this issue can be addressed through multiplexing and operating multiple devices simultaneously on one chip.

A key advantage of the microfluidic ratchet device is its ability to resolve leukocyte subpopulations. While the current iteration of the device failed to resolve granulocytes from monocytes, refinement of funnel filter geometry, including pore size and funnel thickness can ultimately overcome this limitation. Furthermore, since the microfluidic ratchet fractionates cell populations into outlets, these cells are immediately available for downstream molecular characterization, such as immunofluorescence, transcriptome analysis, or cytokine secretion assays.
Table 6.2: Performances specifications of recent microfluidic research in leukocytes separation from whole blood using biophysical methods

<table>
<thead>
<tr>
<th>Method*</th>
<th>Performance Metrics</th>
<th>Leukocyte Recovery Rate</th>
<th>Purity</th>
<th>RBC depletion</th>
<th>Throughput</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfluidic Ratchet Current Study</td>
<td></td>
<td>98%~99%</td>
<td>99.9%</td>
<td>99.9%</td>
<td>5 µL·hour⁻¹</td>
<td>None</td>
</tr>
<tr>
<td>Magnetophoresis [98]</td>
<td></td>
<td>n.d.</td>
<td>n.d</td>
<td>93.5%</td>
<td>5 µL·hour⁻¹</td>
<td>1:10 in PBS</td>
</tr>
<tr>
<td>Magnetophoresis [99]</td>
<td></td>
<td>n.d.</td>
<td>n.d</td>
<td>95%</td>
<td>0.5-0.7 mL·hour⁻¹</td>
<td>1:20 in PBS</td>
</tr>
<tr>
<td>Magnetophoresis [100]</td>
<td></td>
<td>n.d.</td>
<td>n.d</td>
<td>93.7%</td>
<td>0.12-0.92 µL·min⁻¹</td>
<td>1:40 in PBS</td>
</tr>
<tr>
<td>Dielectrophoresis (DEP) [106] [58] DLD [52]</td>
<td>76.9%~92.1%</td>
<td>n.d.</td>
<td>n.d</td>
<td>n.d</td>
<td>50 µL·hour⁻¹</td>
<td>1:5 in PBS</td>
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<tr>
<td>Leukocyte margination [101]</td>
<td></td>
<td>80%</td>
<td>90%</td>
<td>n.d</td>
<td>600 µL·hour⁻¹</td>
<td>0.5%-2% hematocrit</td>
</tr>
<tr>
<td>Leukocyte margination [102]</td>
<td></td>
<td>97%</td>
<td>100%</td>
<td>n.d</td>
<td>1 µL·min⁻¹</td>
<td>1:1000 in PBS</td>
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<tr>
<td>Leukocyte margination [66]</td>
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<td>n.d</td>
<td>n.d</td>
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n.d. Data not available

*References in square brackets
Chapter 7: Conclusion and Future Work

7.1 Thesis Summary

This dissertation described a novel microfluidic method to sort RBCs and WBCs based on deformability using the microfluidic ratchet mechanism. The technique leverages asymmetry in the deformation of single cells through funnel-shaped constrictions to eliminate clogging by periodically reversing flow. Since cells cannot transit against the funnel taper due to the ratchet effect, cells still migrate along a unidirectional path and can be separated based on their ability to deform through progressively narrower funnel microstructures. Furthermore, the novel mechanism was applied to sort the abundant RBCs for Pf-iRBCs. The process dramatically improves the sensitivity of malaria diagnosis, performed using microscopy and rapid diagnostic test, by enriching the concentration of RBCs infected with ring-stage *Plasmodium falciparum* 100-2500X at clinical relevant parasitemia (<0.01%).

Lastly, the microfluidic ratchet was shown to be capable of isolating leukocytes directly from unprocessed whole blood with 100% purity (i.e. no contaminant erythrocytes) and <2% loss of target cells. It was further demonstrated the potential to use this process to phenotypically sort leukocytes to enrich for granulocytes and lymphocytes subpopulations. Together, this process provides a sensitive method to isolate and sort leukocytes directly from whole blood based on their distinct biophysical properties. For the rest of the chapter, the major contributions of both projects are summarized and recommendations for future work are given.

