Multiplexed Fluidic Plunger Mechanism for the Measurement of Red Blood Cell Deformability

by

Marie-Eve Myrand-Lapierre

B.ENG, Université Laval, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF APPLIED SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Biomedical Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2014

© Marie-Eve Myrand-Lapierre, 2014

Abstract

Red blood cell (RBC) deformability plays an important role in the pathology of various diseases, including malaria, hemoglobinopathies, and micronutrient deficiencies. Specifically, in malaria, the analysis of RBC deformability presents new approaches for detecting infections and for rapidly evaluating the response to drugs by patients. A key challenge, however, is that the infected RBCs represent only a small subpopulation of clinical blood specimens. Therefore effective detection of infection and analysis require methods that can measure a large number of individual RBCs. Traditional technologies for measuring RBC deformability either cannot evaluate single cells to identify diseased subpopulations or do not have sufficient measurement throughput to detect rare subpopulations. In additions, they require delicate experiments, expensive equipment, and skilled technicians. To address these issues, we developed a new microfluidic mechanism, known as the Multiplexed Fluidic Plunger (MFP), to measure RBC deformability using many microscale-tapered constrictions in parallel. The deformability of each RBC is determined by the threshold pressure required to squeeze the cell through a constriction. Our mechanism overcomes a key challenge where the pressure applied to each cell is dependent on the presence or absence of other cells and thereby produces in an inconsistent measurement result. We devised a mechanism to avoid this error and showed that a consistent measurement is obtained independent of constrictions occupancy. Furthermore, the sensitivity of the MFP device is comparable or superior to existing techniques since it can distinguish control and 0.0005% glutaraldehyde-treated RBCs ($p < 0.005$). The high sensitivity of this mechanism, potential low production cost, and simplified experimental procedures facilitates its application in a clinical context such as in areas where malaria is endemic. Therefore, we further determined the deformability profiles of RBCs parasitized by *P. falciparum* (concentrations ranging from 1-16%). The MFP device was able to detect the deformability of RBCs with a parasitemia as low as 1.8% and can therefore potentially be used to evaluate the parasitemia of infected samples.

Preface

A version of Chapter 1, Chapter 2, Chapter 3, Chapter 5 and Chapter 6 and Chapter 7 are part of a manuscript currently in preparation: Marie-Eve Myrand-Lapierre, Richard R. Ang, Xiaoyan Deng, Kerry Matthews, and Hongshen Ma (2014) Multiplexed Fluidic Plunger Device for the Measurement of Red Blood Cell Deformability. I conducted most of the experiments and wrote the majority of the manuscript. Co-author Richard Ross Ang developed software to analyse the data. Xiaoyan Deng performed the malaria culture, provided the samples for the malaria experiments, and assisted with device fabrication. Finally, Kerry Matthews and my supervisor, Hongshen Ma, proofread the paper.

Chapter 3 describes the final versions of the Multiplexed Fluidic Plunger Device. The silicon masters were fabricated with the help of Sarah McFaul, Aline Teresa Santoso and JeongHyun Lee as described in section 3.1. The soft lithography of these devices was carried out by Xiaoyan Deng and myself, as described in section 3.2.

Chapter 4 introduces the previous versions of the Multiplexed Fluidic Plunger Device. The silicon masters were fabricated with the assistance of Sarah McFaul.

In section 5.1, Kerry Matthews performed some of the experiments of Figure 5.1.

Xiaoyan Deng determined the parasitemia of the malaria infected samples using Giemsa stain for all the experiments of section 6.2 and section 6.3. She also cultured the malaria parasites, carried out the experiments of the purified parasitized RBCs samples (section 6.2), and performed some of the sample preparation with my assistance (section 3.3.3).

