The Relevance of Red Blood Cell Deformability in the Pathophysiology of Blood Disorders

by

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Submitted to the Department of Electrical Engineering and Computer Science in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

at the

Massachusetts Institute of Technology

September 2014

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ABSTRACT

Red blood cells (RBCs) play a crucial role in delivering oxygen to the body tissues. During the 120 days of their lifespan, average RBCs would circulate for approximately 500,000 times and undergo repeated deformations in small blood capillaries and splenic cords. Increased RBC clearance in the spleen is considered as one of the direct consequences of reduced RBC deformability. On the other hand, deformability is also indirectly impacting on RBC functionality through its complex connections with underlying molecular mechanisms.

With the aid of microfabrication and microfluidic, we are able to perform high-throughput single cell deformability measurement. Overall, we established RBC deformability as an important biomarker for several blood related real world problems such as malaria and blood storage lesion. The ultimate goal is through our quantitative assessment of population-wide single cell deformability, we could aid in the decision-making of various clinical scenarios including drug screening and blood transfusion.

Malaria is the most deadly parasitic disease affecting hundred millions of people worldwide. Infected RBCs are found to be less deformable and therefore more susceptible to splenic RBC clearance. In this thesis, we identified several clinically used anti-malarial drugs that are capable of altering RBC deformability and ultimately modifying RBC retention in spleen. We also employed a rodent malarial model, confirming the important connection of RBC deformability with splenic RBC retention and consequently malarial anemia in vivo.

Blood storage lesion is another important application of our work. Taking the advantage of device high-throughput, we profiled hundreds of single RBC deformability from a large population and identified subpopulations that are less deformable. These subpopulations also exhibited higher osmotic fragility and were therefore predicted to pose higher transfusion risk according clinical standard. Furthermore, a deformability based sorting device was also developed to filter the less deformable blood subpopulations, improving overall blood quality.

Thesis Supervisor: Jongyoon Han
Title: Professor of Electrical Engineering and Computer Science & Biological Engineering
1 Acknowledgements

It has been five years since I first embarked on my journey as a graduate student in MIT. Life has been even more enriching and exciting than I had envisaged. The ample collaboration opportunities amongst dissimilar research groups and the state of the art lab facilities have offered me the maximum research freedom one could possibly have.

First of all, I would like to express my utmost gratitude to my research advisor Professor Jongyoon Han, who introduced me into this amazing field of BioMEMS. I would like to thank Prof. Han for his guidance, support and encouragement throughout. His enthusiasm for science and his insights in conducting scientific research have influenced me greatly and redefined my perspective towards research. In addition, I am also deeply indebted to Prof. Han for the enormous research freedom he endowed me. He encouraged me to explore areas of my own interests and he has always been accepting to my new ideas, even though most of the time they did not work out well. I could not have wished for a better advisor.

The other members on my thesis committee, Profs. Sangeeta Bhatia and Peter Dedon, have also offered me great advice on doctoral research. I thank them for their support and encouragement. It is my great honor to have them on my thesis committee.

I thank all the Han group members who have been so supportive and helpful. It has been a very comfortable and conductive environment to work in. I greatly appreciate Drs. Pan Mao and Hansen Bow, both offered me a lot of hands-on trainings and supervisions and helped me identify possible research directions. The other members in the group are also great people to work with and to spend time with including Aniruddh Sarkar, Lih-Feng Cheow, Lidan Wu, Leon Li, Rhokyun Kwak, Drs. Chia-Hung Chen, Han Wei Hou, Hiong Yap Gan, Sung Jae Kim, Lihong Liu and Yong-Ak Song. During my hard times at MIT, they offered me the warmest companionship.

Dr. Ming Dao and his research groups both at MIT and in Singapore have also offered me a lot of support without which this work could not have been accomplished. Special thanks to Drs. Anburaj Amaladoss, Rou Zhang and Ms Min Liu for their great help on the animal work.

During my research visits to Singapore, Prof. Peter Preiser provided me substantial help on the topic of malaria and host parasite interaction. I thank Prof. Preiser for his insights and knowledge in malaria pathology. His graduate student Ximei Huang was a great pleasure to work with.

I would also like to thank Dr. Huichao Chen. In addition to be a great biostatistician to work with, she is also a great friend.

Singapore-MIT Alliance for Research and Technology (SMART) and Course 6 teaching assistantships provided necessary financial support for my thesis work. Special thanks to Prof. Chwee Teck Lim, Drs. Ali Asgar Bhagat, Guofeng Guan, Weng Kung Peng and the research
program manager Dr. Balasubramanian Narayanan from SMART center for their generous help in many ways.

Last but not least, I would like to thank my dearest parents, my husband Yuan Fang and my lovely friends. I greatly appreciate their support and understanding. I would like to dedicate this thesis to them to express my love and gratitude.
Table of Contents

1 Introduction .......................................................................................................................... 15
  1.1 The importance of red cell deformability: from the perspective of mechanical red cell
clearance in spleen .................................................................................................................. 17
  1.2 Connections between red blood cell deformability and other biophysical and metabolic
factors ....................................................................................................................................... 19
  1.3 Red blood cell deformability measurement techniques ................................................ 22
  1.4 Case study I: Red blood cell deformability in malaria pathogenesis ....................... 28
  1.5 Case study II: Red blood cell deformability in blood storage lesion ....................... 29
  1.6 Thesis scope and outline ................................................................................................. 30

2 The in vitro effect of antimalarial drug on red blood cell deformability ...................... 32
  2.1 Artesunate and current hypotheses on its drug action ................................................. 32
  2.2 Experimental section ................................................................................................. 33
    2.2.1 Microfluidic device design and fabrication ......................................................... 33
    2.2.2 Artesunate drug treatment ............................................................................... 34
    2.2.3 Solution preparation ......................................................................................... 34
    2.2.4 Deformability measurement with microfluidic device .................................. 35
  2.3 Key Results ..................................................................................................................... 36
    2.3.1 Time-dependent effect of artesunate on infected RBC deformability ............. 37
    2.3.2 Concentration-dependent effect of artesunate on infected RBC deformability... 39
    2.3.3 Effect of pentoxifylline on infected RBC deformability.................................. 40
  2.4 Discussions and section summary .................................................................................. 43
  2.5 Section acknowledgement ........................................................................................... 47

3 The in vivo effects of antimalarial drug on Plasmodium yoelii infected mice .......... 49
  3.1 Plasmodium yoelii infected mice model ................................................................. 49
  3.2 Chloroquine and current hypotheses on its drug action .............................................. 50
  3.3 Experimental section ................................................................................................. 51
    3.3.1 Murine model for malaria infection ...................................................................... 52
    3.3.2 Microfluidic deformability measurement ......................................................... 52
    3.3.3 Animal preparation ............................................................................................. 52
    3.3.4 Chloroquine drug treatment ............................................................................. 53
    3.3.5 Sample preparation .............................................................................................. 54
3.3.6 Experimental flow chart ........................................................................................................... 54
3.4 Key results ........................................................................................................................................ 56
3.4.1 Splenic RBC retention based on the RBC deformability profiles ................................................. 56
3.4.2 The effect of malaria infection or/and antimalarial drug on RBC microcirculatory behavior and splenic RBC retention ................................................................. 59
3.4.3 Effect of antimalarial drug on healthy mice's RBC deformability profiles in peripheral blood and in spleen .................................................................................................................. 62
3.4.4 Bimodal estimation of splenic hRBC velocity profiles after CQ treatment ................................. 63
3.4.5 Malaria infection, antimalarial treatment, and blood hemoglobin content .......................... 67
3.5 Discussions and section summary .................................................................................................. 70
3.5.1 RBC deformability, splenic RBC retention and malarial anemia ............................................... 72
3.5.2 Chloroquine decreases RBC deformability and enhances splenic RBC retention .. 72
3.5.3 Threshold prediction for splenic RBC retention .............................................................................. 76
3.5.4 Separation Resolution .................................................................................................................. 77
3.5.5 Statistical Analysis ........................................................................................................................ 78
3.5.6 Section summary ........................................................................................................................ 79
3.6 Section acknowledgement .............................................................................................................. 80

4 Deformability based red blood cell sorting and its application in blood storage ....................... 81
4.1 Blood transfusion and potential storage age related risks ............................................................. 81
4.2 Experimental section ....................................................................................................................... 83
4.2.1 Microfluidic device design and fabrication .................................................................................. 83
4.2.2 Microfluidic device operation and data acquisition ...................................................................... 84
4.2.3 Osmotic fragility ............................................................................................................................ 85
4.2.4 Microparticle quantification.......................................................................................................... 85
4.3 Key Results ....................................................................................................................................... 87
4.3.1 Changes in red blood cell deformability over storage time .......................................................... 87
4.3.2 Changes in red blood cell fragility over storage time ................................................................... 90
4.3.3 Deformability based blood sorting device: principle and characterization ....................... 91
4.3.4 Red blood cell deformability after sorting .................................................................................... 93
4.3.5 Red blood cell osmotic fragility after sorting ............................................................................... 97
4.3.6 Microparticle concentration after sorting ...................................................................................... 99
4.4 Discussions and section summary .................................................................................................. 101
4.4.1 Deformability-based blood sorting vs. conventional blood washing ........................................ 101
4.4.2 The relevance of deformability and spleen RBC clearance ......................................................... 102
4.4.3 Differential sorting benefits on old vs. fresh RBCs ................................................................... 104
4.4.4 Threshold velocity estimation .............................................................................. 106
4.4.5 Changes intracellular Calcium content over storage time .................................. 108
4.5 Section Acknowledgement .................................................................................... 111

5 Changes in red blood cell deformability during ATP depletion ................................ 112
5.1 ATP depletion on RBC deformability and intracellular Calcium concentration .... 112
5.2 Experimental section ......................................................................................... 115
  5.2.1 Blood sample preparation .............................................................................. 115
  5.2.2 Microfluidic device operation and data acquisition ........................................ 115
5.3 Key Results ........................................................................................................ 116
  5.3.1 Simultaneous measurement on RBC deformability and intracellular Calcium .... 116
  5.3.2 Changes in RBC morphology and deformability during ATP depletion ............ 118
  5.3.3 ATP depletion on PKC activated RBCs .......................................................... 120
  5.3.4 PKC activation of banked RBCs .................................................................... 123
5.4 Discussions and section summary ...................................................................... 125
  5.4.1 Spectrin-membrane interaction on whole cell deformability ........................... 125
  5.4.2 PKC pre-activation and RBC deformability ..................................................... 125
  5.4.3 Changes of single RBC deformability and intracellular Calcium level during ATP depletion ...................................................... 127

6 Conclusion ............................................................................................................ 129
6.1 Thesis contribution ............................................................................................. 129
  6.1.1 RBC deformability in Malaria pathogenesis .................................................. 129
  6.1.2 RBC deformability in blood storage ............................................................... 131
  6.1.3 RBC deformability during ATP depletion and PKC activation ....................... 132
6.2 Future works ...................................................................................................... 134
  6.2.1 RBC deformability during iron overload ........................................................ 134
  6.2.2 RBC deformability and splenic RBC clearance for different malaria parasite virulence .......................................................... 134

7 References ........................................................................................................... 136
List of Figures

Figure 1. 1 Schematic diagram of spectrin (adapted from Wikipedia 20) ........................................... 20
Figure 1. 2 RBC passing through narrow constrictions (adapted from Huang et al, 2013 3)...... 26

Figure 2. 1 Device schematics ........................................................................................................ 36
Figure 2. 2 Effect of drug on RBC deformability .......................................................... 37
Figure 2. 3 Time dependent change after drug treatment .......................................................... 38
Figure 2. 4 Concentration dependent drug effect on RBC deformability ..................................... 39
Figure 2. 5 Effect of pentoxifylline on RBC deformability ......................................................... 40
Figure 2. 6 Effect of PTX at different concentrations ................................................................. 41
Figure 2. 7 Effect of PTX at different incubation hours ............................................................... 42
Figure 2. 8 Effect of PTX at different incubation hours (iRBCs) .................................................. 42
Figure 2. 9 Combined effect of ART and PTX on RBC deformability ........................................... 43
Figure 2. 10 Hypothetical spleen retention threshold ................................................................. 46

Figure 3. 1 Experiment flow chart .................................................................................................. 55
Figure 3. 2 Healthy RBC deformability profile in peripheral and in spleen .................................. 56
Figure 3. 3 RBC size comparison between peripheral and splenic blood ....................................... 57
Figure 3. 4 Deformability profile in infected mice .......................................................................... 58
Figure 3. 5 Photo on healthy and malaria infected mice spleen ................................................... 59
Figure 3. 6 Deformability profiles for healthy, uninfected and infected RBCs ............................... 60
Figure 3. 7 RBC deformability with CQ treatment ................................................................. 60
Figure 3. 8 Image of drug treated vs. control infected spleens ..................................................... 61
Figure 3. 9 Spleen mass and size with and without CQ treatment ................................................ 62
Figure 3. 10 Deformability profiles for CQ treated RBCs in spleen .............................................. 62
Figure 3. 11 Healthy RBC treated with CQ (peripheral) ............................................................. 63
Figure 3. 12 Healthy RBC treated with CQ (spleen) ................................................................... 63
Figure 3. 13 Histograms of splenic RBC deformability profile .................................................... 64
Figure 3. 14 Bimodal vs. normal distribution fitting (spleen control) .......................................... 65
Figure 3. 15 Bimodal vs. normal distribution fitting (day 5) .......................................................... 66
Figure 3. 16 Bimodal vs. normal distribution fitting (day 7) ......................................................... 66
Figure 3. 17 Bimodal vs. normal distribution fitting (day 9) .......................................................... 67
Figure 3. 18 Hemoglobin concentration of healthy and infected mice .......................................... 68
Figure 3. 19 Comparison between deformability measurements against reported retention rates ............................................................................................................................. 71
Figure 3. 20 Surface area and volume measurements on infected RBCs ....................................... 73
Figure 3. 21 Sphericity, area and volume characterizations with and without CQ treatment .... 74
Figure 3. 22 Healthy RBC morphology post CQ treatment days .................................................. 75
Figure 3. 23 Estimation of RBC retention percentage ................................................................. 76
Figure 4. 1 RBC deformability over time .................................................................................. 87
Figure 4. 2 RBC deformability over time (single RBC) .......................................................... 88
Figure 4. 3 Projected retention rate ....................................................................................... 89
Figure 4. 4 Morphological changes over storage time ............................................................ 89
Figure 4. 5 Changes in osmotic fragility over time ................................................................. 90
Figure 4. 6 Margination device schematics ........................................................................... 92
Figure 4. 7 RBC deformability in sorted outlets ...................................................................... 94
Figure 4. 8 RBC deformability after sorting (individual donors) ........................................... 94
Figure 4. 9 Projected RBC deformability after sorting ........................................................ 95
Figure 4. 10 Normalized discocyte count after sorting ........................................................ 96
Figure 4. 11 Fraction of discocytes (individual donors) ........................................................ 96
Figure 4. 12 Osmotic fragility after sorting .......................................................................... 97
Figure 4. 13 Osmotic fragility after sorting (old vs. fresh blood) .......................................... 98
Figure 4. 14 Osmotic fragility after sorting (individual) ....................................................... 99
Figure 4. 15 Microparticle concentration after sorting .......................................................... 100
Figure 4. 16 Bimodal fitting of log normalized velocity .......................................................... 107
Figure 4. 17 Intracellular Calcium level over storage days .................................................... 109
Figure 4. 18 Scatter plots on [Ca++] and Velocity (individual) ............................................. 110
Figure 4. 19 RBC deformability measured with CTO dye ................................................... 110

Figure 5. 1 Experimental videos were analyzed by MATLAB program which simultaneously tracks individual RBC velocity and fluorescent intensity .......................................................... 116
Figure 5. 2 When loaded with Calcium ionophore, microfluidic based fluorescent measurement was compared and calibrated against standard FACS machine (left). Calcium loading resulted in a reduced RBC deformability (right) .................................................................................. 117
Figure 5. 3 Morphological changes during ATP depletion ................................................... 118
Figure 5. 4 Fraction of biconcave RBCs after ATP depletion .............................................. 119
Figure 5. 5 Changes in RBC deformability (left) and intracellular calcium level (right) during ATP depletion .......................................................................................................................... 119
Figure 5. 6 Fraction of "slow" RBCs ....................................................................................... 120
Figure 5. 7 RBC morphology after 4h ATP depletion ........................................................... 121
Figure 5. 8 RBC morphology in ATP rich conditions ............................................................. 122
Figure 5. 9 Effect of PMA on ATP depleted RBC morphology (left) and deformability (right) ... 122
Figure 5. 10 PMA treating banked RBCs (morphology) ....................................................... 123
Figure 5. 11 Effect of PMA treatment on RBC morphology and deformability ..................... 124
Figure 5. 12 Correlation between RBC mean velocity and intracellular Calcium content (left). Scattered plot of single RBC deformability and Calcium content (middle) and the deformability of RBCs with low Calcium content .......................................................... 127

Figure 6. 1 Multiplexed RBC deformability sorter (Courtesy to Dr. Han Wei Hou) .......... 131
List of Tables

Table 1 Summary on various RBC deformability measurement techniques................................. 27
Table 2 shear modulus of infected RBCs in peripheral and in spleen .................................... 59
Table 3 hemoglobin concentrations before and after CQ treatment on healthy mice .......... 69
Table 4 parametric fitting parameters....................................................................................... 77
Table 5 microparticle concentration after sorting (individual sample)..................................... 100
Table 6: parameter estimates.................................................................................................. 108
Chapter 1

This section contains short extracts from previous publications by the thesis contributor:

- “Applying a microfluidic ‘deformability cytometry’ to measure stiffness of malaria-infected red blood cells at body and febrile temperatures”, MIT master thesis
- “Dynamic deformability of Plasmodium falciparum-infected erythrocytes exposed to artesunate in vitro”, Integrative Biology, 2013
- “In vivo splenic clearance corresponds with in vitro deformability of red blood cells from Plasmodium yoelii infected mice”, Infection and Immunity, 2014
- “Identification of different red blood cell subpopulations over prolonged blood storage using microfluidic-based deformability cytometry”, under review.