7.2 Contributions

The major contributions of this thesis include the following:

1) Development of a microfluidic device for deformability-based sorting of RBCs and WBCs;
2) Application of the RBCs sorting device to demonstrate improved sensitivity to detect malaria infection using both microscopy and rapid diagnostic tests;

3) Application of the WBCs sorting device to demonstrate phenotypic separation directly from whole blood.

7.3 Future Work

7.3.1 Deformability-based Sorting of Pf-iRBCs
Deformability-based sorting of Pf-iRBCs has the potential to revolutionize antimalarial drug development where target identification has been a major bottleneck. Since *P. falciparum* parasites rapidly evolve drug-resistance, genome sequencing of drug-resistant mutants has been a standard approach for target identification. Drug-resistant mutants are currently obtained through culturing parasites with target compound, and then isolating individual iRBCs that retain fitness using a micropipette. This approach requires a great deal of skill and luck since only a tiny fraction of iRBCs contains mutations that confer drug-resistance. Previously, our group has established that all clinical anti-malarials specifically decrease iRBC deformability [107]. Based on this finding and the unique microfluidic ratchet to sort RBCs based on deformability, it is feasible to separate drug-resistant from drug-sensitive parasites, therefore enabling rapid determination of the target(s) of putative anti-malarials, and ultimately, of their mechanism of action.

7.3.2 Deformability-based Sorting of Leukocytes
Cell deformability can be used to distinguish erythrocytes from leukocytes, as well as to potentially distinguish various phenotypes of leukocytes from each other. Specifically, it has been shown that the microfluidic ratchets can yield pure suspensions of leukocytes and enrich for granulocytes and lymphocytes up to 95% and 68% purity directly from whole blood. Together, the microfluidic
ratchet represents a compelling new method for biophysical characterization of highly relevant leukocyte populations, which can serve as an indicator for a range of pathological conditions, such as hemorrhagic shock stroke [108], [109], diabetics [110], [111] and leukemia [112], [113]. Therefore, the microfluidic deformability-based cell sorting can potentially provide an effective fractionation and collection of pathological leukocytes and serve as a preparation step for downstream transcriptomic or genetic analysis involving leukocytes-related pathologies.

This study also reveals significant plasticity in the deformability profile of granulocytes, relative to lymphocytes. Such phenotypic changes may reflect alterations in neutrophil activation state, associated with cancer progression [114] and viral infection [115]. In this way, the microfluidic ratchet system may represent a powerful and cost effective alternative to flow cytometry, in monitoring and enriching for leukocytes that belong to relevant subtypes or that adopt specific activation states.
Bibliography


85

2009.


M. T. Elghetany and B. H. Davis, “Impact of preanalytical variables on granulocytic


Appendix A  Material and Methods

A.1  Microfluidic Device Fabrication

Photolithography

The microfluidic ratchet device consists of a single fluidic layer fabricated using soft-lithography of polydimethylsiloxane (PDMS) silicone. Molds for the microstructure of both RBCs and WBCs sorting devices consist of two photolithographically defined layers fabricated on a silicon wafer. They are sorting region microstructures and peripheral supporting microchannels.

The sorting region was fabricated using:

1) SU-8 3005 photoresist (MicroChem, Newton, MA, USA) thinned with cyclopentanone at a ratio of 1:0.8 by volume for RBCs sorting device;

2) SU-8 3010 photoresist (MicroChem, Newton, MA, USA) for WBCs sorting device.

The supporting microfluidic channels were made from:

1) SU-8 3010 photoresist for RBCs sorting device;

2) SU-8 3025 photoresist for WBCs sorting device.