Table of Contents

Abstract	ii
Preface.....	iii
Table of Contents	iv
List of Tables	vii
List of Figures	viii
List of Abbreviations.....	xiii
Acknowledgements	xiv
Dedication.....	xv
Chapter 1 Introduction.....	1
1.1 Previous Techniques for Measuring RBC Deformability.....	3
1.1.1 Bulk flow techniques.....	3
1.1.2 Single cell techniques.....	6
1.1.3 Microfluidic techniques	9
1.2 Thesis Goals	13
Chapter 2 Multiplexed Fluidic Plunger Mechanism	15
2.1 Fluidic Plunger Mechanism	15
2.2 Multiplexed Fluidic Plunger	16
2.3 Multiplexing Error	18
2.4 Deformation and Loading Microchannels	20
2.5 Bypass Microchannel	23
2.6 Pressure Attenuator.....	24
2.7 Instrumentation and Throughput Considerations.....	26
2.8 Summary	28

Chapter 3	Materials and Methods.....	29
3.1	Microfabrication	30
3.2	Soft-lithography	33
3.3	Sample Preparation	34
3.3.1	Healthy RBCs	34
3.3.2	Glutaraldehyde (GTA)-treated RBCs	34
3.3.3	Culture of <i>Plasmodium falciparum</i>	35
3.4	Experimental Apparatus	35
3.5	Experimental Protocol	36
3.6	Statistical Analysis	38
Chapter 4	Previous Generations of the MFP Device	39
4.1	First Generation: Two-layer Device with 8 Constrictions	39
4.1.1	First version.....	39
4.1.2	Second version.....	42
4.2	Second Generation: Single-layer Device with 8 Constrictions.....	43
Chapter 5	Design validation	45
5.1	Multiplexing Error	45
5.1.1	Error arising from simultaneous measurements in parallel	45
5.1.2	Error arising from multiple measurements in series	46
5.2	Variability	48
5.2.1	Variability arising from threshold pressure determination	48
5.2.2	Variability arising from device geometry.....	49
5.3	Sensitivity	52
5.4	Throughput	53
Chapter 6	Results: RBC Samples Parasitized by <i>P. Falciparum</i>	55
6.1	Prototype Selection	55
6.2	Purified Parasitized RBCs	57

6.3	Deformability Profile of RBC Samples Parasitized by <i>P. falciparum</i>	58
Chapter 7	Summary and Conclusions	68
7.1	Summary of Thesis	68
7.2	Statement of Impact	69
7.3	Future Work	70
References		71

List of Tables

Table 2.1 Example of natural variability (NV) in different biological systems.....	19
Table 3.1 Resulting thickness obtained with the different photoresists and spin speeds.	31
Table 3.2 Different prototypes and their characteristics.	37
Table 5.1 Summary of the different sources of variability and the corresponding magnitude...	52
Table 5.2 Throughput of the different devices.	54
Table 6.1 Characteristics of the prototypes used for the RBC sample parasitized by <i>P.</i> <i>falciparum</i>	56

List of Figures

Figure 1.1 Graphic representation of (A) human and murine RBCs and (B) microcapillaries and inter-endothelial clefts, as well as (C) characteristics of the human RBCs. <i>Adapted from [1]</i>	1
Figure 1.2 Experimental setup of the Ektacytometer using (A) the couette and (B) the plate-plate system.	4
Figure 1.3 In the micropore filtration technique, the time required for a defined quantity of RBCs to pass through a membrane filter determines their deformability.	5
Figure 1.4 To measure the RBC membrane deformability, individual cells are partially aspirated into a micropipette.	6
Figure 1.5 In the Optical Tweezers technique, two microbeads are attached onto a RBC. A focused laser pulls on one of the beads to cause the RBC to elongate.....	7
Figure 1.6 (A) An AFM tip in contact with one point on the surface of the RBC. (B) A force is applied onto the cantilever, deforming the RBC and bending the cantilever.....	8
Figure 1.7 RBCs move to different locations in the tapered constrictions based on their surface area and volume.	10
Figure 1.8 Top and side sections of (A) a RBC at rest and (B) a RBC deforming through a microscale constriction.	11
Figure 1.9 (A) Overview of the Transit pressure device developed by Guo et al. [28] (B) Magnified view of the row of constrictions	11
Figure 1.10 RBCs have different locations after a period of time since they transit at a different speed through (A) array of triangle-shaped pillars and (B) a capillary network.	13