1 Introduction

Red blood cells (RBCs), also known as erythrocytes, have an average lifespan of 120 days. Mature RBCs are non-nucleated discoid with an average diameter of 7 μm. The biconcave shape is evolved from the multilobulated reticulocytes during 48 hours of maturation first in the bone marrow and then in blood circulation. During their lifecycle, RBCs circulate 500,000 times undergoing repeated deformations in narrow blood capillaries and splenic cordal meshwork. Deformability therefore plays a vital role in blood circulation under both physiological and pathophysiological conditions. On one hand, reduced RBC deformability is believed to be directly responsible for the increased RBC clearance in the spleen; on the other hand, deformability is also tightly associated with other biophysical and metabolic properties of the red cells. The dynamic interactions between cell deformability and spleen response would greatly impact on the microcirculation of blood and may subsequently correspond to the anemic condition suffered by the host. In this section, we would carefully introduce the following aspects relating to RBC deformability:
1) The importance of red cell deformability: from the perspective of mechanical RBC clearance in spleen;

2) Significant connections between red cell deformability and other biophysical and metabolic factors in both physiological and pathophysiological conditions;

3) A brief review on RBC deformability measurement techniques

4) Case study I: the role of RBC deformability in malaria pathogenesis

5) Case study II: changes of RBC deformability during blood storage lesion
1.1 The importance of red cell deformability: from the perspective of mechanical RBC clearance in spleen

In humans, 76-79% of the spleen is made of red pulp, a dense meshwork composed of splenic cords and splenic sinuses. The splenic slits with critical dimensions around 1 μm provide the most stringent mechanical challenge to the RBCs. When RBCs enter the reticular meshwork, passing the interendothelial slits into venous sinuses, the structural and mechanical quality of the RBCs is ascertained by the mechanical constraint imposed by the meshwork in the red pulp where old and abnormal RBCs that are less deformable are retained and eventually removed by phagocytosis.

The connection between RBC deformability and splenic clearance has been demonstrated in a series of ex vivo spleen studies. Splenic retention of both ring-stage malaria infected RBCs (iRBCs) as well as artificially hardened (by heating) uninfected RBCs (uRBCs) was observed via ex vivo perfusion of human spleen. It is evident that, besides possible molecular interactions, the mechanical properties of RBCs play a vital role in the process of splenic RBC clearance. This was further validated by experiments that mimicked splenic retention in vitro using a microsphere filtration system.

In fact, the role of the spleen in influencing the pathogenesis of malaria has been well documented in a number of clinical studies. Splenomegaly (enlarged spleen) is a characteristic clinical consequence of malaria infection, and therefore, the size of spleen has in fact been used to estimate the intensity of malaria transmission. Clinical studies involving radioactively labeled RBCs reveal that patients with an enlarged spleen display a more rapid clearance of RBCs.
compared to patients with a normal spleen. It has been proposed that splenomegaly modifies blood microcirculation and splenic filterability. Studies on splenectomized hosts that show higher fatality rates and delayed parasite clearance after antimalarial treatment also point to the role of the spleen in the clinical outcomes for malaria patients.

Experiments also suggest that splenic retention of RBCs could contribute to malarial anemia, which is a common consequence of severe malaria associated with high mortality. Excessive splenic clearance of RBCs is considered a likely mechanism for malarial anemia. While this process is not fully understood, several mechanisms have been proposed for the increased clearance of uRBCs, including the activation of splenic macrophages and enhanced splenic mechanical retention by altering the mesh size of spleen red pulp.

The significant implication of spleen as a “mechanical blood filter” is not restricted in the pathogenesis of malaria. In other vascular disorders such as thalassemia or transfusion of old stored RBCs, spleen is also believed to be able to sense and remove the poorly deformable RBCs, influencing the overall blood microcirculation. Studies revealed that the transfusion of old stored RBCs, for example, corresponds to a significant increase in spleen mass, indicating a substantial RBC retention in the spleen. Impaired deformability over prolonged storage, therefore, may be an important biomarker for the clearance of old stored RBCs post transfusion.
1.2 Connections between red cell deformability and other biophysical and metabolic factors

Subtle changes in RBC deformability often reveals and reflects alterations in other biophysical or molecular properties in red cells. To gain an in-depth understanding how RBC deformability correlates with other biophysical and metabolic factors, we first discuss the unique structure of RBCs.

Mature RBCs are non-nucleated with average diameter and thickness of 7 μm and 2 μm respectively. In comparison to the biconcave shaped discocytes of healthy RBCs, old or abnormal RBCs often display a variety of geometries such as echinocytes and spherocytes. The plasma membrane is the only structural component of a mature RBC, which encloses a large amount of hemoglobin. The ability of RBC to squeeze through small apertures, or simply deformability, is therefore largely determined by three factors: cell shape, membrane viscoelasticity and cytosol (hemoglobin) fluidity.

The shape of RBCs plays a determining factor on their microcirculatory deformability. Independent studies with different measurement characterizations have demonstrated that discocytes to spher-o-echinocyte transformation would result in significant decrease in cell deformability. Abnormal RBC shapes often link to certain blood disorders or clinical conditions. For example, sickle cell disease manifests with a notable fraction of sickle shaped RBCs; malaria infected RBCs undergo transformations from biconcave to spherical shaped; and aged RBCs comprise a lot more speculated echinocytes than fresh RBCs.
RBC membrane is a composite structure in which the membrane envelope is connected to an elastic network of skeletal proteins via transmembrane proteins. Membrane viscoelasticity is therefore affected by complex interactions of membrane skeletal proteins. The principal skeletal proteins that form the spectrin network are α- and β- spectrin, actin, protein 4.1R, and adducing (Figure 1.1). Any modifications on these proteins would subsequently alter the shape, stability, as well as deformability of RBCs. In this thesis, we note in particular the role of protein 4.1R in maintaining the mechanical properties of RBCs. Studies suggest that any defects in protein 4.1R would result in impaired spectrin-membrane binding, leading to reduced membrane stability overall. Indeed, the post translational modulations on the phosphorylation and unphosphorylation of 4.1R can dynamically regulate RBC membrane properties. For example, Ser-312 in 4.1R has been identified to be on the Protein Kinase C (PKC) phosphorylation site; PKC activation favors the phosphorylation of 4.1R, weakening the spectrin-membrane interaction. ATP depletion, on the other hand, forces 4.1R unphosphorylation and increases spectrin-membrane binding.

![Figure 1.1 schematic diagram of spectrin (adapted from Wikipedia)](20)
Hemoglobin (Hb) is the iron-containing oxygen-transport protein that is involved in oxygen transportation. Abnormal Hb concentration or altered Hb structure can lead to anemia and other genetic disease. Membrane-hemoglobin interactions have been shown to influence RBC deformability\textsuperscript{26}. The effect is believed to be contributed by the peroxidation of heme proteins\textsuperscript{26}. In adult humans, Hb protein typically contains 4 subunit proteins, each consisting of a protein chain associated with a non-protein heme group. The heme group consists of an iron ion held in a heterocyclic ring, known as a porphyrin. Under homeostasis, heme is controlled by its insertion into the “heme pockets” of hemoglobins. However, under oxidative stress, some hemoglobin may release their heme prosthetic groups which are highly cytotoxic. It is believed that the iron ion in the protoporphyrin IX ring undergoes Fenton chemistry to catalyze in an unfettered manner and produces free radicals. This deleterious effect could play an important role in blood storage lesion and malaria pathogenesis.\textsuperscript{27}
1.3 RBC deformability measurement techniques

Over the past decades, several techniques have been developed and matured for RBC deformability characterization. Some tools, such as ektacytometry, provide bulk deformability information, whereas other tools, including micropipette aspiration and optical tweezers, measure single RBC membrane properties. In this section, we introduce several more commonly used deformability measurement methods and discuss their potential applications.

1.3.1 Optical Tweezers

Optical tweezers (OT) generate force via a highly focused laser beam which could trap micron-sized dielectric particles and manipulate sub-nanometer displacements. The force acting on the particle can be modeled as a simple spring which follows Hooke’s law:

\[ F = -kx \]  

(1.1)

where the constant \( k \) is the trap stiffness depending on the OT design and the particle size. Experimentally, \( k \) is usually in the order of 50pN/\( \mu \)m, which corresponds to a resolution of 0.5pN in force measurement.

Though the OT has been used to characterize micron particles for almost 20 years, its application to measure cell deformability was fairly recent. In the paper published by Mills et al. in 2007\(^{28}\), OT was used to measure single RBC force-displacement response, from which membrane shear modulus was derived based on computational modeling\(^{29}\).
One distinct advantage of optical tweezers measuring RBC membrane stiffness is its high precision and specificity. By stretching the beads that are strategically attached to cell surface, optical tweezers can stretch RBCs in one or more directions. Several limitations are also associated to this method. For example, typically the maximum optical force is limited to several hundred pico–Newton, which could be insufficient to induce large deformation in the cells commonly encountered in vivo. Moreover, care needs to be taken to avoid overheating the cells by laser light during operation.

1.3.2 Micropipette Aspiration (MP)

Micropipette aspiration (MP) is one of the most classic methods that measure RBC membrane stiffness. During a typical MP experiment, RBCs are usually diluted by 1000 times from whole blood and suspended in a saline solution with comparable osmolarity. A small micropipette with inner diameter between 1 to 3 µm is carefully inserted in the RBC suspended solution and the target RBC is aspirated into the mouth of the pipette under a known suction pressure ΔP.

Compared to OT, MP is a more versatile method for measuring the mechanical properties of living cells as the suction pressure could ranges from 0.1pN/μm² to almost atmospheric. That means this technique can probe soft, fluid-like cells, such as RBCs, as well as very rigid cells. On the other hand, the high flexibility of red cells and their biconcave shape often cause “buckling” problem while performing MP experiment. That means, even at a low suction pressure, it is very easy for the entire cell to be sucked into the micropipette. Additionally, as a tool aimed to
measure RBC membrane property, a considerable amount of hemoglobin would also be sucked into the pipette, depending on the size of pipette. Though many complex models try to accommodate these variations in pipette inner diameter, it remains a question whether measured membrane stiffness can be consistent with pipettes of different sizes.

1.3.3 Ektacytometry

Ektacytometer is another method to measure cell deformation in Couette flow and was first developed by Bessis and Mohandas in 1975. The basic idea is to suspend cells in the narrow gap between two concentric cylinders and apply various shears on the cells by rotating the outer cylinder at different speed. As a laser beam is passed through the cell solution, the cells scatter the light to form a diffraction pattern which is circular at low shear force and becomes ellipsoidal at higher shear. The ratio of the major and minor axis of the ellipsoid indicates how deformable the cells are under a given shear force, and the deformability index (DI) was properly defined as follows:

\[
DI = \frac{A - B}{A + B}
\]

where A and B are the major and minor axes of the ellipsoid.

Distinct from MP and OT measurement, Laser-assisted Optical Rotational Cell Analyzer (LORCA) ektacytometry measures the average deformability of cell populations. Therefore, as a bulk measurement tool, ektacytometry has a much higher throughput and is capable of producing population-wide trait but it fails to reflect the deformability of individual cells in a given
population. This becomes an important drawback when the target cells to be studied form a minority population in a given sample, such as in malaria culture.

1.3.4 Microfluidic Devices (MF)

Most of the above mentioned measurement methods provide information on how flexible red blood cells are, but it is still difficult to correlate the calculated deformation index (DI) or shear modulus directly with the \textit{in vivo} blood flow. Hence, several microfluidic devices were designed to mimic the RBC deformation in small capillaries.

The first realistic \textit{in vitro} microfluidic realization of \textit{in vivo} RBC deformation was presented by Brody \textit{et al} in 1995 \cite{Brody1995}. Comparing the single capillary system by Shelby \textit{et al}. Brody's pillar array structure has several distinct advantages: 1) the pillar array structure minimizes cell-cell interaction, achieving single cell accuracy measurement; 2) clogging is less likely a problem as there are several channels in parallel and the constriction is fairly short; 3) the pillar array structure could be a better depiction of \textit{in vivo} RBC deformation in microcirculation as the cells have to undergo "repetitive" "severe" deformations while each deformation time is not necessarily long.

In 2003, Shelby \textit{et al} developed a microfluidic model for single-cell capillary obstruction \cite{Shelby2003}. Red blood cells at different stages of malaria infection were passed through the microchannel of 2, 4, 6, and 8\textmu m size. It was found that while Ring-stage infected erythrocytes were able to pass through all constricted channels, cells with later infection stage exhibited decreased ability to squeeze through the microchannels and Schizont stage infected erythrocytes were blocked
even at 6 μm channel. Though this experiment did not derive cell deformability in a very rigorous manner, it demonstrated several *in vivo* concepts such as “pitting” and “capillary blockage”. However, this technique would probably suffer from serious clogging issue and no quantitative data on cell deformability can be obtained directly. Cell-cell interaction may play a significant role and it is difficult to interpret single cell deformability based on this channel design.

Based on the similar design principles first proposed by Brody *et al*, Bow *et al.* optimized the pillar array structure for malaria diagnostic and related deformability studies. The “microfluidic cytometry” could detect the minute deformability shift from healthy to Ring stage malaria infected RBCs. This optimized device is used for this project and Figure 1. 2 depicts how single cells could pass through repeated 3 μm constrictions

![Figure 1. 2 RBC passing through narrow constrictions (adapted from Huang et al, 2013)](image)

Compare the aforementioned measurement tools which are commonly used to probe RBC deformability, each has its own advantages and limitations in terms of throughput, precision, specificity, and ease of operation. However, distinct from many other material stability tests, the need for RBC deformation study is built on its physiological importance. Therefore, when performing these deformation tests, it is important to recreate a physiologically relevant environment, such as shear rate, pressure gradient etc. At very low shear rate, the pseudo-static measurement could overlook the inherent viscoelasticity of cell membrane and leads to
misrepresentation. On the other extreme when very high shear rate is applied, the red blood cells could be permanently damaged. As RBCs pass through the splenic slit in vivo, shear rate is estimated to be \( \sim 10 \text{ sec}^{-1} \) and this number could be important potentially in interpreting the difference in experimental results by different measurement tools\textsuperscript{36}.

More recently, a few other microfluidic based deformability measurements have been reported, most of which operates at much higher flow rates\textsuperscript{37, 38}. For example, Gossett et al. reported microfluidic system which hydrodynamically stretches single cells\textsuperscript{37}. One distinct advantage of this system is its high throughput in the order of 2000 cells/s. However, due to the lack of directionality, such system is more suitable for spherical cells instead of biconcave RBCs. Zheng et al. employed a similar hydrodynamic system to measure RBC deformability by quantifying the extent of RBC elongation under shear stress\textsuperscript{38}. The system operates under a pressure of 2.5kPa and processes approximately 300 cells/min. The table below summarizes several key features of above mentioned measurement tools.

<table>
<thead>
<tr>
<th>Tool</th>
<th>Dynamic /Static</th>
<th>Single cell / Bulk</th>
<th>Throughput</th>
<th>Shear Rate (sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OT\textsuperscript{28}</td>
<td>Static</td>
<td>Single cell</td>
<td>(&lt;1 \text{ cell / min})</td>
<td>7-50</td>
</tr>
<tr>
<td>MP\textsuperscript{39}</td>
<td>Static/dynamic</td>
<td>Single cell</td>
<td>(&lt;1 \text{ cell / min})</td>
<td>(&lt;1)</td>
</tr>
<tr>
<td>Ektacytometer\textsuperscript{32}</td>
<td>Static</td>
<td>Bulk</td>
<td>(~ 1000 \text{ cell/min})</td>
<td>500\textsuperscript{40}</td>
</tr>
<tr>
<td>MF (Bow et al.)\textsuperscript{35}</td>
<td>Dynamic</td>
<td>Single cell</td>
<td>(~ 100 \text{ cell/min})</td>
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</tr>
<tr>
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<td>Dynamic</td>
<td>Single cell</td>
<td>(~ 300 \text{ cell/min})</td>
<td>NA</td>
</tr>
</tbody>
</table>
1.4 Case study I: Red blood cell deformability in malaria pathogenesis

Malaria is the most deadly parasitic disease which affects 200 million people worldwide and accounts for nearly one million annual deaths. The most virulent malarial parasite *Plasmodium falciparum* can lead to severe complications and has the highest mortality rate. During its asexual stage, *P. falciparum* infects red blood cells (RBCs), which then undergo notable morphological and rheological changes from the ring stage to trophozoite and finally schizont stage, constituting a 48 h asexual reproduction cycle. Cyclic febrile attack is a characteristic clinical feature of *P. falciparum* malaria, which corresponds to the release of merozoites in circulation following iRBC rupture in the late schizont stage.

Apart from cerebral malaria, malarial anemia is the most frequent and severe syndrome of *falciparum* malaria. Massive loss of RBCs cannot be entirely attributed to the destruction of infected RBCs (iRBCs), which usually constitute only a small fraction of total RBCs in malaria patients. Instead, the major cause of malarial anemia is believed to be the excessive loss of uninfected RBCs (uRBCs), mostly in the spleen and/or the liver. Malaria-related dyserythropoiesis is likely a minor factor because complete removal of erythropoiesis brings about only a minor decrease in RBC population. On the other hand, it has been suggested that uRBCs exposed to the parasites are slightly less deformable, and/or decorated with parasite molecules, both of which could potentially lead to splenic retention and clearance of a large number of uRBCs, exacerbating malarial anemia. However, the exact causes and mechanisms of malarial anemia are yet to be firmly established.
1.5 Case study II: Red blood cell deformability in blood storage lesion

Blood transfusion is one of the most common and lifesaving medical therapies\textsuperscript{46}. Every year in the United States alone, close to 5 million people need blood transfusion and approximately 14 million units of blood are collected and transfused\textsuperscript{47}. According to the Food and Drug Administration (FDA) regulation, refrigerated red blood cells (RBCs) can be stored up to 42 days. However, strong interindividual differences exist and some stored RBCs were observed to degrade way before six-week limit\textsuperscript{48}, raising concerns that older stored blood may give rise to an increased risk of blood transfusion\textsuperscript{49}.

Significant loss of RBC deformability typically occurs after 3 weeks of storage time due to ATP and 2,3-diphosphoglycerate (DPG) depletion\textsuperscript{50,51}. Poorly deformable RBCs potentially give rise to microcapillary obstruction\textsuperscript{52} and massive post-transfusion RBC clearance\textsuperscript{15,53,54}. However, studies suggest not all stored RBCs are unfit for transfusion: only a subpopulation of transfused stored RBCs were rapidly cleared in mice and the remaining transfused stored RBCs stayed in circulation in the same way as transfused fresh RBCs\textsuperscript{49}. The significant increase in corresponding spleen mass indicates mechanical retention in the spleen may be a likely mechanism for stored RBC subpopulation clearance. Since splenic clearance is highly correlated by RBC deformability\textsuperscript{15,55}, it is therefore important to identify population-wide single RBC deformability over storage time.
1.6 Thesis scope and outline

In this thesis, we based on a microfluidic platform to explore many physiological and pathophysiological situations where RBC deformability can serve as unique and important markers. More specifically, we applied the concept of “threshold velocity (deformability)” to predict how changes in RBC deformability may correlate to \textit{in vivo} RBC clearance. In particular, we employed malaria and blood storage lesion as two interesting case studies to demonstrate the important implications of altered RBC deformability or splenic retention threshold.