The patterns were drawn using AutoCAD. The design files were then sent to Advance Repro. Corps. for the fabrication of chrome mask for the subsequent UV-photolithography. The photolithography process is composed of first fabricating the sorting region microstructures followed by the second layer of microstructure of supporting microchannels precisely aligned using the mask aligner (CANON PLA-501F). The microstructures were fabricated on a cleaned 100 mm silicon wafer. After dehydration baking on a hotplate at 200ºC for 5 minutes,
1) for RBCs sorting device, thinned SU-8 3005 was spun at 500 rpm for 10 seconds, and then spun at 4000 rpm for 30 seconds to remove the edge beads, which yields a thickness of 4.5 µm microstructure measured using a profilometer (Alpha step 200);

2) for leukocyte sorting device, photoresist SU-8 3010 was spread onto the wafer at 1500 rpm to 1800 rpm for 30 seconds to create a thickness of 11 µm to 15 µm, as measured using a profilometer.

The wafer was then soft baked at 95°C for 4 minutes before exposed to UV light in a mask aligner with uniform exposure for 35-40 seconds. The exposed wafer was given a post exposure bake at 65°C for 1 minute, 95°C for 3 minutes and then 65°C for 1 minute. Finally, the wafer was developed using SU-8 developer (MicroChem). The geometry of the sorting region SU-8 photoresist was stabilized by further baking with ramped temperature at the acceleration of 100°C hour\(^{-1}\) from 40°C to 200°C, held at 200°C for one hour, and then gradually cooled to 40°C.

The supporting microchannel microstructures were added to the silicon wafer containing the sorting region microstructures. The SU-8 photoresist was spin-coated on the wafer at 3000 rpm (RBCs sorting device) for 50 seconds; and 4500 rpm (WBCs sorting device) for 30 seconds. The coated wafer was soft baked on hotplates set at 65°C for 1 minute, 95°C for 2 minutes, and then 65°C for 1 minute. The designed mask for the supporting microchannel SU-8 pattern was then aligned with the sorting region pattern and exposed for 4 minutes in 30 seconds bursts (RBCs sorting device) and 55 seconds continuously (WBCs sorting device). After waiting for approximately 30 minutes, the wafer was developed using SU-8 developer (MicroChem). The finished structure was measured to be 10 µm in thickness using profilometer for RBCs sorting device and to be 20 µm for leukocyte sorting device.
Soft-lithography

Replicas of the silicon wafer molds were fabricated using a polyurethane-based plastic (Smooth-Cast ONYX SLOW, Smooth-On) using the process described by Desai, et al. [116] PDMS microfluidic devices were then fabricated from these molds using soft-lithography of RTV 615 PDMS (Momentive Performance Materials).

After PDMS was cured in the polyurethane molds in 60°C convection oven for at least 3 hours, the cured PDMS device was removed from its mold, and holes were punched using a 0.5 mm outer diameter hole punch (Technical Innovations, Angleton, TX, USA) as the fluidic introduction ports including cross flow and cell inlets as well as the oscillation inlets. The outlets are punched using 4 mm diameter puncher. The microfluidic device is then bonded to a blank PDMS layer, which was spin-coated onto a blank silicon wafer at 1500 rpm for 1 minutes and cured beforehand. The device containing a blank layer of PDMS at bottom is then peeled off. The bonding is realized through the exposure of both surfaces to air plasma (Model PDC-001, Harrick Plasma) for 70 seconds before the PDMS device is brought into contact with the blank PDMS layer to create a permanent covalent bond. Subsequently, the double layer device is bonded to the standard microscope slide (50×75 mm, Fisher Scientific) cleaned beforehand with acetone and isopropanol.

A.2 Instrumentation for the Microfluidic Device Operation

The setup of the experimentation is composed of three parts with interfacial connectors and tubes as shown in Figure A.1. They are pressure controllers, fluid reservoirs and microfluidic device. Sample and medium fluids were contained in the 15 ml conical tubes (Falcon, Fisher Scientific), sealed with custom-made caps. The setup acts as pressurized reservoirs through which the fluid is transported into the microfluidic device through 0.5 mm ID flexible Tygon tubing (Cole-Parmer).
At the microfluidic device end, 19 mm long 23-gauge stainless steel tubing (New England Small Tube, Litchfield, NH, USA) connects the Tygon tubing with the device through the custom punched holes in the chip. The connections provide an elastic, watertight seal on both ends.