Figure 2.1 Fluidic plunger mechanism (A) When a cell is not trapped in a constriction, the applied pressure is distributed across the microchannel. (B) When a cell is trapped in a constriction, the applied pressure focuses across the cell.	16
Figure 2.2 A RBC transiting through a constriction for different applied pressure ($P_5 > P_4 > P_3 > P_2 > P_1$).....	16
Figure 2.3 Graphic representation of the RBC loading process and threshold pressure measurement process (A) Cells located in the loading microchannel are loading into the deformation microchannels. (B) Applied pressure waveform. (C) Configuration of streamlines in the loading microchannel when all the constrictions are occupied with cells and its associated hydrodynamic circuit. (D) Configuration of streamlines in the loading microchannel when only one constriction is unoccupied and its equivalent hydrodynamic circuit.	17
Figure 2.4 (A) Equivalent hydrodynamic circuit of the Multiplexed Fluidic Plunger (MFP) Device. (B) Overview of the MFP device. (C) Magnified view of the deformation, loading and bypass microchannels. (D) Pressure in the two loading microchannels (PL) as a function of position. The difference between these pressures is the pressure across the deformation microchannel (PD), which remains constant. (E) 3D model of the loading and deformation microchannels. (F) Schematic representation of the front and side view of a loaded constriction. (G) Micrographs of deformation microchannels near the opening of the constrictions.	18
Figure 2.5 (A) Overview of the fourth device generation. (B) Magnified view of the two inverted device designs with the alignment marks.....	27
Figure 2.6 Interface of the video analysis software developed to identify the threshold pressures of each individual cell.	28
Figure 3.1. Schematic representation of the fabrication process for a positive photoresist.....	29
Figure 3.2 Interface of the constriction size measurement software developed by Richard Ang.	36

Figure 4.1 Overview of the first version of the first generation device.	40
Figure 4.2 Bar graph of the measured threshold pressure for single and multiple cell measurements of the first version of the first generation. The values are normalized to the median of the single cell measurements. $p < 0.05$, $n \geq 27$ for Single and $n \geq 26$ for Multiple.	41
Figure 4.3 Overview of the second device version of the first generation.	42
Figure 4.4 Bar-graph showing no distinction between single and multiple cell measurements of the second version of the first generation device. <i>The values are normalized to the median of the single cell measurements.</i> $p > 0.7$, $n \geq 35$ for Single and $n \geq 30$ for Multiple.	43
Figure 4.5 Bar graph showing no distinction between single and multiple cell measurements of the second version of the second generation. The values are normalized to the median of the single cell measurements. $p > 0.1$, $n \geq 23$ for Single and $n \geq 24$ for Multiple.	44
Figure 5.1 Graphs showing no distinction between single and multiple cell measurements for (A) 4 different paired experiments ($p \geq 0.29$, $n \geq 27$ for Single and $n \geq 91$ for Multiple) and (B) the combination of the 4 different experiments ($p = 0.39$, $n = 114$ for Single and $n = 472$ for Multiple). <i>The values are normalized to the median of the single cell measurements.</i>	46
Figure 5.2 Micrographs of the deformation microchannels showing the different quantities of RBCs present in each microchannels.	47
Figure 5.3 Scatter plot showing similar normalized threshold pressures for deformation microchannels containing different number of RBCs (combination of 12 different experiments). The values are normalized to the median of 2 RBCs per channel. $p = 0.26$, $n \geq 132$	47
Figure 5.4 Scatter plot showing measured threshold pressures of the same healthy RBC sample using devices of the prototype #3 having different constriction size. $n \geq 44$	49