Chapter 2 was essentially building the groundwork before we formally introduced RBC clearance threshold. We used a popular anti-malaria drug to demonstrate that drug treatment could alter the deformability of malaria infected RBCs, which may eventually impact on their clearance mechanism in spleen.

In chapter 3, we further validated our deformability related splenic RBC clearance model in a malarial mice model. We investigated how RBC deformability profile changes after parasite invasion and/or antimalarial drug treatment \textit{in vivo}. For the first time, we associated RBC deformability with \textit{in vivo} RBC retention in spleen and anemic conditions suffered by the host. We formally introduced the notion of “threshold velocity” and how it could dynamically change under pathophysiological conditions. Furthermore, we employed statistical models to resolve different RBC subpopulations based on cell deformability. The notion of RBC subpopulation and dynamic threshold velocity allowed us to project \textit{in vivo} clearance mechanism in malaria pathogenesis.
Chapter 4 served as an extension of our previous findings on different RBC subpopulations. We applied the same concept in another clinical scenario of blood storage lesion and found that different deformability subpopulations coexist in old stored RBCs: while significant fraction of old stored RBCs were notably stiffer than fresh RBCs, some old stored RBCs remained deformable. We then attempted to enrich and separate the stiffer subpopulation using another microfluidic cell sorting system to demonstrate potential clinical benefits with prefiltration of stiff RBCs.

In chapter 5, we attempt to connect RBC deformability with the metabolic state of RBCs. The morphological and mechanical changes of RBC were examined during irreversible ATP depletion. PKC activation by PMA during ATP depletion was found to provide beneficial effect on the red cells.

RBC deformability can serve as an important and more direct biomarker for various vascular disorders and a few other on-going projects and potential applications are outlined in chapter 6.
Chapter 2

This section is an excerpt from our paper entitled "Dynamic deformability of Plasmodium falciparum-infected erythrocytes exposed to artesunate in vitro", published in Integrative Biology, 2013.

2 The in vitro effect of antimalarial drug on red blood cell deformability

Artesunate (ART) is widely used for the treatment of malaria, but the mechanisms of its effects on parasitized red blood cells (RBCs) are not fully understood. We investigated ART's influence on the dynamic deformability of red blood cells infected with ring-stage Plasmodium falciparum malaria (iRBCs) in order to elucidate its role in cellular mechanobiology. The dynamic deformability of red blood cells was measured by passing them through a microfluidic device with repeated bottleneck structures. The quasi-static deformability measurement was performed using micropipette aspiration. After ART treatment, microfluidic experiments showed 50% decrease in iRBC transit velocity whereas only small (~10%) velocity reduction was observed among uninfected RBCs (uRBCs). Micropipette aspiration revealed similar ART-induced stiffening in RBC membranes. These results demonstrate, for the first time, that ART alters the dynamic and quasi-static cell deformability, which may subsequently influence blood circulation through microvasculature and spleen cordal meshwork, thus adding a new aspect to artesunate's mechanism of action.

2.1 Artesunate and current hypotheses on its drug action
Clinical studies show that malaria patients with artesunate (ART) treatment exhibit a more rapid decline in the parasitemia and also that the accelerated parasite clearance is delayed in splenectomized patients. The involvement of spleen is therefore believed to be responsible for rapid parasite clearance after ART drug treatment. The in vivo parasite clearance following ART treatment has often been attributed to a process known as pitting, whereby spleen removes intraerythrocytic parasites without destructing the host RBCs. However, pitting might not be the only mechanism pertaining to splenic parasite clearance. Studies by Newton et al. noted that the average lifespan of pitted RBCs (i.e. RESA-RBC) is only 183 hours, significantly shorter compared to normal RBC life of 1027 hours. Furthermore, ART treatment was found to further shorten the pitted RBC life, suggesting that other mechanisms facilitating splenic parasite clearance may exist. Since spleen is also known as a "mechanical filter" that removes old and stiffened RBCs from microcirculation, the rapid splenic parasite clearance after ART treatment might be attributed to ART altering the mechanical properties of iRBCs and possibly of uRBCs. However, there is currently a lack of experimental evidence to confirm such an effect of ART on RBC deformability.

2.2 Experimental

2.2.1 Microfluidic device design

The microfluidic device was designed with layout program and it consists of 500 μm x 500 μm inlet/outlet reservoirs and parallel capillary channels with triangular pillar arrays. Two
different inter-pillar gap sizes of 3.0 and 4.0 μm were tested for optimum deformation condition.

Detailed fabrication steps was described by Bow et al\textsuperscript{59}.

2.2.2 Artesunate drug treatment

The stock ART solution (1.25 mg/ml) was prepared by dissolving ART powder (Sigma A3731) in aqueous sodium bicarbonate. A highly synchronized culture of 6 hour old rings with ~ 15% parasitemia was resuspended at 0.1% hematocrit in malaria culture medium containing various concentration of ART drug. In the control group, sodium bicarbonate solution without ART was added. Both drug and control groups were incubated at 37 °C for 2-6 h.

2.2.3 Solution preparation

Phosphate buffered saline (PBS) with 1% w/v Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was fresh made on every experimental day as stock solution. For experiments measuring healthy RBC deformability, fresh whole blood (Research Blood Components, Brighton, MA) was diluted 100 times with PBS/BSA buffer. For experiments measuring malaria infected cells, 1ml of cultured cells were centrifuged at 350g for 5 min; 1 μl of the pellet was then aliquot to 200 μl stock solution.

To distinguish infected cells from uninfected RBCs, 10 μl of 1 x 10\textsuperscript{-6} M thiazole orange (Invitrogen, Carlsbad, CA), which stains the RNA of the cell, was added to the aforementioned 200 μl iRBC containing solution 20 min before the experiment. The iRBCs would appear fluorescent under the GFP filter set whereas the uRBCs were seen as dark shadows.
2.2.4 Deformability measurement with microfluidic device

To accurately control the ambient temperature, the microscope surface was replaced by a heating chamber (Olympus), which was preheated to a desired temperature for 30 min before the beginning of every experiment. Meanwhile, the PBS/BSA stock solution was injected into the device to coat the PDMS walls to prevent adhesion. This filling step needs not to be done inside the heating chamber, but the PBS/BSA filled device needed to be placed into the heating chamber at least 5 min before loading 5 μL of diluted blood sample. During temperature calibration phase, a thermal meter was used to probe the exact temperature inside the heating chamber. When the temperature needed to be adjusted to a different value, at least 5 min of waiting time was required to ensure a new stable ambient temperature.

The microfluidic device was driven by a constant pressure gradient across. The inlet reservoir was connected to a vertically held 60-ml syringe which was partially filled with PBS/BSA buffer solution. A CCD camera (Hamamatsu Photonics, C4742-80-12AG, Japan) was connected to the inverted fluorescent microscope (Olympus IX71, Center Valley, PA) to capture the movement of the RBCs in the microchannels. Images were automatically acquired by IPLab (Scanalytics, Rockville, MD) at 100 ms time interval and the post-imaging analysis was done using imageJ. The velocity of individual RBCs was defined as the distance the cells moved divide by the time in seconds. To better illustrate drug-induced percentage change in iRBC/uRBC velocity, the term “normalized velocity” was adopted. Normalized velocity was obtained by dividing the measured velocity of individual RBCs from various experimental conditions by the average uRBC velocity of the control group on the same experimental day.
2.3 Key Results

The microfluidic device comprises a series of equally spaced triangular pillar arrays with gap sizes ranging from 2.5 to 4 μm (Figure 2.1A illustrates the system with 4 μm gap size). The gap sizes were designed to impose mechanical constraints similar to those encountered by the RBCs (with an average diameter of about 8 μm) when traversing blood capillaries and splenic meshwork. Driven by a constant pressure gradient that is smaller than the Pa/μm level, RBCs undergo large elastic deformation at each constriction while traversing the channel. The dynamic deformability of RBCs is then characterized by their velocity at a given pressure gradient which signifies their ability to deform repeatedly in order to pass through many successive constrictions.

Figure 2.1B presents sequential freeze-frame images of an uRBC moving inside the microchannel. The velocity of individual RBCs can then be derived by recording the time of passage of each cell through 10 constrictions in series (i.e. for a total travel distance of 200 μm). The typical pressure gradient (~0.5 Pa/μm) and shear rate (~100 s⁻¹) applied in this device as well as the resulting RBC flow rate (20 - 200 μm/s) are comparable to those of physiological flows; they are also of the same order of magnitude as RBCs passing through splenic inter-endothelial slits (IES).
2.3.1 Time-dependent effect of artesunate on infected RBC deformability

Ring-stage *P. falciparum* cultures were exposed to ART, an artemisinin derivative. The deformability of both iRBCs and co-cultured uRBCs was measured 2, 4 and 6 h after ART drug treatment. Cultures of iRBCs and uRBCs without ART treatment were used as control. All experiments were performed at 37 °C.

Figure 2.2 demonstrates pronounced decrease in iRBC velocity after 4 h of ART exposure. Compared to the control iRBCs (40 μm/s), the average transit velocity of ART-exposed iRBCs was halved (20 μm/s), indicating a statistically significant reduction of the dynamic deformability induced by ART (*p* < 0.001). On the other hand, a much smaller decrease in the average transit velocity of uRBCs was observed after ART exposure.

![Figure 2.2 Effect of drug on RBC deformability](image-url)
To assess the time-dependent effects of ART treatment, the dynamic deformability measurements were performed after 2, 4, and 6 h of ART exposure. The results are given in Figure 2. While no significant difference in the average iRBC velocity could be observed after 2 h ART incubation, iRBC velocity dropped significantly by approximately 40% after 4 h of ART exposure (p < 0.001).

![Figure 2.3 Time dependent change after drug treatment](image)

The differential deformability between uRBCs and iRBCs was previously noted as the key parameter for efficient splenic filtration of iRBCs.\(^7,9,10,62\) The deformability difference between uRBCs and iRBCs can be assessed through the index, \(R_s\):

\[
R_s = \frac{X_2 - X_1}{\frac{2(\sigma_1 + \sigma_2)}{2}}
\]

(2.1)

Here \(X_1\) and \(X_2\) are the mean values of velocities of iRBCs and uRBCs, respectively, and \(\sigma_1\) and \(\sigma_2\) denote the standard deviations of velocities of iRBCs and uRBCs, respectively. A higher \(R_s\) value implies larger separation between the velocities of iRBCs and uRBCs.
While in both control and ART-exposed groups the iRBC velocities are significantly slower than that of cocultured uRBCs (p < 0.001), the velocity separation resolution $R_s$ was enhanced by 2.3 times from 0.33 to 0.77 after ART exposure. Additionally, the average iRBC velocity after ART exposure was $3.13\sigma_2$ ($\sigma_2$: standard deviation of uRBC velocity distribution) away from the average uRBC velocity. This result suggests a more specific dynamic deformability differentiation between uRBCs and iRBCs after ART exposure, which may consequently lead to more efficient splenic parasite clearance.

2.3.2 Concentration-dependent effect of artesunate on infected RBC deformability

The *in vivo* serum concentration of ART varies with time after injection $^{63, 64}$. Typically, the half-life of ART in patients is approximately 3 h with peak serum concentration of 0.1 $\mu g$/ml. Therefore, the dynamic deformability assay was further expanded to different concentrations. Figure 2.4 indicates that from 0.01 $\mu g$/ml to 0.1 $\mu g$/ml, there is no statistically significant dose dependency on ART induced alteration of the RBCs deformability.
2.3.3 Effect of Pentoxifylline on infected RBC deformability

Pentoxifylline (PTX), a hydroxyl radical scavenger, is believed to be able to improve impaired blood flow. Several studies also suggest it can be used as an ancillary treatment for severe falciparum malaria, in combination with ART. In this section, the effect of PTX alone, as well as the combined effect of PTX and ART on the dynamic deformability of *P. falciparum*-infected RBCs are studied *in vitro*. All experiments were performed at 37 °C.

Figure 2. 5 demonstrates that PTX has no statistically significant effect on the deformability of ring-stage iRBCs even after 4 h of incubation at a concentration of 100 μg/ml. The average transit velocity of iRBCs after PTX treatment was 33 μm/s, which was very similar to that of the control sample (34 μm/s, p = 0.54). Simultaneous measurements were also performed on co-cultured uRBCs. Whereas the average uRBC velocity in the control sample was 43 μm/s, the value after PTX treatment was statistically different (46 μm/s, p = 0.034), exhibiting a significant albeit mild increase on the dynamic deformability of uRBCs.

![Figure 2. 5 Effect of pentoxifylline on RBC deformability](image)
The effect of PTX on the difference in response between uRBC and iRBC was calculated in the similar way as introduced before. The $R_s$ value for the control sample was 0.33 compared to 0.40 after PTX treatment. The result suggests a marginal (if any) effect of PTX drug on RBC deformability.

Typical clinical dosage of PTX is between 5 and 20 μg/ml. To investigate whether the effect of PTX on uRBCs is dose-dependent, the co-cultured uRBCs were exposed to 20 μg/ml and 100 μg/ml PTX for 2 h. Compared to the control sample (42.79 μm/s), the average uRBC velocity exhibited significant improvement at both concentrations (52.03 μm/s and 51.33 μm/s, $p < 0.01$) and no statistically significant concentration dependence could be concluded. Figure 2.6 suggested that the effect of PTX on uRBCs was not dose-dependent ($p=0.67$) in the concentration range between 20 to 100 μg/ml.

After 2 h incubation with PTX, the uRBC dynamic deformability seemed to be slightly enhanced (from 42.79 to 52.03 μm/s). The effect remained for incubation up to 6 h (Figure 2.7).
Simultaneous deformability measurement was obtained for iRBCs and no statistical significant effect was observed up to 6 h PTX incubation (Figure 2.8).

The combined effect of PTX and ART is shown in Figure 2.9. Compared with ART only experiments, PTX does not seem to provide additional influences to the dynamic deformability of both iRBC and uRBC when applied in combination with ART.
2.4 Discussions and section summary

It is widely accepted that ring-stage *P. falciparum* parasites are the only asexual intra-erythrocytic parasite forms able to travel through human circulation without being recognized by the human host. Antimalarial drugs affecting ring-stage iRBC membrane mechanical properties would help the host with early identification of parasitized red blood cells, and consequently their effective clearance by the human spleen.

In the present study we explored the effect of two different drugs (artesunate and pentoxifylline) on the microcirculatory velocity of RBCs after malaria infection. Both drugs are used alone or in combination for the treatment of *p. falciparum* malaria: artesunate (ART) is known to facilitate splenic parasite clearance 68, and pentoxifylline (PTX) has been suggested as an effective adjunctive therapy with ART 69, but their relevant effects on the RBC mechanical properties are not yet well understood.
Our results demonstrate that ART treatment significantly stiffens ring-stage iRBCs based on both quasi-static and dynamic single cell deformability assays. Additionally, our microfluidic set up mimicking RBC microcirculatory behavior revealed that ART has a significant impact on the velocity separation resolution between iRBCs and uRBCs. Both observations suggest the role of ART in influencing the dynamic circulation of iRBCs and uRBCs. Since human spleen is well known as a "mechanical filter" that removes old and abnormal RBCs, and splenic retention of artificially hardened uRBCs has been demonstrated by Buffet et al. using an ex vivo spleen, we speculate ART induced alternation in RBC membrane stiffness and dynamic microcirculatory behavior would have a significant effect on splenic RBC filtration.

Pentoxifylline, on the other hand, was initially developed to improve blood flow in the microvasculature. It was suggested as a possible ancillary therapy for cerebral malaria but clinical trials showed conflicting results. In this study, we attempted to evaluate how PTX and PTX-ART combinational therapy impact on the microcirculatory behavior of RBCs infected with P.falciparum malaria. The microfluidic based dynamic deformability assay with ring-stage iRBCs treated with PTX suggested a marginal albeit significant enhancement on uRBC deformability (p<0.05). This result is consistent with several other deformability studies using different measurement platform. However, we did not observe any significant effect of PTX on iRBC deformability. In terms of separation resolution, the effect of PTX is subtle if any (from 0.33 to 0.40 after PTX treatment). Experiments with ART-PTX combinational therapy did not reveal any beneficial effect of adding PTX: it showed no effect on iRBC deformability; neither did it compensate ART’s adverse effect on uRBC deformability as we earlier hypothesized.
Currently the underlying molecular mechanism of ART-induced RBC stiffening is undetermined. Clinical studies revealed that the drug action is mainly attributed by the endoperoxide-bridge. Deoxyartemisinin, an Artemisinin analog lacking the endoperoxide bridge, is clinically proven devoid of antimalarial activity, indicating the important role of peroxide in Artemisinin’s and its derivative ART’s drug action. Artesunate induced oxidative stress, a likely consequence due to its endoperoxide structure, was also suggested by Krungkrai et al to be the key for the drug’s antimalarial activity. It is therefore possible that the endoperoxide-bridge could also be responsible for ART-induced RBC stiffening. This is confronted by our microfluidic experiments with iRBCs treated with deoxyartemisinin, which revealed that deoxyartemisinin has no effect on the deformability of both uRBCs and iRBCs.

Our study attempted to understand the impact on drug-related changes on the ring-stage mechanical properties of P. falciparum iRBCs, by recreating the microcirculatory blood flow in vitro using a microfluidic device, and measuring their quasi-static membrane behavior using a micropipette device. By putting iRBCs through repeated physical and mechanical barriers, compared with the quasi-static data, we were able to quantify the drug related dynamic deformability modification for iRBCs and uRBCs.

Spleen is believed to work as a mechanical filter that removes stiffer cells from a large population. The splenic retention model has recently been hypothesized by Buffet et al. In this work, to quantitatively illustrate splenic clearance,
Figure 2. 2 is re-plotted into Figure 2. 10 with and without ART treatment. A normalized threshold value is drawn by assuming 5% of the aged healthy RBC population would occupy the lower 5% percentile of the velocity plot and are consequently removed by human spleen at each passage.

In our experiment, the normalized threshold velocity is assumed to be 0.67, such that in the control group only 5 out of 92 uRBCs fall below this value. Among the infected RBC population in the control group, 18 out of 42 iRBCs traverse below the threshold velocity, indicating the efficiency of splenic filtration of iRBC in the control group without ART treatment is only 43%. However among the drug treated group, all 18 iRBCs have traverse velocity below 0.67, suggesting a possibility of close to 100% iRBCs clearance after ART treatment.