Two pneumatic pressure control systems were used to pressurize the reservoirs in order to introduce fluids into the device. The first system is a custom pressure controller designed to supply pressure from 0 to 4 bar using manual pressure regulators (Omega, McMaster-Carr). On/off pressure control is enabled using solenoid valves (Pneumadyne, McMaster-Carr) activated by MOSFET switches that are controlled using a MSP430 microcontroller (Texas Instruments) integrated on a printed circuit board. The MSP430 is controlled using a Visual Basic user interface program on a PC. The second pressure control system is the MFCS-4C system (Fluigent SA, Paris, France). This system supplies precise pressure with a resolution of 30 mbar (30 Pa) up to 1000 mbar.
Figure A.1: Instrumentation of the microfluidic device. 
(A) Schematics of the experimental setup composed of pressure controllers, fluidic reservoir and microfluidic device. (B) image of the three components

A.3 Blood Preparation

Packed RBCs

Blood from healthy donors was obtained via venipuncture in tube containing EDTA anti-coagulant, following informed consent and approval from the University of British Columbia
(UBC) Research Ethics Board. The whole blood was spun down at 3000 g for 10 minutes. The plasma, the buffy coat and the top layer of the cells were then removed. The remaining cells are packed RBCs. For the glutaraldehyde (GTA) study, the packed RBCs were used within the same day. Packed RBCs were also used to feed the *Plasmodium falciparum* parasites.

**Glutaraldehyde Treatment.**

Packed RBCs were suspended in Phosphate Buffered Saline (PBS; CaCl$_2$-free and MgSO$_4$-free; Invitrogen) with 0.2% Pluronic$^	ext{TM}$ F-127 (Invitrogen) to achieve desired hematocrit. Glutaraldehyde (GTA; Alfa Aesar, MA) solution was diluted in PBS to achieve the desired concentration. Diluted RBCs were then incubated for 30 minutes at 25$^\circ$C with 0.000% to 0.050% Glutaraldehyde. After incubation, the RBCs suspension was washed three times in PBS and then re-suspended in PBS with 0.2% Pluronic.

**A.4 Malaria Culture and Parasite Preparation**

*P. Falciparum Culture*

The 3D7 strain of *P. falciparum* parasites was cultured under standard *in vitro* conditions with modifications [117]. Type A+ or O blood was collected from healthy donors with written informed consent and approval from the Research Ethics Boards of UBC and Canadian Blood Services (CBS) by the CBS’s Network Centre of Applied Development. Cultures were maintained at approximately 5% hematocrit in malaria culture medium (1640 RPMI with HEPES; 0.2% sodium bicarbonate; 100 µM hypoxanthine; 10% heat-inactivated human serum; 1 mg·mL$^{-1}$ gentamicin). Parasites were incubated in an atmosphere of 5% CO$_2$, 3% O$_2$ and 92% N$_2$ at 37$^\circ$C and 95% humidity.
Synchronization with Sorbitol

5% (w/v) sorbitol solution was dissolved thoroughly in distilled water and warmed at 37°C for 5 minutes. Malaria culture at 50% was added to the sorbitol solution at 1:9 ratios. The mixture was incubated for 8 minutes at 37°C following 30 seconds of vigorous vortex to rupture old and mature parasites. Then the sample was washed using centrifugation at 250 g for 5 minutes at 37°C three times. The prepared culture sample was considered Hour 0 and was kept in the culture condition.

Giemsa Staining using Thin Film

Blood smears of the cell cultures of approximately 50% hematocrit were prepared onto a slide. The specimens were air-dried, fixed in methanol and stained with 10% Giemsa solution (Sigma Aldrich) to evaluate the stages of the iRBCs. Parasitemia was determined by counting at least 1000 RBCs under regular light microscope, equipped with a 100X oil-immersion objective. Microscopic pictures were taken with Nikon camera mounted on the microscope. Images of the Giemsa stained Pf-iRBCs are shown in Figure 5.6B.