Figure 5.5 Scatter plot showing measured threshold pressures of the same healthy RBC sample using devices of the prototype #4 having different constriction size. $n \geq 31$	51
Figure 5.6 (A) Scatter plot of the combination of 3 experiments showing the measured threshold pressure for the control and varying GTA-treated cultured RBCs ($p < 0.0001$, $n \geq 367$), and (B) bar graph of the results of each experiment for the control and 0.0005% GTA treatment ($p < 0.005$, $n \geq 98$). The values are normalized to the median of the control.	53
Figure 6.1 Bar graphs showing the proportion of RBCs of the infected samples too rigid to be measured with their corresponding parasitemia for the prototype #4. $n \geq 100$ for control and $n \geq 198$ for infected samples.	56
Figure 6.2 Bar graphs showing the proportion of RBCs of the infected samples too rigid to be measured with their corresponding parasitemia for the prototype #7. $n \geq 345$ for control and $n \geq 872$ for infected samples.	57
Figure 6.3 Deformability difference between control and purified parasitized RBCs. Bar graph showing a decrease of deformability of the RBCs in the late-stages of <i>P. falciparum</i> infection (late trophozoite and schizont) for the combination of 3 experiments. The values are normalized to the median of the control. $p < 0.0001$, $n = 177$ for control and $n = 300$ for Purified.	58
Figure 6.4 (A) Histogram, (B) Box plot and (C) Cumulative histogram for the control and infected sample. The values are normalized to the median of the control. $p < 0.0001$, $n = 622$ for control and $n = 1609$ for infected sample, combination of 5 paired experiments, parasitemia = $11 \pm 2\%$	59
Figure 6.5 Scatter plot showing the median of the control RBCs and infected samples having different parasitemia. The values are normalized to the median of the control.	60
Figure 6.6 (A) Histogram and (B) Box plot for the control and infected samples. The values are normalized to their respective median. $p = 0.08$, $n = 622$ for control and $n = 1609$ for infected samples, combination of 5 different experiments, parasitemia = $11 \pm 2\%$...	61

Figure 6.7 (A) Histogram and (B) Box plot for subsets of RBCs having normalized pressure above the cut-off of 1.196 for the control and infected samples. <i>The values are normalized to the respective median of the corresponding samples.</i> $p=0.07$, $n=193$ for control and $n=558$ for infected samples, combination of 5 different experiments, parasitemia= $11 \pm 2\%$	62
Figure 6.8 Scatter plot and logarithmic trendline of the deformability score in function of the parasitemia of the infected samples.	63
Figure 6.9 Histograms and lognormal fit for (A) the control and (B) the infected samples having a parasitemia of 6.4%.....	64
Figure 6.10 Lognormal fit of the control and infected samples having a parasitemia of 6.4% and the resulting iRBCs distribution.	65
Figure 6.11 Simulated mixture distribution for a parasitemia of 10% and the corresponding control and iRBCs contributions.	66
Figure 6.12 Simulated DS vs parasitemia obtained from the control and infected samples distribution for a parasitemia of 6.4%.	67

List of Abbreviations

MFP = Multiplexed Fluidic Plunger Device

RBCs = Red Blood Cells

EI = Elongation Index

DI = Deformability Index

MPA = Micropipette Aspiration

AFM = Atomic Force Microscopy

YM = Young's Modulus

ROI = Region Of Interest

NV = Natural Variability

UV = Ultraviolet

PDMS = Polydimethylsiloxane

PBS = Phosphate Buffered Saline

DI = Deionized

SD = Standard Deviation

n = Number of data

iRBCs = Infected RBCs

uRBCs = Uninfected RBCs exposed to malaria parasites

DS = Deformability Score

Acknowledgements

I would like to first thank my supervisor, Dr. Hongshen Ma, for his patience, support and encouragement. I am glad that he welcomed me in his laboratory. Dr. Ma gives a lot of time to each student, which is always greatly appreciated. He also helped me to develop my skills and define my ambitions and gave me tools for my future career.