The significant improvement in splenic filtration efficiency (from 48% to close-to-100%) suggests a possible ART drug mechanism in the pathophysiology of *P. falciparum* infection and in splenic clearance in general. On the other hand, as we compare the splenic retention of uRBCs
with and without drug treatment, we found that whereas only 5% of uRBCs would be removed from blood stream in the normal control group, 12% (12 out of 101) drug treated uRBCs are below the threshold velocity. The findings suggest ART may have adverse effects on uRBCs as well. The mildly reduced uRBC deformability may lead to significant increase in uRBC removal and could be the source of malarial anemia.

In conclusion, this work demonstrates that in vitro dynamic and quasi-static deformability measurements can be used to study subtle cell deformability changes resulting from various environmental factors such as in vitro drug treatment. ART drug treatment on malaria infected RBCs shows significant alternations in RBC's membrane stiffness and microcirculatory behavior. Since human spleen is a “mechanical filter”, reduced RBC stiffness may enhance splenic clearance of less deformable parasite-iRBCs from the circulation. On the other hand, the drug stimulus may also aggravate the loss of uRBCs as significant decrease in uRBC deformability was also observed in vitro. These hypotheses, while insightful and important, are certainly yet to be further corroborated by future in vivo and clinical studies. From the bioengineering perspectives, these measurements could provide a well-controlled in-vitro experimental platform to test novel anti-malarial compounds, or clarify the drug’s mode of action in relation to splenic clearance, which is generally difficult to do in vivo due to the lack of viable animal models and ethical considerations.

2.5 Section Acknowledgement
The thesis contributor would like to acknowledge all collaborators for their significant contributions. The micropipette measurement was performed by Dr. Andreas Undisz and the malaria sample prepared by Dr. Monica Diez-Silva. The manuscript was prepared and edited among all authors including Dr. Ming Dao and Prof. Jongyoon Han. Special thanks to Prof. Subra Suresh for the fruitful discussions.
3 The in vivo effects of antimalarial drug on *Plasmodium yoelii* infected mice

Recent experimental and clinical studies suggest a crucial role of mechanical splenic filtration in the host’s defense against malaria parasites. Subtle changes in red blood cell (RBC) deformability, caused by infection or drug treatment, could influence the pathophysiological outcome. However, *in vitro* deformability measurements have not been directly correlated *in vivo* with the splenic clearance of RBCs. In this paper, mice infected with malaria-inducing *Plasmodium yoelii* revealed that chloroquine treatment could lead to significant alterations to RBC deformability and increase clearance of both infected and uninfected RBCs *in vivo*. These results have clear implications for the mechanism of human malarial anemia, a severe pathological condition affecting malaria patients.

3.1 *Plasmodium yoelii* infected mice model

Although *ex vivo* studies of human spleen show retention of both iRBCs and artificially hardened uRBCs, little is known about the RBC splenic clearance during the course of malaria infection and antimalarial treatment. Furthermore, a better understanding from *in vivo* studies of possible connections between the mechanical retention of RBCs in the spleen and excessive blood loss or anemia is needed. The dynamic splenic response interweaves host, parasite and...
antimalarial drugs in such a complex manner that ex vivo spleen studies alone are insufficient to predict the role of splenic retention in influencing any anemic response in the host. Therefore, a quantitative and more direct measurement of the deformability of both spleen minced blood (SMB) and peripheral venous blood (PVB) in healthy and malaria infected host is highly desirable.

Rodent malarial model has been commonly used to complement the research on Plasmodium falciparum. Splenic clearance of parasitized RBCs was determined to play an important role in both human and mice. In this paper, we identified Plasmodium yoelii as the most relevant rodent model to study in vivo splenic RBC clearance for it shares similar “invasion characteristics” with Plasmodium falciparum. Careful consideration was also given to the structural similarities and differences between human and mice spleens: human spleen is sinusal; human RBCs (8 μm) have to squeeze through the interendothelial slits (≈1 μm) in venous sinus walls which act as a mechanical filter to abnormal or stiffened RBCs. In comparison, though mouse spleen is arguably classified as nonsinusal, the fenestrations in the walls of mouse pulp venules are so small (1-3μm) compared to murine RBCs (~6μm) that they still function just like “venous sinus” and mechanically trap less deformable RBCs. Additionally, unlike human spleen where both “open” and “closed” microcirculation exist, all mice RBCs need to undergo the same pathway, i.e. mechanical entrapment in spleen.

3.2 Chloroquine and current hypotheses on its drug action

How antimalarials influence RBC retention in the spleen could consequently impact on the therapeutic outcomes of malaria; however the mechanical impact of chloroquine on host RBCs
has not been explored. In the past, the inhibition of hemozoin formation was inferred as a key mechanistic consequence of chloroquine treatment. Hemozoin is a non-toxic crystal synthetized by the parasites as they digest RBC hemoglobin and release highly toxic (ferriprotonporphyrin IX) α-hematin. Since hematin may lead to RBC membrane disruption and eventually host cell lysis, the parasites need to convert hematin to hemozoin for their own survival. It is known that chloroquine can prevent hematin polymerization, but whether it also modifies host RBC deformability and consequently alters splenic RBC retention is unknown.

3.3 Experimental

To assess the deformability of RBCs in mouse spleen, we extracted mouse RBCs from both PVB and SMB and the deformability of the RBCs was quantitatively evaluated using a microfluidic deformability cytometer. Several important aspects relating to splenic RBC retention were explored: first, we established the correlation between in vitro deformability assay and in vivo splenic retention; secondly, the effects of malaria infection and/or antimalarial drug treatment on RBC deformability as well as on splenic RBC retention were then investigated; thirdly, we attempted to use 2 different approaches to estimate splenic retention threshold based on RBC deformability. Physically, "retention threshold" is decided by the effective pore size of the reticular meshwork or the splenic slits, as well as the RBC geometry (size, shape) and membrane stiffness. In this study, we estimate "retention threshold" below which RBCs are considered to be "most likely retained", expressed as a fraction of normalized PVB velocity. Finally, the possible
anemic effect related to increased splenic retention, both in infected and uninfected mice and with or without drug treatment, was investigated.

3.3.1 Murine model for malaria infection

4 to 6 weeks male or female Balb/C mice were infected with $1 \times 10^5$ parasites of *Plasmodium yoelii* YM by i.p. injection. This blood smear made from these infected mice showed 1-10% parasitemia approximately 4 days post-infection. Mice were then injected with drug or PBS for 3 consecutive days by i.p. injection as described below.

3.3.2 Microfluidic deformability measurement

The deformability of single RBC was assessed using microfluidic deformability cytometer (Figure 2.1) as described $^{59}$. Only 3 - 4 μl of blood samples with ~1% hematocrit was required for loading and the system was operated at pressure driven mode. RBC movement in the main channel was captured by CCD camera and the video could be post-analyzed using ImageJ software. The deformability for every RBC was characterized by its average traverse velocity across repeated bottleneck structures. It is noted that the device channel height is 4.2μm which ensures white blood cells and other cells from splenic minced blood to stay in the reservoir and not enter the main channel.

3.3.3 Animal preparation
Adult BALB/c mice each weighing approximately 20g were used for our experiments. Four treatment conditions were included: healthy mice/saline, healthy mice/CQ, malarial-infected mice/saline, and malaria-infected mice/CQ. Prior to the experiments, approximately 10 µl venous blood was taken out from all mice for baseline measurements of both RBC deformability studies and Hb assays. During the experiments, the healthy mice were bled approximately 10 µl on alternative days and the malarial-infected mice were bled only on the first and the last experimental days. The spleens of all mice were harvested when they were culled. Spleens were minced with sterile scissors and forceps. The extracted blood washed three times before being loaded to device.

3.3.4 Chloroquine drug treatment

Chloroquine diphosphate salt (Sigma-Aldrich) was dissolved in DI water with a final concentration of 100mM, stored at -20 °C freezer. Working dose was then prepared weekly by diluting the stock solution with 1X PBS buffer at the ratio of 2:11, stored at 4 °C. The mice were received 100µl diluted CQ solution daily via IP injection. In the control groups, mice were injected with 100 µl sterile PBS buffer solution. 3 consecutive days of CQ/ or saline treatments were carried out for all malaria infected mice and their spleens were harvested. For healthy mice group, drug effect was studied over CQ treatment days. Mice received 4, 6, or 8 days of consecutive CQ/ or saline injection before being culled on the following day.
3.3.5 Sample preparation

To differentiate iRBCs from uRBCs, Hoechst dye (33342, sigma) was added to the sample 20 minute prior to the microfluidic experiment so that the infected cells were fluorescent under UV excitation. A fixed inter-pillar gap size of 3μm was used for all mice RBC deformability measurements to achieve best deformation differentiation. The microfluidic device is pre-coated with 1% BSA (or 20% FBS) to minimize confounding effects due to RBC adhesion.

3.3.6 Experimental flow chart

The deformability of red blood cells was investigated in uninfected or *P. yoelii* (YM) infected balb/c mice. Figure 3.1 describes the flow chart of one single experimental round, consisting of 6 healthy mice and 6 *P. yoelii* infected mice. For the experiments involving only healthy mice, three of them received 750 μg (i.e. approximately 30mg/kg) of chloroquine (CQ) drug via intraperitoneal (i.p) injection daily \(^85,86\) and were therefore referred as healthy-drug (HD) mice. The remaining three healthy mice received equal volume of 1X phosphate buffered saline (PBS) solution and were termed as healthy-control (HC) mice. On experimental day 1, approximately 10μl of blood was extracted from all healthy mice to establish baseline measurement. After 4 consecutive days of CQ or PBS treatment, one mouse of HC and HD group each was culled on day 5. Both peripheral venous blood (PVB) and splenic minced blood (SMB) \(^87\) were collected for subsequent measurement. CQ and PBS treatment continued for the remaining four healthy mice and the procedure was repeated on day 7 and 9 until all mice were culled. For the experiments involving infected mice, 10⁵ parasites of *P. yoelii* YM were injected in all 6 mice.
2-3 days prior to the experiment. The parasitemia was monitored by Giemsa stained thin blood smear every 24 hours. When parasite levels first reached > 1% for all infected mice, 10μl blood was taken for baseline measurement (i.e. experimental day 1). At this stage three infected mice received CQ drug treatment (the Malaria-Drug (MD) group), and the remaining three mice received a PBS placebo (the Malaria-Control (MC) group) for 3 consecutive days. All mice were culled on day 4, and the PVB and SMB were collected. At this stage the MD mice had an average parasitemia less than 1% while the MC mice had an average parasitemia between 50 - 90%.

Figure 3. 1 experiment flow chart
3.4 Key Results

3.4.1 Splenic RBC retention based on the deformability profiles

RBC deformability profiles were characterized by a cell deformability cytometer, a microfluidic device which consists of triangular pillar arrays as described by Bow et al. The inter-pillar gap size was selected to be 3 μm for all mice RBC measurements. Individual RBCs deformed considerably as they passed through the device and their traverse velocities were recorded to describe a population-wide deformability profile (Figure 3.2). It is understood that higher traverse velocity corresponds to higher deformability and vice versa.

![Healthy Mice]

Figure 3.2 Healthy RBC deformability profile in peripheral and in spleen

The deformability (velocity) profiles of RBCs sampled from PVB were compared to RBCs from SMB of the same mice. As the red pulp of the spleen is capable of retaining the abnormal and hardened RBCs, a fraction of splenic minced RBCs were attributable to the splenic trapped RBCs, while the remainder would represent healthy normally circulating RBCs. Therefore, two significant RBC subpopulations in the spleen minced RBCs were assumed: 1) “flow through” RBCs
that share very similar mechanical properties to the peripheral RBCs; 2) abnormal or senescent RBCs that were trapped in the splenic meshwork. Mathematically the population wide splenic RBC velocity profile can be expressed as follows:

\[ V_{SMB} = (1 - a_1)V_{PVB} + a_1V_{trapped} \]  

(3.1)

where \( V_{SMB} \), \( V_{PVB} \), and \( V_{trapped} \) represent the average deformability (or velocity) of SMB, flow through, and splenic trapped RBCs; \( a_1 \) denotes the fraction of SMB that came from spleen-trapped cells. Comparison of the normalized SMB velocity against the average velocity of the PVB sample of the same healthy mouse (Figure 3.2) showed that RBC velocity of SMB was on average 15% lower than that of PVB (\( p<0.001 \)), reflected as a very gentle left shift in the velocity histogram (Figure 3.2). This difference in blood deformability profiles was likely contributed by the second subpopulation in SMB samples, reflecting the fraction (i.e. \( a_1 \)) of less deformable RBCs trapped in the splenic meshwork. No significant difference in the size distributions of PVB and SMB RBC populations was identified (Figure 3.3)
Similar analysis was also performed on *P. yoelii* infected mice and the infected RBCs (iRBCs) were differentiated from uninfected RBCs (uRBCs) by Hoechst staining. Figure 3.4 illustrated different deformability profiles of both uRBCs and iRBCs extracted from SMB and PVB samples. The average uRBC and iRBC velocities of SMB were respectively 21% and 30% slower than respective uRBCs and iRBCs in the PVB \( (p<0.001) \), indicating *in vivo* splenic retention, which mechanically removed stiffened RBCs from circulation. The reduced iRBC velocity SMB reconciled with increased membrane shear modulus analyzed by micropipette aspiration (Table 2). Compared to Figure 3.2, the differences in RBC (both uRBC and iRBC) velocities between PVB and SMB were more pronounced (Figure 3.4) in the infected mice, indicating a possibly increased splenic retention in mice after *P. yoelii* infection. This was further supported when examining the size of the healthy and *P. yoelii* infected mice spleens: the length of an infected mouse spleen (Figure 3.5) was approximately 1.6X of a healthy mouse spleen (i.e. ~4X volume enlargement), suggesting more intense splenic RBC retention.
### Table 2 shear modulus of infected RBCs in peripheral and in spleen

<table>
<thead>
<tr>
<th>Shear modulus (pN/μm)</th>
<th>Ring</th>
<th>Trophozoits</th>
<th>Schizonts</th>
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<td>Control Peripheral</td>
<td>2.70±1.42</td>
<td>2.18±0.84</td>
<td>1.83±0.67</td>
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<tr>
<td>Chloroquine Peripheral</td>
<td>-</td>
<td>6.24±1.70</td>
<td>16.67±17.0</td>
</tr>
<tr>
<td>Control Spleen</td>
<td>2.79±0.96</td>
<td>3.05±0.47</td>
<td>4.65±1.30</td>
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#### 3.4.2 The effect of malaria infection or/and antimalarial drug on deformability and retention

There are clear differences in the deformability of RBCs obtained from PVB from healthy and infected mice (Figure 3.6). Normalized against average velocity of healthy RBCs (i.e. hRBCs) from healthy mice, the average velocity of uRBCs from malaria infected mice was 0.91, 9% slower than hRBCs (p<0.001), and the average velocity of iRBCs was only 0.58, 42% slower than hRBCs (p<0.001). These results provide strong in vivo evidence that malaria parasite infections not only significantly stiffen diseased RBCs, but also have a visible impact on uninfected RBCs of the host.

![Figure 3.5 photo on healthy and malaria infected mice spleen](image-url)
This direct impact on uRBCs could result in an increased retention of these cells in addition to iRBCs in the spleen. The mechanical impact of the antimalarial drug chloroquine (CQ) on infected mice was investigated. In the peripheral blood, the average velocity of both uRBCs and iRBCs dropped by 17% and 42%, respectively, after CQ treatment (p<0.01) (Figure 3.7).
The spleen harvested from an infected mouse after 3 days of CQ treatment was 21% longer and 53% heavier than the spleen of the other infected mouse receiving PBS placebo (Figure 3.8 and Figure 3.9), indicating a drastic increase in splenic retention after CQ treatment. This observation was in good agreement with our studies on splenic uRBC velocity (Figure 3.10): the average velocity of splenic uRBCs declined from 0.72 to 0.54 after CQ treatment (p<0.001) and the percentage of splenic uRBCs moving at a velocity below 0.65 increased from 29% to 62% after CQ treatment, a sharp contrast as compared to only 2.2% (19 out of 873) of untreated peripheral hRBCs have velocity less than 0.65. Details on the threshold value (0.65) determination are discussed later.
3.4.3  Effect of antimalarial on healthy RBC deformability

Chloroquine was reported to be concentrated within malaria parasites by the formation of hematin, explaining the selective drug toxicity \(^9\)\(^1\). On the other hand, high dose and/or prolonged chloroquine exposure showed detrimental effects on the spleen and brain tissues of a healthy host: significant increase in the protein and cholesterol level was found in mice with prolonged CQ exposure; disorganization in red pulp was also reported \(^9\)\(^2\). With our observed effect of CQ on uRBCs from \(P.\) yoelii infected mice, it would be interesting to investigate the effect of CQ on the deformability of hRBCs from healthy mice.

Healthy mice were treated with CQ for 8 consecutive days. For hRBCs sampled from PVB, mean hRBC velocity dropped by 9% and 15% respectively after 4 and 6 days of consecutive CQ treatment \((p<0.001)\). No further velocity change was seen on day 9 \((p>0.1,\) Figure 3. 11). For hRBCs sampled from SMB, in additional to a gradual decrease, the velocity profile appears to
assume bimodal distribution, in which two normal distributions seem to start separate at normalized velocity close to 0.7 (Figure 3.12). Throughout the experiment, no significant size changes in the mice spleens were observed.

Figure 3.11 healthy RBC treated with CQ (peripheral)

Figure 3.12 healthy RBC treated with CQ (spleen)

3.4.4 Biomodal estimation of splenic healthy RBC deformability profiles after drug treatment
To study the distributions of hRBC velocity, Figure 3.12 was re-plotted as histograms (Figure 3.13). We fit data with both bimodal density functions and single normal distributions (Figure 3.14 to Figure 3.17). A comparison of the two models via likelihood ratio test (LRT) suggested that the bimodal distribution fit better to all SMB data ($p < 0.05$).

![Figure 3.13 histograms of splenic RBC deformability profile](image)

Since RBCs from SMB consist of two significant subpopulations of "flow through" and trapped RBCs, the contribution of each subpopulation to overall velocity profile was assessed using probability density estimation, where $a_1$ denotes the fraction of cells coming from the splenic retained population; $b_1$, $c_1$ denote the mean and standard deviation of the spleen retained RBC velocities; and $b_2$, $c_2$ denote the mean and standard deviation of the normal RBC velocities. Parameters were estimated by the maximum likelihood (ML) method, and all fitted results were listed in Table 4.

\[
f(x) = \sum_{i=1}^{2} \left( a_i \frac{1}{\phi(b_i)} \frac{1}{\sqrt{2\pi c_i}} \exp \left(-\frac{(x-b_i)^2}{2c_i^2}\right) \right)
\]  

(3.2)
Based on the parametric fittings, (red curves in Figure 3.14 to Figure 3.17), \( a_1 \) was estimated to be 0.39 in the control sample and increased to 0.68 after 6\(^\text{th} \) days of consecutive \( \text{CQ} \) treatment. \( \text{CQ} \) induced alteration in RBC microcirculatory behavior was hence suggested by the increased fraction of splenic retained RBCs. Besides, except for the control sample, the values of \( b_1 \) and \( b_2 \) remained largely unchanged, centered on 0.65 and 1.0 respectively. This result suggests a fairly constant splenic retention threshold. The value of \( b_2 \) agrees well with the average peripheral hRBC velocity of 1.0.