Hoechst Fluorescence Staining

Synchronous sample was stained with Hoechst 33342 (Sigma Aldrich) before introduced through the device. Hoechst (5 µg·ml⁻¹) were added to the falciparum blood sample (~20% hematocrit) at 1:100 (v/v) in PBS solution with 2% heat-inactivated fetal bovine serum (HI-FBS). The stained falciparum blood sample was incubated for 20 minutes in room temperature in the dark. The sample, after sorting, was observed under fluorescence microscope and images of stained Pf-iRBCs are shown in Figure A.2.
Figure A.2: Hoechst DNA stain of *falciparum* parasites. (A) fluorescence image of the parasites stained with Hoechst 33342. (B) Multichannel-image of RBCs containing parasites (blue) stained with Hoechst 33342.

Rapid Diagnostic Tests (RDTs)

Rapid diagnostic tests detect malaria infection based on the presence of parasite specific antigens, which produces a color change on an absorbent test strip. For the RDT tests, CareStart™ test strips for pLDH antigen were purchased from AccessBIO. The image of a RDT strip is shown in Figure 5.8, which contains a reservoir for sample input, quality control marker (CON) and positivity marker (*Pf*).

Malaria samples containing ultra-low density ring stage *Pf*-iRBCs were tested before and after the microfluidic enrichment. Pre-sorting samples were prepared at 40% hematocrit mimicking the hematocrit of whole blood; and 5 μL aliquot was transferred into the test strip’s reservoir for testing. Post-sorting samples were prepared by pooling samples from outlets 4-9 together, and then
centrifuged to remove the excess supernatant. The remaining cells, suspended in 5 µl of PBS, are then transferred into the reservoir of the RDT for testing.

**Magnetic Column Purification**

Magnetic column purification was used in conjunction with sorbitol treatment to achieve tighter synchronous sample. A magnetic purification stand was fabricated based on the design by Charles C. Kim [118] with some modifications to fit super magnets. LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) which are designed for MACS were used. They were initially washed once with 5 ml incomplete RPMI medium (10.4 g·L⁻¹ RPMI-1640, 25 mM HEPES, 0.5% AlbuMAX I (w/v), 100 µM hypoxanthine, 12.5 µg·mL⁻¹ gentamicin) before loading sorbitol synchronized sample (2% hematocrit). The subpopulation trapped by the magnet was discarded while the eluted sample containing late-stage-parasite-depleted iRBCs was transferred into a 15 mL Falcon tube (Corning Life Science, Tewksbury, MA, USA), which was washed twice by centrifuging at 2000 rpm for 5 minutes without brake.

**A.5 Leukocyte Preparation**

**DNA Staining of Leukocytes**

The whole blood (200 µL) was stained with Hoechst 33342 (Sigma Aldrich) to label the DNA of leukocyte before the leukocyte sorting validation experiment (Section 6.3). Hoechst (5 µg·mL⁻¹) was added at 1:100 (v/v) to the sample and incubated for 30 minutes at room temperature in the dark. Subsequently, the sample was washed three times in PBS with 2% HI-FBS. After the last wash, the supernatant was replaced with PBS containing 0.2% Pluronic™ F-127 (Invitrogen) at 1:1 (v/v) ratio with the packed blood cells.
Leukocyte Immunophenotyping

Lymphocytes were stained with Alexa Fluor® 488 Mouse Anti-Human CD3, clone SP34-2 (557705, BD) and Alexa Fluor® 488 Mouse Anti-Human CD19, clone HIB19 (557697, BD). Granulocytes were stained with Alexa Fluor® 647 Mouse Anti-Human CD66b, clone G10F5 (561645, BD) and monocytes with Alexa Fluor® 488 Mouse Anti-Human CD14, clone M5E2 (557700, BD), all according to manufacturer’s instructions. After staining and washing, the supernatant was replaced with PBS containing 0.2% Pluronic™ F-127 (Invitrogen) at 1:1 (v/v) ratio with the packed blood cells. The images of the stained granulocytes and lymphocytes are shown in Figure 6.5.
Appendix B  Publications and Presentations (2012 ~ 2016)

B.1  Journal Publications


B.2  Conference Presentations