Next, I would like to thank NSERC, Engineer in Scrubs and the Department of Mechanical Engineering for their monetary support. Also, I want to thank the granting agencies CIHQ, Bill and Melinda Gates Foundation and Grand Challenges Canada for their contributions.

I also want to thank my colleagues at the Multi-Scale Design Laboratory and in Bio-MEMS. They were all very helpful and supportive. Specially, I want to thank Jackie Chan who first trained me in the fabrication protocols, and Quan Guo who helped me understand the mechanism of his device, which my project was based on. Also, I would like to thank Aline Teresa Santoso and Sunny Zhong who fabricated devices for me when I was away doing experiments in Toronto and San Francisco. The fabrication of the wafers of my devices wouldn't have been possible without the help of Sarah McFaul, Aline Teresa Santoso and JeongHyun Lee who spent a lot of time with me in the cleanroom. I want to thank Kerryn Matthew as well for proofreading this thesis and my paper. I would also like to thank Simon Duffy and Aline Teresa Santoso for proofreading this thesis. I also want to thank Richard Ang, who developed the software that allowed me to save a significant amount of time in the data analysis. Lastly, I would like to thank Xiaoyan Deng for her wonderful patience and great help with the malaria culture.

Finally, I would like to thank my family and close friends for their love and support. I also want to thank the Varsity Outdoor Club at UBC who made my time spent in Vancouver so enjoyable and unforgettable.

*To my family and closest friends
who always supported and encouraged me*

In memory of my Grandmother and Godfather

Chapter 1 Introduction

Red blood cells (RBCs) perform the critical function of transporting oxygen and carbon dioxide between different tissues in our bodies. This capability is enabled in part by their extraordinary mechanical deformability. Specifically, normal discoid shaped RBCs are $8\ \mu\text{m}$ in diameter and $2\ \mu\text{m}$ in thickness, but can repeatedly deform through microcapillaries $<2.5\ \mu\text{m}$, as well as inter-endothelial clefts in the spleen ranging from $0.5\text{--}1\ \mu\text{m}$ (Figure 1.1)[1]. A loss of deformability can result in microvascular occlusion and impairment of blood flow, resulting in tissue necrosis and ultimately, organ failure [2]. Not surprisingly, the loss of RBC deformability is known to be associated with the pathology of many diseases including malaria [3]–[6], hemoglobinopathies (hereditary RBC abnormalities such as sickle cell disease, thalassemia, and G6PD deficiency) [4]–[7], and micronutrient deficiencies [8], [9]. The analysis of RBC deformability presents a potential means to rapidly analyze the status and severity of these diseases in order to select the most appropriate treatment and evaluate treatment efficacy. This thesis focuses on the development of a new microfluidic mechanism for measuring RBC deformability and its application to the detection and analysis of malaria.

Figure 1.1 Graphic representation of (A) human and murine RBCs and (B) microcapillaries and inter-endothelial clefts, as well as (C) characteristics of the human RBCs. *Adapted from [1].*

Malaria is a severe disease caused by a microscopic parasite transmitted by mosquitos that affects more than 200 million people and is responsible for one million deaths annually [10]. The most common and deadly form of this parasite is *Plasmodium falciparum*, which is responsible for ~85% of the cases and nearly all of the deaths. Part of the life cycle of these parasites is spent inside human RBCs where it metabolizes haemoglobin and reproduces asexually [11]. During this process, infected RBCs (iRBCs) are known to become progressively less deformable than unparasitized RBCs. This loss of deformability has been attributed to several causes including chemical modification of the RBC membrane and cytoskeleton resulting from the metabolism of haemoglobin, deposition of parasite proteins onto the RBC membrane, as well as presence of the parasite bodies and digestive vacuole [12], [13]. The distinct deformability of iRBCs presents the potential to detect infections, as well as analyze disease status and drug efficacy.