![Figure 3.14 Bimodal vs. normal distribution fitting (spleen control)](image)

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<tr>
<th>param</th>
<th>estimate</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a_1 )</td>
<td>0.39</td>
<td>(0.22, 0.56)</td>
</tr>
<tr>
<td>( b_1 )</td>
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<td>(0.67, 0.82)</td>
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<tr>
<td>( c_1 )</td>
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<td>(0.24, 0.32)</td>
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<tr>
<td>( b_2 )</td>
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<td>(0.81, 1.02)</td>
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<tr>
<td>( c_2 )</td>
<td>0.14</td>
<td>(0.11, 0.16)</td>
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</tbody>
</table>

Let \( a_2 = 0 \). We reduce the bimodal distribution to one single normal distribution.

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<tr>
<th>param</th>
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</thead>
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<tr>
<td>( b_1 )</td>
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</tr>
<tr>
<td>( c_1 )</td>
<td>0.22</td>
<td>(0.21, 0.23)</td>
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</tbody>
</table>
Normal distribution: Let $a_2=0$. We reduce the bimodal distribution to one single normal distribution.

<table>
<thead>
<tr>
<th>param</th>
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<th>95% CI</th>
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<tbody>
<tr>
<td>$a_1$</td>
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<td>(0.18, 0.87)</td>
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<tr>
<td>$b_1$</td>
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<td>(0.49, 0.84)</td>
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<td>$c_1$</td>
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<td>(0.19, 0.33)</td>
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<td>$b_2$</td>
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<tr>
<td>$c_2$</td>
<td>0.16</td>
<td>(0.11, 0.21)</td>
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</tbody>
</table>

Spleen 5 day
Sample size = 227

Figure 3.15 Bimodal vs. normal distribution fitting (day 5)

Normal distribution: Let $a_2=0$. We reduce the bimodal distribution to one single normal distribution.

<table>
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</tr>
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<td>$b_1$</td>
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<td>(0.80, 0.88)</td>
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<tr>
<td>$c_1$</td>
<td>0.29</td>
<td>(0.26, 0.31)</td>
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</table>

Spleen 7 days
Sample size = 510

Figure 3.16 Bimodal vs. normal distribution fitting (day 7)
It is noticed that the mean velocities of both subpopulations seem to vary in malaria infected mice. It is speculated that the enlargement of malarial spleen, and the further spleen enlargement after CQ treatment have modified the pore size in the splenic meshwork and altered RBC microcirculation.

3.4.5 Malaria infection, antimalarial treatment, and blood hemoglobin concentration

Malarial anemia is one of the most common complications of Plasmodium falciparum malaria and splenic RBC retention has been suggested to be a potential contributing mechanism. The possible anemic effect relating to increased splenic retention was hence investigated.
In mice infected with parasites the average hemoglobin level had dropped from 19.3 ± 2.2 g/dl to 16.0 ± 1.3 g/dl when the parasitemia first reached 1-10% (day 1, Figure 3.18). Infected mice were then treated with either PBS placebo or CQ for three consecutive days. By day 4, the parasitemia of MC (Malaria-Control, i.e. PBS treated) mice reached 50-90%; all displayed severe anemic syndrome with average hemoglobin concentration of 3.8g/dl (day 4, Figure 3.18). In comparison, all MD (Malarial-Drug, i.e. CQ treated) mice had parasitemia well below 1%. Despite the very low parasite burden, the average hemoglobin concentration of these MD mice was 10.3g/dl, still significantly lower than that on day 1 before CQ treatment (16.0g/dl, p<0.01). It is also noted that several MC mice from different experimental batches died on day 4 and were disregarded in all measurements.

Figure 3.18 hemoglobin concentration of healthy and infected mice
It is believed that the significantly lowered hemoglobin concentration (from 16.0g/dl to 10.3g/dl after CQ treatment) is highly correlated with the increase in the size and mass of mice spleens (Figure 3. 9). This result is consistent with reported clinical studies on human patients. On the other hand, the changes in hemoglobin concentrations were much less pronounced in CQ treated healthy mice (Table 3). No significant change in spleen size or mass was observed.

Table 3 hemoglobin concentrations before and after CQ treatment on healthy mice

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<th>Healthy mice + CQ</th>
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<td>18.2</td>
</tr>
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<th>Healthy mice + CQ</th>
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<td><strong>Day 1</strong></td>
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<tr>
<td>I</td>
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69
3.5 Discussions and section summary

While there have been several experimental results suggesting a significant role of splenic RBC clearance in human malaria pathology, this has never been validated in an in vivo setting with actual disease progression. This work aims to study in vivo filtration of RBCs directly in a mouse malaria model, and to correlate it with in vitro microfluidic based deformability measurement. Clinical studies have shown both similarities and differences in the malaria pathology between human and mouse in terms of invasion characteristics and malaria anemia\(^1\). For the scope of our study, in which mechanical RBC filtration spleen was concerned mainly, \textit{Plasmodium yoelii}-infected mice were chosen as our mouse model for two reasons: first, mouse spleen trapping of \textit{Plasmodium yoelii}-infected RBCs was demonstrated by prior experimental studies\(^9\); second, similar to \textit{Plasmodium falciparum} infection in human that the massive destruction of uninfected RBCs play a significant role in malarial anemia, non-parasitized RBCs in \textit{Plasmodium yoelii}-infected mice also believed to contribute to murine malaria anemia\(^9\). Additionally, it should be noted that for a typical nonsinusal spleen, adhesion (rather than deformability) is believed as the predominant mechanism for RBC removal, as the critical mesh size of the spleen is significantly larger RBC. However, mice spleen has very small fenestrations (1-3\(\mu\)m) that mechanically function like “venous sinus” and RBC deformability therefore plays a major role in the splenic retention in mice\(^8\). We also note that the microfluidic device for deformability measurement was pre-coated with 1% BSA or 20% FBS to minimize confounding effects from RBC adhesion.

It is worth mentioning that the deformability measured by a microfluidic device may not be equivalent to other measurement of cell deformability, such as micropipette and ektacytometry.
Cell deformability is a complex characteristic, depending on many factors such as shear rate that cannot be reduced to a single number. Still, our deformability cytometer is arguably better than other static deformability measurements, in terms of mimicking the splenic filtration process and predicting individual cells' filterability in vivo. Indeed, the percentages of “most likely retained” RBCs as predicted using our velocity-based single-cell measurements are in good agreement with reported retention rates of *Plasmodium falciparum* RBC cultures (Figure 3.19). The relatively low shear rate (<100 sec⁻¹) and slow RBC flow velocity (up to 200 μm/s) used in our system are consistent to those reported in vivo and the strong correlation between in vitro deformability and in vivo splenic filtration profile, demonstrated in our data, also support this argument.

![Figure 3.19 comparison between deformability measurements against reported retention rates](image)

<table>
<thead>
<tr>
<th>Infection stage</th>
<th>0-8 h</th>
<th>8-16 h</th>
<th>16-30 h</th>
<th>30-48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention %</td>
<td>30.0</td>
<td>37.5</td>
<td>83.3</td>
<td>100.0</td>
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</tbody>
</table>

Figure 3.19 comparison between deformability measurements against reported retention rates
3.5.1 RBC deformability, splenic RBC retention and malarial anemia

Based on experimental results on both healthy and malaria infected mice blood, SMB displayed significantly lowered transit velocity (Figure 3.2, Figure 3.4, Figure 3.7) in line with the spleen filtering less deformable RBCs. This observation along with the earlier ex vivo results by Buffet et al. ⁸, clearly establishes that in vivo splenic clearance of RBCs is determined by cells’ deformability.

Additionally, for the first time, we demonstrate the important links connecting RBC deformability, splenic RBC retention and malarial anemia within the in vivo mouse setting. Several earlier studies have eluded the possibility that intensified RBC retention in spleen exacerbates malarial anemia ⁷, ⁹. Here we demonstrate that reductions in RBC deformability and increased splenic RBC retention after malaria infection and CQ treatment exhibit strong correlations with the hemoglobin concentration of the test subject. It needs to be highlighted that CQ treatment, though successfully reduced the parasite count in the infected mice, did not alleviate malarial anemia completely (Figure 3.18) suggesting that instead of parasite loading alone, splenic retention of uRBCs could be directly responsible for the excessive blood loss in malarial anemia.

3.5.2 Chloroquine decreases RBC deformability and enhances splenic RBC retention

Another key finding of this study is that CQ has a direct impact on the red blood cell deformability. This decrease in RBC deformability provides another mechanism of action of CQ
as it leads to increased retention of iRBC in the spleen. On the other hand the fact that uRBCs show reduced deformability and consequently enhanced retention in the spleen can explain the high level of anemia seen at low parasitemia levels. To the best of our knowledge, we provide here the first demonstration of CQ induced iRBC alteration on RBC deformability in vivo by measuring RBC velocities under flow.

![Graph](image)

Figure 3. 20 Surface area and volume measurements on infected RBCs

The exact mechanism for CQ induced reduction in RBC deformability (or velocity) is still unclear. Drug induced changes on RBC size and shape is one possible factor, that could have direct impact on RBC deformability. Indeed, significant changes in the size and sphericity of iRBCs (trophozoites and schizonts) were observed (Figure 3. 20 and Figure 3. 21), and slight
increase in stomatocytes appears to be evident after 5 days of consecutive CQ treatment (Figure 3.22).

Figure 3.21 sphericity, area and volume characterizations with and without CQ treatment

On the other hand, membrane stiffening can be another important source for drug related iRBC rigidification as CQ causes hemin-induced oxidative damage on RBC membrane. This possibility has been validated using micropipette aspiration; the average membrane shear modulus of iRBCs (trophozoites and schizonts) was found to have increased by 2-8 folds after CQ treatment (Table 2). The impact on uRBC and hRBC in both healthy and malaria infected mice could be due to a general increase of the oxidative stress in the system. An earlier study injecting chloroquine to healthy swiss mice reported that, after 5mg/kg chloroquine i.p injection (approximately 8 fold lower dose than our 0.8mg/mice) both Glucose-6-phosphate dehydrogenase (G6PDH) and NADH diaphorase activities of normal RBC increased significantly, suggesting that the oxidative stress induced by chloroquine needs to be compensated by increasing the activity of protective enzymes. On the other hand, RBCs deficient in G6PDH are more susceptible to oxidative damages, so that drug induced oxidative stress could drastically decrease the deformability of these RBCs. It is also possible that chloroquine impacts RBC
deformability through accelerating hemoglobin denaturation. In all, the mild albeit significant reduction in hRBCs and uRBCs transit velocity (i.e. deformability) after CQ treatment is not unique to chloroquine: in a separate work, we also observed similar stiffening (in terms of both higher shear modulus and lower microcirculatory velocity) on uRBCs in vitro, from *Plasmodium falciparum* malaria infected human blood incubated with artesunate, another popular antimalarial drug containing an endo-peroxide bridge.

![Figure 3. 22 healthy RBC morphology post CQ treatment days](image-url)
3.5.3 Threshold prediction for splenic RBC retention

We are able to establish a clear relationship between RBC deformability and splenic RBC retention in a more quantitative manner, enabling us to study the effective threshold for retention. The term “retention threshold” was previously introduced to describe the stringency of spleen as a mechanical filter. It was postulated that different retention thresholds may exist in healthy and malaria infected host.

The average lifespan of red blood cells (RBCs) in balb/c mice is approximately 40 days. Aged mouse RBCs, which are generally stiffer, are trapped by mice spleen for phagocytosis. The spleen is therefore estimated to filter the least deformable 2.5% red blood cells during microcirculation each day. Based on this approximation, a cutoff line was drawn on Figure 3.2, marking the bottom 2.5% of PVB. The corresponding velocity range allows a rough estimation of splenic retention threshold in healthy animals, which is around 0.65 of normalized PVB velocity (Figure 3.23).

![Graph showing healthy mice retention](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control Peripheral</th>
<th>Control Spleen</th>
<th>CQ (days) 5</th>
<th>CQ (days) 7</th>
<th>CQ (days) 9</th>
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</thead>
<tbody>
<tr>
<td>% retention</td>
<td>2.2</td>
<td>16.1</td>
<td>24.7</td>
<td>39.4</td>
<td>28.1</td>
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</table>

Figure 3.23 Estimation of RBC retention percentage
This threshold is cross-validated by the maximum likelihood parametric fitting (S4), which resolved the SMB into two potential subpopulations according to the velocity distributions. The average velocity of the spleen-retained subpopulation, as suggested by parametric fitting, is also around 0.65 (i.e. b₁, Table 4).

It is interesting to note that a shift in the “retention threshold” was observed in mice after malaria infection. In contrary to healthy mice, of which the threshold was estimated to be centered at 0.65 (i.e. b₁ Table 4), malaria infected mice seem to have a lower threshold centered at 0.4 (Figure 3. 10). Lowered spleen retention threshold could imply enlarged pore size in malaria infected mice, which is consistent with several independent studies ¹⁰⁷⁻¹⁰⁹ using intravital microscopy and magnetic resonance imaging, which reported “dilated venous sinuses” in the red pulp of enlarged spleen.

<table>
<thead>
<tr>
<th>Table 4 parametric fitting parameters</th>
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<tr>
<td>Control Spleen</td>
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<tr>
<td>c₁</td>
</tr>
<tr>
<td>c₂</td>
</tr>
</tbody>
</table>

3.5.4 Separation Resolution

The term separation resolution Rs as adopted to describe how well two populations were separated or distinguished from each other as defined in equation (2.1). Based on Figure 3. 1, Rs
between the velocities of peripheral uRBCs and splenic iRBCs ($R_s = 0.78$) was almost doubled from that of peripheral uRBCs and peripheral iRBCs ($R_s = 0.44$).

Though CQ stiffens iRBCs to a larger extent than uRBCs, the deformability $R_s$ between iRBCs and uRBCs only improved marginally after CQ treatment (from 0.44 to 0.53). This was mainly due to an increase in the spread of uRBC velocity after CQ treatment.

3.5.5 Statistical Analysis

To account for the two distinct subpopulations of splenic minced hRBCs, and considering the bimodality of splenic hRBC velocities observed experimentally, the following bimodal normal mixture probability density function was proposed for the velocity:

$$f(x) = \sum_{i=1}^{2} \left( a_i \frac{1}{\sqrt{2\pi c_i}} \exp\left(-\frac{(x-b_i)^2}{2c_i^2}\right) \right)$$

where the mixture weights $a_i > 0$ ($i = 1, 2$), $a_1 + a_2 = 1$, $a_1$ denotes the fraction of cells coming from the splenic retained population, and $a_2$ denotes the fraction of cells from splenic blood vessel. By standard computation, the mean and variance of two types of hRBC velocities are

$$b_i + \frac{c_i}{\sqrt{2\pi} \Phi\left(\frac{b_i}{c_i}\right)} e^{-\frac{b_i^2}{2c_i^2}}$$

and

$$c_i^2 - \frac{b_i c_i}{\sqrt{2\pi} \Phi\left(\frac{b_i}{c_i}\right)} e^{-\frac{b_i^2}{2c_i^2}} - \frac{c_i^2}{2\pi \Phi^2\left(\frac{b_i}{c_i}\right)} e^{-\frac{b_i^2}{2c_i^2}}$$

respectively, where $\Phi(.)$ represents the cumulative distribution function of the standard normal distribution. In this study, we claimed that $b_i$ and $c_i^2$ correspond to the mean and variance of the hRBC velocities for the $i^{th}$ subpopulation ($i = 1, 2$) because the rest parts of the true mean and
variance were close to 0. The maximum likelihood based approach was adopted to estimate the \( a_i, b_i \) and \( c_i \) parameters in the bimodal density function \(^{110}\). The 95% confidence intervals were constructed based on the asymptotic normality of the estimators. In addition, the likelihood ratio test was used to compare the fit of the bimodal density function and a single normal model. All the above statistical computing was conducted using R version 2.13.2 (a free software GNU project) \(^{111}\).

3.5.6 Section summary

By employing a microfluidic single cell deformability cytometer, we investigated the "filterability" or deformability of PVB and SMB from healthy and malaria infected mice. Several interesting observations were made from the population-wide distribution of single RBC deformability: 1) RBCs extracted from SMB are less deformable compared to cells from PVB, indicating passive splenic RBC retention of less deformable RBCs, though the retention rate is fairly low for healthy test subjects; 2) CQ has a general stiffening effect on hRBCs, uRBCs and iRBCs, leading to an increased splenic RBC retention of all RBCs. It is likely that CQ induced alteration in RBC microcirculatory behavior is attributed to increased oxidative stress. 3) Increased splenic RBC retention strongly correlates to anemic condition for both healthy and malaria infected mice. Malaria related anemia is likely caused by the direct impact of intense splenic RBC retention, rather than high parasite loading. These new insights are expected to be useful in developing strategies to deal with malarial anemia, which is one of the severe pathological outcomes of (chronic) malaria infection.
3.6 Section Acknowledgement

The thesis contributor would like to acknowledge all collaborators for their significant contributions. Dr. Anburaj Amaladoss and Ms. Min Liu performed substantial animal work. The micropipette measurements were done by Dr. Rou Zhang and the biostatistical analyses were done by Dr. Huichao Chen. The manuscript was prepared and edited among all authors including Dr. Ming Dao and Profs. Peter Preiser and Jongyoon Han. Special thanks to Prof. Subra Suresh for the fruitful discussions.
Chapter 4

Significant portion of this section is an excerpt from our paper entitled “Identification of different red cell subpopulations over prolonged blood storage using microfluidic-based deformability cytometry”, currently under review.

4  Deformability based blood sorting and its application in blood storage

4.1 Blood transfusion and potential storage-age related risks

The metabolic, biochemical and molecular changes in aged RBCs, collectively termed as storage lesion, produce irreversible functional damage on the cells and ultimately limit blood shelf life. RBCs after prolonged storage period become less deformable and more fragile. Transfusion of these aged RBCs lead to increased RBC clearance in spleen, hemolysis and formation of microparticles.

The loss of RBC integrity plays another important risk factor for old stored blood transfusion. Reduced membrane integrity leads to RBC hemolysis and microparticle formation, a potentially important mechanism for transfusion associated adverse clinical outcomes, including increased risk of infection, multiple organ failure and death. Experiments with canine RBC concentrates noted a 1.8-fold increase in microparticle concentration over 35 storage days, confirming the effect of prolonged storage time on the formation of microparticle-encapsulated hemoglobin.

With all the potential harmful effects related to old stored RBC transfusion, a pre-wash that removes “bad” RBCs prior to transfusion would be desirable. Standard RBC wash procedure only
reduces the supernatant hemoglobin \(^{121}\), having little effect on the cells directly. Old and abnormal stored RBCs still give rise to long term adverse effects post transfusion. In this paper, we propose a cell-based sorting technology which efficiently remove both "bad" RBCs and microparticles. The simple microfluidic system employs Fahraeus effect and margination to laterally move stiff RBCs away from axial center and enrich microparticles in the cell free layer \(^{122}\). We believe this novel blood wash technology can potentially extend blood shelf life and improve clinical outcome.
4.2 Experimental

4.2.1 Microfluidic device design and fabrication

Two novel microfluidic devices were introduced in this project, namely the deformability cytometer\(^5\) and the deformability sorter\(^12\). Briefly, the deformability cytometer consisted of repeated constrictions with gap size of 3 µm and channel height of 5.3 µm. The gap size enabled RBCs to undergo substantial deformation, mimicking in vivo microcirculation\(^4\) and the channel height was carefully chosen such that all RBCs including the spherocytes would not be constrained by the device height, entering the main channel, whereas other blood cell types with diameter above 6 µm would be stopped in the reservoir, minimizing potential clogging downstream.

The deformability sorter attempted to mimic the in vivo margination effect in blood capillaries\(^12\). The critical channel length (i.e. between A to B in Figure 4. 6) was 2 cm, to allow sufficient lateral displacement. The expansion zone between B and C in Figure 3 was introduced to clearer separation into two distinct compartments. Channel width and height were chosen to be 20 µm X 10 µm, which were comparable to in vivo capillary size.

All microfluidic devices were fabricated in polydimethylsiloxane (PDMS) using standard microfabrication and soft-lithographic techniques described previously\(^5,\)\(^12\). Briefly, patterned silicon wafers were silanized with trichloro (1H,1H,2H,2H-perfluorooctyl) silane (Sigma Aldrich, St Louis, MO) and PDMS prepolymer (Sylgard 184, Dow Corning, Midland, MI) mixed ratio with curing agent in 10:1 (w/w) were poured onto the wafer to cure for 1-2 h at 80 °C. For the deformability sorter, a second cast was required to give the final PDMS microchannels. Holes (1.5
mm) for fluidic inlets and outlets were punched and the PDMS devices were irreversibly bonded to glass slides using an air plasma machine (Harrick Plasma Cleaner, Ithaca, NY).

4.2.2 Microfluidic device operation and data acquisition

Blood bank units from healthy donors containing packed RBCs (60~70% hematocrit) (Research Blood Components, Brighton, MA) were stored in sterile conditions at 4 °C to different aging period. Prior experiments, blood samples were carefully withdrawn from the package with a 23 gauge needle and adjusted to physiological hematocrit (~45%) in sample buffer containing 1× PBS.

The deformability sorting device was mounted on an inverted phase contrast microscope (Olympus IX71) equipped with a Hamamatsu Model C4742-80-12AG CCD camera (Hamamatsu Photonics, Japan) for imaging of blood flow. Blood samples were pumped through the device using a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA) at 5 μLmin⁻¹. After the flow had stabilized, samples were collected from the centre and side outlets for subsequent analyses.

RBC samples were then further diluted to between 0.1 – 1% hematocrit for deformability analysis. When the deformability cytometer was mounted on the same microscope set up, diluted RBC samples were loaded to the inlet reservoir and the flow was driven by pressure gradient as described 59. RBC flow was recorded by the camera and individual cell velocity (or deformability) was post-analyzed with image J.
4.2.3 Osmotic fragility

Evaluation of RBC osmotic fragility in unsorted and sorted (center/side) RBC specimens was determined using a modified Pink Test assay. Unsorted or sorted RBC specimens were washed one time (1500xg, 10 min, 18 °C) to remove supernatants, and packed RBCs were suspended with Pink Test solution (a hypotonic Bis-Tris buffer containing 25 mmol/L sodium chloride, 70 mmol/L Bis-Tris buffer, and 135 mmol/L glycerol; pH 6.6) to a final HCT of approximately 1.6 %. The RBCs were incubated at room temperature for 24 h. After the incubation period, each RBC sample was centrifuged (1500xg, 10 min, 18 °C) and the concentration of free hemoglobin (µmol/L) in samples supernatants was determined by the Drabkin’s reagent method. Percent osmotic hemolysis was calculated as follows:

\[
\text{Osmotic hemolysis (\%)} = \frac{Hb_{\text{supernatant}}}{Hb_{\text{total}}} \times 100
\]

whereas \(Hb_{\text{supernatant}}\) is the concentration of free Hb (µmol/L) from RBC supernatants following Pink Test, and \(Hb_{\text{total}}\) is the total amount of Hb (µmol/L) of each RBC sample.

4.2.4 Microparticle quantification

Megamix™, a mixture of monodisperse fluorescent beads of three diameters (0.5, 0.9 and 3 µm), was purchased from BioCytex (Marseille, France). Flow Cytometry Absolute Counting Standard microbeads (7.6 µm) were purchased from Bangs Laboratories, Inc. (Fishers, IN). Annexin V FITC, Glycophorin A-PE, Mouse IgG2b-PE isotype control, and annexin V-binding buffer concentrate were purchased from BD Pharmingen (San Jose, CA).
A detailed method for isolation and quantification of red cell microparticles (RBC MPs) has been previously described\textsuperscript{127,128}. Briefly, each sample consisted of 25 μL plasma, 1μL mouse anti-human CD235a-PE, 5 μL Annexin V FITC and 69 μL of Annexin V-binding buffer for a total volume of 100 μL. Each sample was incubated for 30 minutes at 4°C, protected from light and 400 μL of Annexin V-binding buffer was subsequently added to the sample preparations. Immediately prior to flow cytometric analysis, 150 μL of a master bead mixture consisting of one volume of absolute counting beads (7.6 μm) to two volumes of Megamix\textsuperscript{TM} was vortexed and added to each tube for a final volume of 650 μL. Acquisition for each sample was set to achieve 1000 events of the 3 μm beads. Red cell MP enumeration was obtained on a LSRFortessa flow cytometer (BD Biosciences, San Jose, CA).

RBC MPs were defined as CD235a-PE+ Annexin V+ events conforming to a light scatter distribution within the 0.5 μm to 0.9 μm bead range. A discrete population of CD235a-PE+ Annexin V+ events was first identified in the PE x FITC dot plot. By back gating, the position of the CD235a+ Annexin V+ population could be confirmed relative to the distribution of calibration beads in both the PE x FSC window and the SSC x FSC window. Isotype control labelling of samples with mouse IgG\textsubscript{2}k-PE confirmed specificity of Glycophorin A-PE (CD235a- PE+) labelling of MPs. Calculation of MP counts was based upon the following equation: absolute number of 7.6 μm counting beads added to each sample × (number of MP events /number of 7.6 μm bead events).
4.3 Key Results

4.3.1 Changes in RBC deformability over storage time

Banked RBCs from three healthy donors were analyzed with a microfluidic deformability cytometer consisting repeated bottleneck structures as described by Bow et al.\textsuperscript{129}. High transit velocity indicates better deformability and \textit{vice versa}. Significant RBC stiffening was observed for all three donors (black, blue and red circles) between 21 and 28 days of storage (Figure 4.1). The result was consistent with other bulk measurement tools such as ektacytometry\textsuperscript{48}. 

![Figure 4.1 RBC deformability over time](image)

Figure 4.1 RBC deformability over time

Figure 4.2 revealed the population-wide single cell information obtained from the same experiments that each dot represents one RBC passing. It is found that not only the average RBC velocity decreased drastically between week 3 and 4, the velocity distribution also shifted from unimodal normal to heavy tailed. It is evident that even after 5 weeks storage period, there still
are a significant proportion of RBCs with similar deformability value as compared to the mid-ranged fresh stored RBCs.

Suppose the post-transfusion survival for fresh (1 week) stored RBCs is 95% \(^{49}\), we then drew a velocity threshold at 2.5, marking the stiffest 5% of fresh stored RBCs. It is presumed that RBCs with velocity below the threshold value are likely to be cleared post-transfusion through splenic mechanical retention \(^{4,15}\). The percentage of RBC clearance was then estimated in Figure 4. 3 that a sharp increase in retention percentage was seen after 21 days of storage. Furthermore, old RBCs stored over 28 days were projected to have a survival rate around 50%, surprisingly consistent with an independent mice RBC transfusion study in which 60% of old stored RBC post-transfusion survival were reported \(^{49}\).
The significant deformability drop is believed at least partially caused by the time-dependent morphological changes. After 5 weeks of storage, majority of stored RBCs transformed into echino-spherocytes which are considerably stiffer as compared to the discocytes commonly seen in 2-week-old blood (Figure 4.4).
4.3.2 Changes in RBC fragility over storage time

Osmotic fragility refers to the percentage of hemolysis that occurs when RBCs are subjected to osmotic stress. The measurement reflects the extent of membrane integrity and is heavily affected by RBC surface area to volume ratio (S/V)\textsuperscript{130}. An abnormally high osmotic fragility often associates with clinical conditions such as spherocytosis or hypernatremia\textsuperscript{131}; in both situations the blood contains a significant fraction of crenated RBCs (i.e. echino-spherocytes). In fact, a strong and positive correlation between RBC surface transformation and osmotic fragility has been established that a sharp increase in osmotic fragility was observed during discocyte-echinocyte transformation\textsuperscript{132}.

![Figure 4. Changes in osmotic fragility over time](image)

Figure 4. Changes in osmotic fragility over time

During storage lesion, increased fragility can lead to a higher likelihood of \textit{in vivo} RBC breakdown, contributing to the added risk of blood transfusion. We therefore investigate the changes in RBC osmotic fragility during the course of storage. Time-dependent increase in the
osmotic fragility was found in all four healthy donors as shown in Figure 4.5. As we draw a linear regression line, the rate of increase was fairly similar across all donors (slope = 0.62± 0.13). However, the initial (i.e. fresh RBC) fragility level varies largely (from 10% to 20%). This diverse inter-individual RBC fragility is expected according to normal clinical result, whereas the fairly consistent increase rate across donors may be something interesting worth further exploration.

The increase in osmotic fragility over storage period probably correlated with RBC surface transformation as discussed above. Significantly increased proportion of echino-spherocytes were observed towards late storage period (Figure 4.4), leading to a reduced S/V and membrane integrity.

4.3.3 Deformability based blood storing device: principle and characterization

A microfluidic RBC sorter was developed to remove stiffened RBCs and microparticles through Fahraeus effect and margination. The device consists of an inlet, a filtration region, a margination region of 2 cm x 20 μm x 10 μm (length x width x height) microchannel, an expansion region, followed by the center and side outlets (Figure 4.6). The margination effect took place in the 2 cm long microchannel, where the more fluidic-like deformable RBCs occupied channel center, displacing stiff RBCs away from axis center. As the stiff RBCs marginalized to the side walls, they then could be collected from the side outlet, achieving high throughput RBC sorting. The width of outlet channels were designed to be 1:8:1, so that over 80% of unsorted RBCs would enter center outlet whereas less than 20% of unsorted RBCs would center side outlets. A similar version of this device employing the same principle has been reported earlier by Hou et al.122.
The same device could efficiently remove microparticles as well. As blood flows through the margination region, mechanical collisions between RBCs and microparticles displaces microparticles to the channel side walls. The expansion region then enhanced the separation, enriching the microparticles to the side outlets. The system has demonstrated efficient isolation of bacteria from sepsis blood.\(^{123}\)

To demonstrate the device is also capable of sorting old stored RBCs, we pre-stained some old RBCs (31 storage days) with calcium Fluo 4 dye and then mixed well with fresh RBCs (4-day-old) at 1:9 ratio. When passing the blood mixture through the margination device, old RBCs appeared as fluorescent dots whereas fresh RBCs were seen as dark backgrounds (Figure 4. 6). Fluorescence-based flow cytometry (FACS) then confirmed that the sample indeed comprised of...
two distinct subpopulations in which 89.8% of the RBCs showing extremely low fluorescence signal (i.e. the fresh RBCs, unstained) whereas 10.2% of RBCs exhibiting high level of fluorescence intensity (i.e. the old RBCs, Fluo 4 stained).

Initially, the blood mixture was first loaded into the device inlet, and the fluorescent RBCs (i.e. old RBCs) were evenly distributed across the channel width (Region A, blue). As RBCs moved along the straight channel (margination zone), the fluorescence RBCs slowly migrated towards the channel sidewalls (Region B, green). Finally, the fluorescent RBCs were concentrated to the side channels (Region C, orange). Standard FACS confirmed that the sample collected from the side channel consisted of 26% of fluorescent RBCs (i.e. old RBCs), a 3-fold enrichment as compared to 10.2% in the original blood mixture.

4.3.4 RBC deformability after sorting

Between 30 to 40-day old banked RBCs from different healthy donors were passed through the microfluidic deformability sorter as described above. RBC velocities at center and side outlets were normalized against the inlet velocity specific to each donor sample. The pooled effect on RBCs deformability after sorting was shown in Figure 4.7: cells collected from the center outlet exhibited a normalized velocity of 1.13, slightly higher than the unsorted control, whereas the normalized velocity for cells collected from the side outlet was 0.73, 23% less deformable as compared to the unsort control (p<0.001). The effect of sorting on individual blood deformability was presented in Figure 4.8.
Following the same hypothesis earlier, a threshold velocity was drawn at 0.8 so that around 40% of the unsort RBCs fell below this threshold (Figure 4.9, see discussion for more details) \(^{49}\). The fraction of RBCs moving below threshold velocity was similar as we compare
unsort and center outlet (41.2% and 38.9%). However, in the side outlet, 65.5% of RBCs fell below
given threshold velocity, over 1.6 fold higher compared to inlet control. The result again
confirmed that through margination and Fahraeus effect, less deformable RBCs were
concentrated in the side outlet.

![Graph showing % below threshold velocity for Unsort, Centre, and Side outlets]

**Figure 4. 9 projected RBC deformability after sorting**

The effect of sorting on RBC morphology were also investigated. The percentage of
discocytes in the center and side outlets were normalized against the inlet control for each blood
sample and the final discocyte count were presented as the relative increase or decrease
compared to corresponding control (Figure 4. 10). Significant decrease in the percentage of
discocytes was observed in all samples collected from side channel (p<0.05, paired t-test).
However, no significant difference in the discocyte count can be concluded between unsort and
center outlets. The un-normalized fraction of discocytes in individual blood samples were shown
in Figure 4. 11.
Figure 4. 10 normalized discocyte count after sorting

Figure 4. 11 fraction of discocytes (individual donors)
4.3.5 RBC osmotic fragility after sorting

Although the normal range of osmotic hemolysis percentage is fairly wide and varies from individual to individual, prolonged storage time typically associate with an increase in RBC fragility as evidenced from Figure 2A 130. The increase in osmotic hemolysis is likely associated with time dependent changes in RBC morphology that a larger fraction of echino-spherocytes formed towards old storage time. Since osmotic fragility is linked to RBC S/V and membrane integrity, spherocytic RBCs with reduced S/V and increased membrane fluctuation would be more fragile as compared to the normal discocytes 89. On the other hand, it has been fairly established that these spherocytic cells exhibit significantly lower deformability as well 19. We therefore believe that the deformability-based sorting device can enrich fragile RBCs at side outlet, improving overall blood quality.

Figure 4. 12 Osmotic fragility after sorting
Figure 4. 12 shows the osmotic fragility percentage measured from the unsorted inlet as well as the center and side outlets. RBCs collected from the side outlets exhibited much higher osmotic fragility as compared to the control and center outlet (p<0.0001, paired t-test). Additionally, the sorting was also found to be more effective on late storage RBCs (>28 day storage, Figure 4.13). Even though over 80% of unsorted RBCs would eventually enter center outlet by the design of microchannel outlet splitting, a slight but significant improvement on RBC fragility collected from center outlet was still observed for RBC stored over 28 days (p<0.05, paired t-test). The effect of sorting on individual blood fragility was presented in Figure 4.14. Note that top row illustrates the sorting effect for blood older than 4 weeks whereas bottom row illustrates the sorting effect for blood less than 4 weeks.

Figure 4. 13 osmotic fragility after sorting (old vs. fresh blood)
4.3.6 Microparticle concentration after sorting

The effect of sorting on microparticle count has been assessed. Total number of microparticles collected from each inlet/outlet was normalized against its corresponding cell count (Figure 4. 15). A slight but not yet significant decrease in microparticle concentration was observed in center outlet, whereas a 5-folds increase in the microparticle count was found in the side outlet (p<0.05). The effect of sorting on individual blood microparticle content was presented in Table 5.
Figure 4. 15 microparticle concentration after sorting

Table 5 microparticle concentration after sorting (individual sample)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cell number (10^6/ml)</th>
<th>Cell number /uL</th>
<th>Normalized MPs #</th>
</tr>
</thead>
<tbody>
<tr>
<td>0815 Unsort</td>
<td>172.00</td>
<td>172000</td>
<td>0.04</td>
</tr>
<tr>
<td>0815 Center</td>
<td>194.40</td>
<td>194400</td>
<td>0.09</td>
</tr>
<tr>
<td>0815 Side</td>
<td>22.24</td>
<td>22240</td>
<td>0.19</td>
</tr>
<tr>
<td>0815B Unsort</td>
<td>123.2</td>
<td>123200</td>
<td>0.08</td>
</tr>
<tr>
<td>0815B Center</td>
<td>170.4</td>
<td>170400</td>
<td>0.01</td>
</tr>
<tr>
<td>0815B Side</td>
<td>16.48</td>
<td>16480</td>
<td>0.42</td>
</tr>
<tr>
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<td>184.8</td>
<td>184800</td>
<td>0.07</td>
</tr>
<tr>
<td>0819 Center</td>
<td>187.2</td>
<td>187200</td>
<td>0.03</td>
</tr>
<tr>
<td>0819 Side</td>
<td>22.72</td>
<td>22720</td>
<td>0.36</td>
</tr>
</tbody>
</table>
4.4 Discussions and section summary

In this study, the effects of prolonged storage on several biophysical properties of RBCs were investigated. Storage time dependent changes in cell deformability, morphology, osmotic fragility as well as microparticle concentration were identified. A novel deformability-based microfluidic cell sorter was also developed which demonstrate the ability to enrich the older subpopulations of stored blood in a high throughput manner. This sorting system has promising potential to improve banked blood quality and thereby reducing risks related to blood transfusion.