This thesis describes the development of a novel, sensitive, and multiplexed microfluidic mechanism to measure the deformability of individual RBCs. We demonstrated that this device is able to consistently measure multiple individual RBCs independent of constrictions occupancy while maintaining similar or better sensitivity as other techniques. The sensitivity of the device was compared to existing techniques by measuring the deformability of various concentration of glutaraldehyde(GTA)-treated RBCs. This device was found to have sufficient throughput to detect the presence of rare subpopulations RBCs parasitized by *P. falciparum*.

The subsequent sections of this chapter will first present the previous techniques for measuring RBC deformability. Chapter 2 introduces the design and mechanism of the Multiplexed Fluidic Plunger device. Chapter 3 describes the materials and methods. Afterward, chapter 4 discusses about the previous versions of the final design. Chapter 5 introduces the results for the mechanism validation. Chapter 6 focuses on the application of our mechanism to the measurement of *P. falciparum* parasitized blood sample, and presents the potential of the

device to detect subpopulations. Finally, chapter 7 presents the conclusion and contribution of the thesis research.

1.1 Previous Techniques for Measuring RBC Deformability

This section reviews previous techniques for measuring RBC deformability. Traditional techniques can be divided into bulk flow methods and single cell methods. Bulk flow methods, such as ektacytometry [14]–[16] and micropore filtration [17], [18], provide a measure of the average deformability of thousands of cells. Single cell techniques, such as micropipette aspiration [19], [13], [20], optical tweezers [21]–[23], and atomic force microscopy [24], [25], allow the precise measurement of a few individual cells. Recent advances in microfluidics have produced several new methods for measuring RBC deformability including wedging in tapered constrictions [26], [27], transit pressure through microscale constrictions [28], and transit time through microscale constrictions [45], [46].

1.1.1 Bulk flow techniques

1.1.1.1 Ektacytometer

In ektacytometry, RBCs are suspended in a high viscous medium and flowed in front of a polarized laser beam, as shown in Figure 1.2 [14]–[16]. The RBCs shape and spatial arrangement creates a diffraction pattern collected on a projection screen. To measure the deformability of the RBCs, a shear stress is applied to the RBCs via a rotating apparatus. Ektacytometers use different rotating apparatus, such as the couette and plate-plate systems. In the couette system, the RBC suspension is contained between a stationary cylinder and an outer-rotating cylinder (Figure 1.2A). In the plate-plate system, the suspension is confined between two disks, where the lower one is stationary and the upper one rotates (Figure 1.2B). Under stress, the RBCs elongate and align with the fluid streamlines. The ellipticity of the diffraction pattern changes proportionally with the degree of deformation. The shape of the ellipse characterizes the deformability of the RBCs, expressed as the elongation index (EI). The EI is defined by Equation 1.1, where L is the length of the long axis and W is the length of the short axis of the elliptical diffraction pattern (Figure 1.2A).

Figure 1.2 Experimental setup of the Ektacytometer using (A) the couette and (B) the plate-plate system.

$$EI = \frac{(L - W)}{(L + W)} \quad 1.1$$

The sensitivity of the Ektacytometer is sufficient to measure change in deformability induced by 0.001% glutaraldehyde (GTA) treatment [15]. However, only the average deformability is obtained and therefore, subpopulations can't be distinguished with this technique [31]. In addition, they require the use of expensive and specialized equipment.

1.1.1.2 Micropore filtration

In this technique, the deformability is measured using a membrane filter having pores with a diameter smaller than RBCs (Figure 1.3) [17], [18]. The RBC sample is positioned on one side of the filter and is pushed to the other side via an applied pressure. Poorly deformable RBCs take longer to cross the filter than highly deformable RBCs. The crossing time of the whole sample therefore defines the average RBC deformability, expressed as the deformability index (DI). In the work of Reid et al., the DI is calculated from the volume of the blood sample processed (V), the hematocrit and the time, in minutes, needed to filter the whole sample (Equation 1.2) [17].