4.4.1 Deformability-based blood sorting vs. conventional blood washing

Blood washing has long been performed in clinical practice to remove RBC debris and reduce plasma proteins. However only recently, hemolysis and cell-free hemoglobin were identified to play a significant risk factor during blood transfusion, and the clinical benefit of blood washing can be largely attribute to the reduction of plasma iron content. Nevertheless, standard RBC washing also raises certain clinical concerns: it puts cells through high mechanical shear stress and could increase subsequent post-transfusion hemolysis in vivo. For example, a recent study by Cortés-Push et al revealed that washing fresh blood could indeed increase plasma cell-free hemoglobin in vivo, worsening clinical outcomes. Besides, the inability to specifically remove old or abnormal RBCs is another shortcoming with conventional RBC washing:
the procedure is designed to remove only plasma iron, but failing to filter really fragile RBCs that are likely to release toxic heme in vivo post transfusion.

Our deformability-based sorting approach neatly overcomes both limitations of standard blood washing and has the potential to make a real beneficial impact in clinical setting: firstly, the deformability-based sorting device imposed minimum mechanical damage to the cells. The device was operated at 5 μl/min, mimicking in vivo leukocyte margination in blood capillaries. Secondly, the system works on both cell-free hemoglobin in the fluid (i.e. the lysed RBCs) as well as old and stiffened RBCs which are likely to break down and release free heme in vivo.

With the prefiltration directly on old cells, we anticipate our sorting system to achieve even better outcomes than standard washing method. Preliminarily in this study, we demonstrate promising results after sorting more than 4-week old blood that the side outlet (i.e. waste channel) successfully enriched the really bad cell subpopulations with highest osmotic fragility. Significantly higher microparticle concentration was observed in the side outlet. On the other hand, no considerable adverse sorting effect was detected on fresher (i.e. less than 4-week-old) RBCs as shown in Figure 4. 13.

4.4.2 The implication of deformability in spleen RBC clearance

Besides cell-free hemoglobin, another important mechanism for RBC removal is the "mechanical sensing" of old and abnormal RBCs in spleen. Deplaine et. al demonstrated that the in vivo clearance of old or abnormal RBCs in human spleen can be closely mimicked in vitro using microbead filtration method, highlighting the important role of deformability in splenic RBC
In the same study, the author also established a clear correlation between RBC deformability and retention rate by comparing fresh and old banked RBCs. Deformability is therefore critical in better our understanding of in vivo RBC clearance after transfusion.

In our population-based single RBC deformability analysis, RBC deformability was found to be fairly stable over the first three weeks (i.e. 21 days) of storage time. Significant decrease in overall deformability only became apparent starting week four where the mean transit velocity dropped by more than 50%. The result is similar with a separate study by Frank et al using ektacytometry, though they reported a smaller deformability decrease in terms of elongation index. Assuming the clearance rate for fresh RBCs to be 5%, we approximated a clearance rate around 50% after 4-6 week old storage period (Figure 4.3). The finding again was in good agreement with Deplaine et al and Hod et al. in which RBC retention rates of 30% and 40% were reported in human and mice RBCs after prolonged storage time.

The advantage of our deformability measurement system is that not only we were able to observe the population-wide change in RBC deformability, or identify the fraction of poorly deformable RBCs which were likely to be cleared in vivo, we could also provide single RBC deformability information. The statistics helped us to determine quantitatively how changes in the deformability distribution correlate to RBC retention rate. For example in Figure 4.7, although the normalized velocity in side outlet was 0.73, only 27% slower than the unsorted RBCs, we observed a significant change in the velocity distribution. In the retention rate analysis the projected retention rate increased from 41% in the unsort sample to 66% in the side outlet (Figure 4.9). On the other hand, even we observed a 12% velocity increase in center outlet as
compared to the unsorted inlet, the changes in projected retention was insignificant (from 41% to 39%).

Finally, it should be noted that the deformability we reported using a microfluidic device with multiple constrictions may not be equivalent as compared to other deformability measurements such as ektacytometry, micropipette aspiration, or optical tweezers. Our deformability measurement is more similar to \textit{in vivo} filtration process through probing RBCs under flow \textsuperscript{4}. Besides membrane shear modulus, which is typically reported by micropipette aspiration and optical tweezers, RBC geometry also plays a significant role in influencing our measurement.

4.4.3 Differential sorting benefits on old vs. fresh RBCs

The sorting effects were found to be different with different ages of stored RBCs. For example, a consistently lower velocity in the side outlet was only observed when processing RBCs older than 28 days. Similarly, only for older blood sample (>28days), osmotic fragility test showed both improvement in center outlet as well as worsening in side outlet (Figure 4. 13). The differential sorting benefits on old vs. fresh RBCs can be understood from the sorting device design and operation principle, as well as time dependent differences in RBC subpopulations.

The operation principle for the deformability based sorting device is to allow the more fluidic-like soft RBCs to flow at the axis center, pushing the more particle-like stiff RBCs to the side walls. The mechanical differentiation between “soft” and “stiff” is therefore important to achieve optimal sorting result. In freshly stored blood, most RBCs retained their biconcave
morphology and were reasonable “soft”, that very few cells could be classified as “stiff” enough for the margination effect to take place. For older blood stored for more than 28 days, a significant subpopulation underwent discocyte-echinocyte transformation. These echino-spherocytes not only have significantly stiffened cell membrane 19, their spherical shape made them more particle-like, differentiating them further from normal biconcave RBCs. With a reasonable fraction of echino-spherocytes, the margination device could then efficiently push these significantly stiffer RBCs to the side walls and eventually concentrated them into side outlets.

Finally, we note that the sorting device was designed to demonstrate the ability to enrich old RBCs in the side (waste) outlet. The side outlets were therefore purposely made very narrow to ensure a fairly pure population of old RBCs would be directed to the side. Consequently, we observed very promising differences in terms of RBC deformability, morphology, osmotic fragility and microparticle concentration when we compared samples collected from side outlets with unsorted inlet sample. However, the drawback of the current design was that over 80-90% of RBCs would enter the center outlet, causing the samples collected from center outlet very similar as compared to the unsorted controls. This may be the primary reason that we cannot conclude significant improvement on RBC deformability based on the cells collected from center outlet. In future studies, more focus should be placed on enhanced blood quality in the center outlet rather than efficient removal in the side outlet. To achieve that, we should re-design the split towards the outlet to make the center outlet much narrower and more selective.
4.4.4 Threshold velocity estimation

One important RBC clearance mechanism \textit{in vivo} is through the mechanical trapping of cells in spleen. In this work, we employed a microfluidic RBC deformability cytometer to simulate the microcirculatory system and thereby estimate the rate of RBC retention in spleen.\textsuperscript{55} To translate our velocity measurements to the filterability in spleen, the concept of threshold velocity was conceived below which RBCs were considered more likely to be removed through splenic trapping.\textsuperscript{10,55}

To determine the value of threshold velocity, we attempt to use two independent approaches: 1) rely on other clinical studies to set an initial value, or 2) use statistical modelling to resolve the velocity distributions. For example, the threshold velocity in Figure 4.3 and Figure 4.9 was estimated based on previous studies by Hod et al.\textsuperscript{49}, in which the authors reported approximately 5% of RBC clearance after fresh RBC transfusion. We therefore marked the slowest 5% of RBCs based on our fresh RBC velocity profile and applied the same value for subsequent stored RBCs. This crude threshold velocity estimation worked surprisingly well that the projected percentage of old stored RBC survival matched with reported values from independent sources.\textsuperscript{15,49}

The second approach to estimate threshold velocity is through statistical analysis. We observed that the velocity distribution in Figure 4.7 appeared to have two separate scatters. For that reason, we attempted to fit the velocity profile with mixture model distributions and estimate the retention rate based on the relative ratio of each distribution. More specifically, the following probability density function was used for our estimation:
\[ f(x) = a_1 \frac{1}{\sqrt{2\pi c_1}} e^{-\frac{(x-b_1)^2}{2c_1^2}} + a_2 \frac{1}{\sqrt{2\pi c_2}} e^{-\frac{(x-b_2)^2}{2c_2^2}} \]  

(1)

where \( 0 < a_1 < 1; a_1 + a_2 = 1; b_2 > b_1; c_1 > 0; \) and \( c_2 > 0 \).

After log transformation, the normalized velocity data in Figure 4.7 was replotted into Figure 4.16, in which the velocity of RBC collected from unsort, center and side channels can each be graphed into a bimodal distribution. The fraction of stiff RBCs (i.e. likely to be cleared subpopulations) was denoted by \( a_1 \) and all the parameters were estimated by the maximum likelihood (ML) method. 

Based on this estimation, the fraction of stiff RBCs in the unsorted inlet was 33%, similar to the RBC retention rates reported by other literature (30% - 40%). The fitting also suggested a significantly larger fraction of 58% of stiff RBCs in the side outlet, consistent with our predicted 60% retention using approach 1 (Figure 4.9, black: unsorted, red: center, blue: side).

It is also interesting to note that based on approach 2, though the fraction of stiff RBCs (i.e. \( a_1 \)) is similar for unsorted and center RBCs (0.33 vs. 0.38), the mean value of the high velocity subpopulation \( b_2 \) improved significantly from 0.27 to 0.51 after passing through the sorting
device. The data suggests the promising potential of further purify and enrich the more deformable RBC in the center outlet.

Table 6: parameter estimates

<table>
<thead>
<tr>
<th>parameter</th>
<th>estimate</th>
<th>95% CI</th>
<th>estimate</th>
<th>95% CI</th>
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<th>95% CI</th>
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<td>(0.26, 0.40)</td>
<td>0.38</td>
<td>(0.31, 0.46)</td>
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<td>(0.44, 0.73)</td>
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<tr>
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<td>-2.31</td>
<td>(-2.49, -2.12)</td>
<td>-2.23</td>
<td>(-2.68, -1.79)</td>
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<td>(0.67, 1.10)</td>
<td>0.73</td>
<td>(0.59, 0.87)</td>
<td>1.03</td>
<td>(0.72, 1.33)</td>
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<tr>
<td>b2</td>
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<td>(0.19, 0.36)</td>
<td>0.51</td>
<td>(0.45, 0.57)</td>
<td>0.29</td>
<td>(0.03, 0.54)</td>
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<tr>
<td>c2</td>
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<td>0.31</td>
<td>(0.27, 0.35)</td>
<td>0.50</td>
<td>(0.31, 0.69)</td>
</tr>
</tbody>
</table>

4.4.5 Changes intracellular Calcium content over storage time

Intracellular Ca$^{2+}$ plays an important role in RBC physiology, though the exact links between Ca$^{2+}$ and blood related medical conditions remain unclear. During blood storage, as adenosine 5′-triphosphate (ATP) decreases over time, an increase in RBC Ca$^{2+}$ develops irreversibly. The elevated Ca$^{2+}$ concentration is believed to be correlated with oxidative damage and deformability drop suffered by old RBCs.

In this work, we also investigated single RBC Ca$^{2+}$ concentration in parallel to the deformability measurement (Figure 4.17). The intracellular Ca$^{2+}$ level was found to increase steadily for the first 2 – 3 weeks of storage, and then remained fairly unchanged up to week 5. Scatter plots were also generated mapping out both Ca$^{2+}$ and velocity (deformability) of individual RBCs from the same blood source but measured at different storage days (Figure 4.18). For the first 3 weeks, most RBCs were in the velocity range between 5 and 15; and an elevated Ca$^{2+}$ was weakly correlated to reduced deformability. The Ca$^{2+}$ concentrations on week
2 or 3 were also significantly higher than those measured during the first week. Beyond week 3, most RBC scatters fell in lower velocity range between 0 and 5 and the Ca\(^{2+}\) concentrations remained at similar levels with the more deformable RBCs measured in week 3 (around 35 arbitrary units).

On the other hand, it is observed that sorting of more than 4 week old blood does not have significant impact on their intracellular Ca\(^{2+}\) concentrations. No consistent changes in Ca\(^{2+}\) level were observed in the side outlet, suggesting that beyond 4 weeks of storage, no strong correlation between RBC deformability and Ca\(^{2+}\) can be concluded.

Lastly, we also noted that the Ca\(^{2+}\) staining protocol may introduce additional stiffening effect on banked RBCs given it required additional hours for sample preparation and it exposes RBCs to a buffer containing 1.8mM Ca\(^{2+}\) ions (i.e. Calcium influx). For that reason, we verified our
deformability measurement using cell tracker orange dye (Figure 4. 19). RBCs collected from side outlet exhibited significant stiffening similar to that observed with Ca$^{2+}$ staining.

Figure 4. 18 scatter plots on [Ca++] and Velocity (individual)

Figure 4. 19 RBC deformability measured with CTO dye
4.5 Section acknowledgement

The thesis contributor would like to acknowledge all collaborators for their significant contributions. Dr. Han Wei Hou contributed the margination based deformability device. The osmotic fragility measurement was done by Dr. Tamir Kanias and Mr. Derek Sinchar. The microparticle quantification was done by Dr. J.T. Sertorio. Dr. Huichao Chen performed the bimodal statistical analysis. The manuscript was prepared and edited among all authors including Dr. Mark Gladwin and Prof. Jongyoon Han.
Chapter 5

Significant portion of this section is an excerpt from our paper entitled “Changes in red blood cell deformability during ATP depletion”, in preparation.

5 Changes in red blood cell deformability during ATP depletion

5.1 ATP depletion on RBC deformability and intracellular Calcium concentration

The exceptional ability for RBCs to deform is mainly attributed to its unique structure: the biconcave shape as well as the membrane-cytoskeleton interaction, both believed to be highly regulated by the presence of adenosine 5’ -triphosphate (ATP). ATP depletion in RBCs could result in a drastic morphological transformation from discocytes to echino-spherocytes. Marked increase in RBC membrane bending rigidity was also observed following ATP depletion.

Although the connection between ATP and RBC deformability has been previously established, the underlying mechanism of how biochemical energy influences biomechanical membrane stiffness remains unclear. Previously, the ATP-dependent membrane biomechanics was hypothesized to be related to the phosphorylation of the 4.1R protein. Protein 4.1 plays a significant role in the shape, stability and deformability of RBCs, and any defects in 4.1R would result in impaired spectrin-membrane binding, leading to reduced membrane stability overall. Post-translational modifications on the phosphorylation and unphosphorylation of 4.1R have also been shown to dynamically regulate RBC membrane properties. For example, Ser-312 in 4.1R has been identified to be the protein kinase C (PKC) phosphorylation site, and PKC

112
activation favors the phosphorylation of 4.1R, weakening the spectrin-membrane interaction.\(^2\) \(^2\) ATP depletion, on the other hand, forces 4.1R unphosphorylation and increases spectrin-membrane binding.\(^1\) \(^9\) However, it remains unclear how effects on spectrin-membrane interaction could translate to overall deformability of RBCs during microcirculation.

Additionally, recent studies also demonstrated that glucose depletion or PKC activation could stimulate the Ca\(^{2+}\) entry to the RBCs.\(^{138,139}\) Intracellular Ca\(^{2+}\) not only controls the redox state of RBCs, it also regulates RBC biophysical properties such as deformability and morphology and modulates protein activity including PKC. Under physiological condition, the plasma membrane Ca\(^{2+}\) pump (PMCA) could efficiently extrude Ca\(^{2+}\) to maintain a basal intracellular Ca\(^{2+}\) concentrations around 50nM in healthy RBCs.\(^{140}\) The ability of PMCA Ca\(^{2+}\) transport is however limited by ATP availability: an elevated intracellular Ca\(^{2+}\) level may be achieved through the inhibition of energy supply.\(^{141}\) On the other hand, calcium-calmodulin (Ca-CaM) complex interacts with major cytoskeletal elements such as protein 4.1R and adducing, playing a key role in maintaining RBC cytoskeletal flexibility and stability.\(^1\) \(^142\) Indeed, old and senescent RBCs of low ATP contents have been found to associate with increased intracellular Ca\(^{2+}\) concentrations and reduced RBC deformability.\(^{143}\) However, how ATP depletion and PKC activation correlate to single RBC deformability as well as intracellular Ca\(^{2+}\) level remains unexplored.

In this paper, we employed a microfluidic deformability cytometer which simultaneously measure the intracellular Ca\(^{2+}\) level as well as deformability of single RBCs during ATP depletion.\(^3\) \(^59\) The effect of PKC activation on RBC deformability was also investigated to examine whether cell deformability can be restored in part through regulating the membrane-spectrin interactions. This work has important clinical implications in blood storage lesion during which both significant
biochemical and biomechanical changes are observed as RBC ages. In particular, we are interested in the following aspects: how biomechanical changes such as reduced cell deformability correlate with biochemical changes in terms of ATP depletion and intracellular calcium accumulation; and whether it is possible to recover the deformability of senescent RBCs through the activation of PKC.
5.2 Experimental

5.2.1 Sample preparation

Fresh blood (Research Blood Components, Brighton, MA) were washed one time (300xg, 3 min) to remove the white blood cells and platelets prior to the experiment. Packed RBCs were then diluted in 1X phosphate buffered saline (PBS) solution so that the standard sample solution contained 1% hematocrit.

Irreversible ATP depletion was achieved by treating RBC samples with 3 mM iodoacetamide and 5 mM inosine for up to 4 h at 37 °C as described \(^{19,25,144}\). PKC activation was achieved by treating the sample solutions with 1 μM PMA for 1 h at 37 °C \(^{22}\). Cell Tracker Orange (Life Technologies) was used to stain all the cells when Calcium staining was not applied. All chemicals were purchased from Sigma-Aldrich, unless specified otherwise.

5.2.2 Intracellular calcium measurement

Intracellular Calcium level was measured as described in other reference \(^{145}\). Briefly, fluorescent dye-based calcium indicator Fluo-4 (Life technologies, Carlsbad, CA) was used and standard staining protocol was followed. Intracellular calcium content was then assessed by measuring the fluorescence intensity after Fluo-4 treatment, using fluorescence-activated cell sorting (FACS) machine (Accuri™ C6 flow cytometer, BD Biosciences, San Jose, CA) or fluorescent microscope (Olympus, IX71).
5.3 Key Results

5.3.1 Simultaneous measurement on RBC deformability and intracellular Calcium

The deformability and intracellular Ca\(^{2+}\) were simultaneously measured using a microfluidic system described by Bow et al.\(^5\). RBCs were first stained with Fluo4 Calcium dye and then passed through the microchannels with repeated pillar array structures. RBC deformability was quantified by the velocity of cells passing through the channel constrictions whereas the intracellular Ca\(^{2+}\) concentration was measured by their fluorescence intensity. Figure 5.1 is a snapshot from an experimental video processed by MATLAB. The time and number of cells in screen were displayed on top left corner (534; 3C), whereas the intracellular Ca\(^{2+}\) intensity were traced at the same time. All data could then be exported to an excel sheet summarizing population-wide single RBC velocity and corresponding Ca\(^{2+}\) level.