A dimensionless value could also be obtained by processing 1 ml of blood. In this case, the Equation 1.2 is divided by 1 ml and multiplied by the equivalent of one minute [18].

Figure 1.3 In the micropore filtration technique, the time required for a defined quantity of RBCs to pass through a membrane filter determines their deformability.

$$DI = \frac{V \times \text{hematocrit}}{\text{time}} \quad 1.2$$

The experimental apparatus needed is simpler than in the case of Ektacytometry. However, several characteristics of the sample condition have a great impact on the measurements. For example, the presence of other blood components such as white blood cells and platelets reduces the filtration rate since they tend to be trapped and therefore clog the membrane filter [31], [32]. These constituents need to be removed in order to get accurate results, which may also affect the RBC population. Also, as in Ektacytometry, Micropore filtration offers no information on the deformability of individual RBCs.

Bulk flow methods provide a measure of the average deformability of thousands of cells, but obscure information on subpopulations of diseased cells [31]. Single cell techniques can therefore overcome this issue by measuring cells individually.

1.1.2 Single cell techniques

1.1.2.1 Micropipette aspiration (MPA)

Micropipette aspiration is a single cell technique used to measure the RBC membrane extensional rigidity or shear modulus (μ) [19], [13], [20]. With this method, individual RBCs are partially aspirated into a micropipette with an internal radius (R_p) smaller than the radius of the RBC (Figure 1.4). It was found that μ is proportional to the derivative of the suction pressure (P) with respect to the protusion length (L), as expressed in Equation 1.3. To find the rate dP/dL , P is gradually increased while L is simultaneously recorded.

Figure 1.4 To measure the RBC membrane deformability, individual cells are partially aspirated into a micropipette.

$$\mu \propto R_p^2 \frac{dP}{dL} \quad 1.3$$

The pressure entry (P_e) is another parameter that can be obtained with this technique. It corresponds to the threshold pressure in which the RBCs are completely aspirated into the micropipette [13]. The acquisition of these different parameters is time consuming and labour intensive. Experienced technicians are thus required to perform the experiments. Also, the throughput of this technique is relatively low. A typical experiment is only able to measure 20 to 40 RBCs [13].

1.1.2.2 Optical tweezers

In this method, the RBC deformability is measured by pulling on silica microbeads attached to the RBC membrane [21]–[23]. The RBC sample is first mixed with a solution containing approximately two microbeads per cell. After an hour of incubation, nonspecific adhesion between the beads and the RBCs then occurs (Figure 1.5). Afterwards, the sample is deposited on a microscope slide and covered by a coverslip. Stationary RBCs, with one microbead fixed on the microscope slide, and one free microbead, are selected for the measurements. Then, a highly focused laser is directed to the free beads. The microbeads are trapped by the laser since they are attracted by its focus point. The attraction force is determined by calibration. Briefly, a free bead is first captured by the focused laser. Next, the laser is moved at different velocity until the bead exit the trap. The force of the trap then corresponds to the known drag force experienced by the bead. To apply a force on the individual RBCs, the laser is moved apart from them. This force causes the elongation of the RBCs. It is incrementally increased until the RBCs exit the trap. At this moment, the force (F), and the transverse (D_t) and axial (D_a) diameters are recorded. From these parameters, the RBC deformation, defined as the elastic shear modulus of the membrane, can be determined by computational simulations [21], [22].

Figure 1.5 In the Optical Tweezers technique, two microbeads are attached onto a RBC. A focused laser pulls on one of the beads to cause the RBC to elongate.