![Time: Cell in Cell No. 1 Pillar](image)

Figure 5.1 Experimental videos were analyzed by MATLAB program which simultaneously tracks individual RBC velocity and fluorescent intensity.

The microfluidic-based calcium fluorescent measurement was compared with standard Fluorescence Activated Cell Sorting (FACS) machine as shown in Figure 5.2. Different intracellular calcium concentrations were achieved by treating healthy RBCs with calcium ionophore A23187 which allows the transport of extracellular calcium ions through the otherwise impermeable RBC.
membrane. RBCs were then suspended in Phosphate buffered saline (PBS) buffer containing calcium ions up to 1.8mM for efficient calcium loading. The mean fluorescent intensity measured on microfluidic device (blue circle) was then plotted and calibrated against the FACS data (black square). Sharp increase in intracellular Ca\(^{2+}\) concentrations was observed when RBCs exposed to PBS buffer containing 1.4 – 1.8 mM of Ca\(^{2+}\) ions and the microfluidic based fluorescent measurement was in good agreement with FACS data.

Single RBC deformability vs. intracellular Ca\(^{2+}\) result was plotted in Figure 5.2. Each dot represented one RBC measured. In PBS solution containing 0.4mM of extracellular Ca\(^{2+}\) ions, the Calcium intensities were close to basal level (between 10-20 units) and the corresponding RBC deformabilities were between 10-22 units, whereas in PBS solution containing more than 1.4mM of Ca\(^{2+}\) ions, significant drop of RBC deformability to less than 5 units was observed accompanied with elevated intracellular Ca\(^{2+}\) concentrations around 35 units. It is possible that the sharp RBC deformability decrease was associated with morphological transformations from discocytes to echino-spherocytes.

![Graph](image1)

**Figure 5.2** When loaded with Calcium ionophore, microfluidic based fluorescent measurement was compared and calibrated against standard FACS machine (left). Calcium loading resulted in a reduced RBC deformability (right).
5.3.2 Changes in RBC morphology and deformability during ATP depletion

Irreversible ATP depletion was achieved by treating fresh RBCs with inosine and iodoacetamide. Time dependent changes in the morphology of RBCs were traced over a period of 2 h (Figure 5.3), post-incubation. Approximately 55% of biconcave RBCs were transformed to echino/spherocytes after 2 h of ATP depletion (Figure 5.4).

The morphological transformations may partly due to elevated intracellular Ca\(^{2+}\) concentrations during ATP depletion. Average intracellular Ca\(^{2+}\) concentrations were found to increase from 20.8 to 31.5 units after 1 h of ATP depletion (p<0.0001) and remained fairly stable during the remaining course of the experiment. The increase in Ca\(^{2+}\) level was likely due to
inability of PMCA to extrude Ca$^{2+}$ ion with limited energy source. ATP depletion appeared to have less significant effect on PMCA activity after 1 h of ATP depletion.

![Figure 5. 4 Fraction of biconcave RBCs after ATP depletion](image)

Consistent with the morphological transformations, the average RBC deformability was also found to decrease by 55% (from 2.2 to 1.0) after 2 h of ATP depletion (p<0.01, Figure 5. 5). Additionally, the overall RBC deformability profile changed significantly from unimodal normal to bimodal and eventually heavy tailed. This may suggest inherent heterogeneity of the primary RBC population obtained from donors, potentially due to age of individual RBCs.

![Figure 5. 5 Changes in RBC deformability (left) and intracellular calcium level (right) during ATP depletion](image)
Since average RBC deformability was largely affected by an increase in the fraction of “slow” RBCs, which was quantified by assuming the slowest 5% of RBCs from the control sample. This concept was used previously to explain individual RBCs ability to survive splenic filtration in vivo microcirculation. With a threshold velocity of 1.3 determined using healthy RBCs population (red line in Figure 5.6), the projected percentage of “slow” RBCs after 2 h of ATP depletion was 66.5% (Figure 5.6).

![Graph showing fraction of "slow" RBCs over time]

Figure 5. 6 fraction of "slow" RBCs

5.3.3 ATP depletion on PKC activated RBCs

Impaired RBC deformability during ATP depletion was believed to be associated with increased membrane tension through increased spectrin-membrane binding. PKC activation, on the contrary, favors spectrin-membrane dissociation and consequently decreases membrane tension. We therefore investigated the morphological and mechanical effects of ATP depletion on PKC activated RBCs.
Prior to ATP depletion, RBCs were pre-incubated with PKC activator Phorbol 12-myristate 13-acetate (PMA), PKC inhibitor calphostin C, or phosphate buffer solution (PBS) only (Materials and Methods). After 4 h of ATP depletion, almost all RBCs transformed into echino-spherocytes in both PBS and calphostin C treated groups (Figure 5. 7). In contrast, in the PMA treated group, 41% of RBCs retained their initial discoid shape after ATP depletion (Figure 5. 9).

To further validate the morphological changes were ATP-dependent, a set of positive controls were prepared by treating RBCs with PBS, calphostin C, or PMA in ATP rich buffer. All RBCs were able to retain biconcave shape throughout the course of the experiment (Figure 5. 8).
The change in RBC deformability was consistent with morphological transformations: after 4h ATP depletion comparing to the PBS controls, calphostin C treated RBCs were 17% stiffer, whereas PMA treated RBCs were 37% more deformable (p<0.05, Figure 5.9). The result suggests that PMA treatment may be able to provide a certain level of "mechanical protection" to RBCs during ATP depletion.
5.3.4 PKC activation of banked RBCs

ATP depletion and RBC rigidification are two important biochemical/mechanical markers during RBC storage lesion. Since PKC activation appears to have protective effect during in vitro irreversible ATP depletion, it would be interesting to examine the effect of PMA treatment on old banked RBCs.

Five to six week old banked RBCs (Blood Research Components, ) were first diluted to 1% hematocrit in PBS solution and then treated with PMA or PBS placebo for 30-40 h at 4 °C. The morphology and deformability of PBS- or PMA-treated RBCs were compared. Almost all RBCs suspended in PBS solution became spherocytes whereas 28% of PMA-treated RBCs were still able to retain their original discoid shape (Figure 5.10).

Figure 5.10 PMA treating banked RBCs (morphology)
Parallel experiments measuring RBC deformability found that PBS-treated control RBCs were significantly stiffer as compared to PMA-treated cells (Figure 5.11, $p<0.01$). “Slow” RBC subpopulation was defined cells that traverse below the mean velocity of the PBS treated ATP depleted group. In the PMA-treated samples, only 36% RBCs were determined as “slow” RBCs, significantly lower than 76% “slow” RBCs as seen in the control sample (Figure 5.11).

![Figure 5.11](image.png)

Figure 5.11 Effect of PMA treatment on RBC morphology and deformability
5.4 Discussions and section summary

5.4.1 Spectrin-membrane interaction on whole cell deformability

Given the important clinical and biological relevance of RBC deformability, a lot of effort has been devoted previously to study the ability of RBCs to “change shape under deforming force”\(^{26}\). Conventional single cell measurement tools such as diffraction phase microscopy, optical tweezers, and micropipette aspiration, mainly focused on the membrane properties of RBCs. The interactions between RBC membrane and underlying cytoskeleton are therefore considered to be the dominating factor influencing outcomes in those measurements. However, besides membrane properties, RBCs’ overall ability to pass through narrow blood capillaries and splenic slits is the most relevant for their survival during \textit{in vivo} microcirculation\(^{147}\), which we aim to monitor directly by ‘mimicking’ splenic clearance \textit{in vitro} using microfluidic bottleneck structure. In the previous study\(^{25}\), ATP depletion and PKC activation were both shown to affect spectrin-membrane interactions. The question remains whether whole cell deformability, measured by the probing of cells under flow, would be regulated by the association and dissociation of spectrin-membrane. This is the first study to directly connect spectrin-membrane interaction with whole cell’s ability to survive various clearance / filtration challenges \textit{in vivo}.

Taking into account of additional factors including cell size and shape, the whole cell deformability measurement using a microfluidic platform still showed good consistency with previous studies measuring RBC membrane properties\(^{19, 25}\), although our results are showing single cell level population distribution along with more drastic changes.
5.4.2 PKC pre-activation and RBC deformability

Results in Figure 5.9 suggest that ATP depletion may impact on RBC deformability via PKC related pathways. During ATP depletion, PMA treatment appeared to be able to provide certain level of protection to RBC deformability: PMA treated RBCs, though still less deformable than ATP-rich RBCs, displayed a significantly higher velocity when compared to the control or calphostin C treated RBCs. Besides, PMA-treated RBCs were also less susceptible to morphological transformations: after 4 h ATP depletion, close to half of the PMA-treated RBCs still maintained their biconcave shape whereas almost all RBCs in the control or treated with calphostin C became echinocytes.

The regulatory role of PKC supports conventional belief that RBC deformability is highly dependent on spectrin-membrane interaction \(^{26}\) and is consistent with existing model proposed by Betz et al.: ATP depletion forces 4.1R unphosphorylation and increases spectrin-membrane binding; PKC activation, on the other hand, favors 4.1R phosphorylation and weakens the spectrin-membrane interactions. \(^{21,22,25}\)

However in this study, we provide the first experimental evidence that PKC activation may provide beneficial effect on RBCs during ATP depletion. PKC is commonly known to be associated with cell death following ATP depletion. \(^{139}\) Treating RBCs with PMA, a commonly used PKC activator, was also found to induce phosphatidylserione (PS) exposure \(^{148}\) which typically associate with reduced cell deformability \(^{149}\). In this study, we pretreated fresh RBCs with PMA prior to ATP depletion and found that PKC preactivation could mitigate the detrimental effects of extreme ATP depletion. The exact cellular mechanism remained unclear, but similar protective
effect of PKC preactivation was also observed in cardiomyocytes. It is suggested that PKC preactivation may be able to reduce energy demand and delay ATP depletion.

5.4.3 Changes of single RBC deformability and intracellular Calcium level during ATP depletion

Elevated Ca\(^{2+}\) concentration was observed during irreversible ATP depletion as shown in Figure 5.5. Overall, the increase in bulk Ca\(^{2+}\) level appeared to have a strong correlation with the drop in RBC deformability (Figure 5.12 left, r=-0.9156). However, at single cell level, the correlation between individual RBC deformability and its corresponding intracellular Ca\(^{2+}\) content was fairly weak. Based on the scattered plot in Figure 5.12, the correlation coefficient for RBC deformability and its corresponding Ca\(^{2+}\) concentration was only -0.2875 in the control sample. After 2 h of ATP depletion, the correlation became even weaker with a negative r value of -0.0767. The weak correlation suggests Calcium may not be directly involved in the regulation of RBC deformability.

![Figure 5.12](image_url)

Figure 5.12 Correlation between RBC mean velocity and intracellular Calcium content (left). Scattered plot of single RBC deformability and Calcium content (middle) and the deformability of RBCs with low Calcium content.

To further investigate the changes of RBC deformability and Ca\(^{2+}\) content during ATP depletion, the deformability of the control and ATP depleted RBCs were compared again at
specific calcium intensity intervals. Selecting only a subpopulation of RBCs with Calcium intensity between 19 to 21 units (Figure 5. 12, right), the average RBC deformability in the ATP depleted group was still significantly stiffer than the control group (0.816 vs. 2.29, p<0.001). We also note that, only 15% of control RBCs exhibited calcium intensity above 22 units, which is significantly lower fraction compared to over 80% of ATP-depleted RBCs. Besides, close to 40% of control RBCs in contrast to less than 1% of ATP depleted cells possessed Calcium level below 19 units.

In short, under normal physiological condition, the intracellular Ca\(^{2+}\) concentrations among RBCs are fairly similar (i.e. at basal level). ATP depletion induced a significant increase in both bulk level Ca\(^{2+}\) concentration as well as Ca\(^{2+}\) variations among individual RBCs. At single RBC level, a high Calcium content does not necessarily correlate to poor deformability. Calcium loading alone cannot explain the deformability loss in RBCs during ATP depletion.

Finally, we would also note that the Calcium staining procedure exposed RBCs to a calcium rich buffer and required additional incubation time during sample preparation, both potentially reduced the overall deformability of RBCs. For that reason, we used cell tracker orange for the investigation of PKC activation on RBC deformability.
Chapter 6

6 Conclusion

6.1 Thesis Contribution

With the aid of microfluidic system, we are able to perform high-throughput single cell deformability measurement on RBCs and subsequently provide meaningful biomechanical insights to several real-world clinical and biological problems. More specifically in this thesis, we report 1) the macroscopic correlation between RBC deformability and in vivo blood loss as exemplified by splenic RBC retention; 2) the engineering and mechanical sorting of different RBC "subpopulations" in blood storage lesion; 3) experimental correlation between several biochemical markers (ATP, Ca++ influx) with deformability, demonstrating in vivo biomechanical-biochemical coupling in general RBC circulation.

6.1.1 RBC deformability in Malaria pathogenesis

In the case study of malaria pathogenesis, we attempt to better understand malaria pathology through the mechanical retention of RBC in spleen. Although prior work have suggested the major role of RBC deformability in malaria pathology\(^8\), in vitro measurements have not been directly correlated in vivo with the splenic clearance of RBCs. In this work, we independently studied the in vitro and in vivo impacts of malaria infection and/or drug treatment
on RBC deformability. With the microfluidic platform, the dynamic deformability of RBCs were quantitatively measured. Several important aspects pertaining to anemia and splenic retention were explored.

**Key findings:**

1. Established the correlation between *in vitro* RBC deformability and *in vivo* splenic retention;
2. First *in vivo* demonstration of RBC stiffening and consequently increased splenic retention as a result of malaria infection and/or antimalarial drug treatment;
3. Numerically estimated the mechanical threshold for splenic retention;
4. Relate anemic condition to increased splenic RBC retention both in infected and uninfected mice, with and without drug treatment was evaluated. The results have clear implications on the mechanism of human malarial anemia.

**Future work:** The work provides a new perspective in the understanding of malaria pathogenesis. Potentially we want to employ the platform in the following fields:

1. Drug screening for emerging antimalarials in clinical settings;
2. Apply RBC deformability as a diagnostic marker to understand various anemic conditions suffered by the hosts.
3. Apply RBC deformability as a biomarker in the understanding of malaria parasite virulence.
6.1.2 RBC deformability in blood storage

The project on blood storage lesion focuses on the identification of subpopulations of bad RBCs after prolonged storage time. In this thesis, we identified whether reduced RBC deformability is the shared attribute for this rapidly cleared subpopulation. Moreover, we developed a mechanically pre-sorting system for old stored RBCs for improved blood quality and potentially better clinical outcome.

Key Findings:

1. Identified that RBCs with reduced deformability indeed have higher osmotic fragility, an important clinical parameter associated with higher transfusion risk.

2. Designed and applied a microfluidic-based “mechanical sorter” which filters a “less deformable” RBC subpopulation from 30-42 day-old banked blood. These sorted “less
deformable" blood are confirmed to have higher microparticle content and higher osmotic fragility, both are important clinical parameters associated with increased transfusion risk.

**Future work:** The ultimate goal of the project is to apply our label-free mechanical wash to old banked RBCs prior to transfusion for better clinical outcome. To achieve that, two immediate short term tasks are:

1. Increase the throughput of the "mechanical sorter" so that it could be used for clinical trials. A simple demonstration of such multiplexed high throughput device is shown in Figure 6.1.
2. Perform animal trials on rat to show real clinical benefits of the "mechanical blood wash".

6.1.3 RBC deformability during ATP depletion and PKC activation

RBC's exceptional ability to deform is mainly attributed by its biconcave shape and membrane-cytoskeleton interaction, both believed to be highly regulated by the presence of adenosine 5'-triphosphate (ATP) \(^{19}\). ATP depletion would result in reduced deformability in RBC membrane, possibly through increased membrane-spectrin binding. In this work, we are interested in preserving RBC deformability during ATP depletion through activating Protein Kinase C (PKC) \(^{151}\), which favors membrane-cytoskeleton unbinding. This work sees potential applications in RBC storage lesion: during blood storage, ATP depletion and reduced deformability are two key bio-chemical and -mechanical attributes exhibited by old blood.
Key Findings:

The primary objective of this work is to understand how biochemical energy microscopically influences biomechanical membrane stiffness. A more practical implication of the work is to further improve and optimize current blood storage conditions. The key findings include:

1. Validate, on an *in vitro* microfluidic setting, that irreversible ATP depletion corresponds to an altered RBC shape and reduced whole cell deformability;

2. First demonstration that PKC activation provides certain level of “mechanical protection” on RBC during ATP depletion.

Future work: The ultimate goal of the project is to apply finding #2 to standard banked blood and demonstrate that old banked RBC exhibited improved deformability after PKC activation.
6.2 Other on-going works

6.2.1 RBC deformability during iron overload

Iron metabolism acts as a double-edged sword in human health and disease: on one hand, it is essential for the function of iron-containing cellular proteins, such as the heme group; on the other hand, excess iron could be highly toxic, catalyzing ROS production\(^{153}\). In this thesis, we investigate how iron overload directly impact on RBC deformability. This work can potentially have important implications on several iron deficient or iron overload diseases, including iron deficiency anemia and β-thalassemia.

**Acknowledgement:** The work is in collaboration with Dr. Lihong Liu, a visiting scientist from South Medical University, China.

6.2.2 Deformability and Splenic RBC clearance for different malaria parasite virulence.

The spleen controls the course of rodent malaria partly through mechanical trapping of circulating RBCs\(^{154}\). Lethal and non-lethal rodent malaria strains associate with very different spleen response as evidenced from the size of the spleen. Since one important function of spleen is the mechanical retention of old and abnormal RBCs, we hypothesize that parasite virulence may depend on the mechanical properties of RBCs as well. We believe the rate of clearance of
infected RBCs in spleen may rely on the changes in RBC deformability after parasite infection. Therefore, in this work, we measured the changes in RBC deformability during the course of both lethal and non-lethal parasite infections. We attempted to correlate RBC deformability change with parasitemia growth and spleen response over the post infection days.

**Acknowledgement:** The work is in collaboration with Prof. Peter Preiser and Ms Ximei Huang from Nanyang Technological University, Singapore.
Chapter 7

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