Skilled technician are needed to operate optical tweezer systems because the measurement process is extremely delicate. In addition, the throughput of this technique is similarly low since only 15 to 40 individual RBCs can be measured in a typical experiment [21], [23].

1.1.2.3 Atomic force microscopy (AFM)

In atomic force microscopy measurements, RBC deformability is determined by applying a force on the RBC surface with a sharp tip mounted on a cantilever (Figure 1.6) [24], [25]. The RBCs are initially fixed on a microscope slide via a glass surface treatment. First, the tip of the AFM is brought into contact with individual RBCs (Figure 1.6A). Then, a force is incrementally applied. This force generates a local deformation on the RBC surface and the deflection of the cantilever (Figure 1.6B). The deflection of the cantilever is measured using a laser and a photodetector. The laser reflects off the cantilever and is directed onto a different position on the detector depending on its angle. These deflections are converted into surface positions and are compared with the ones obtained from a hard surface. The difference between the two positions gives the indentation. From the indentation versus force curve, the Young's modulus (YM) of the surface is determined. The mean YM of each RBC is found by measuring several points on the surface of the cell.

Figure 1.6 (A) An AFM tip in contact with one point on the surface of the RBC. (B) A force is applied onto the cantilever, deforming the RBC and bending the cantilever.

Similarly to the other single cell techniques presented, the throughput is very low. Indeed, only 20 RBCs are measured in one experiment. The equipment needed is also costly and the

experimental procedures are done by skilled technicians. In addition, only localized deformation is obtained. Therefore, information about the overall deformability of the RBCs is incomplete.

In summary, single cell techniques typically require complex experiments performed by trained personnel using expensive equipment [33], and thereby cannot easily provide sufficient throughput to profile a large heterogeneous cell population, such as in cases where diseased cells are a subset of the entire population.

1.1.3 Microfluidic techniques

The previously described techniques all require delicate procedures, expensive equipment, and trained personnel to operate. Consequently, they are not suitable for routine clinical analysis or more difficult conditions in areas where malaria is endemic. Microfluidic technologies have the potential to address these challenges by enabling portable, low cost, and disposable sample handling devices. In addition, microfabrication and microfluidics present the potential to create structures at the length scale of single cells, as well as the ability to precisely control the flow of minute volumes of liquid. Recently, there have been significant new developments in microfluidic mechanisms for measuring RBC deformability including those based on wedging in tapered constrictions [26], transit pressure through microscale constrictions [28] and transit time through microscale constrictions [29], [30].

1.1.3.1 Wedging in tapered constrictions

Wedging in tapered constrictions is performed using microfluidic devices containing a row of tapered constrictions (Figure 1.7) [26], [27]. Individual RBCs are suctioned into the constrictions due to a flow that runs parallel to them. Since the cells have different volume and surface area, they reach different positions in the tapered constrictions. Their position and the geometry of the constrictions give information for the calculation of their area-to-volume ratio. This characteristic defines the RBCs deformability.

Figure 1.7 RBCs move to different locations in the tapered constrictions based on their surface are and volume.

This device is easy to operate. However, since the readout of this technique relies on optical measurements of the position of compressed RBCs with sub-micrometer accuracy, the sensitivity of this technique is limited. In addition, this method investigates only the area-to-volume ratio. Therefore, no conclusion can be obtained about the overall deformability of the cells since crucial information might be missing.

1.1.3.2 Transit pressure through microscale constrictions

Transit pressure through microscale constrictions involves measuring the pressure required to squeeze single cells through a microscale constriction (Figure 1.8B). Transit pressure measurements mimic the physiological transport of RBCs through the microvasculature, as well as the mechanism of splenic clearance. Consequently, this process has been shown to be highly sensitive [28].

Figure 1.8 Top and side sections of (A) a RBC at rest and (B) a RBC deforming through a microscale constriction.

This device contains one row of constrictions having different sizes (Figure 1.9B). Therefore, RBCs with different characteristics can be measured with a different constriction size. The cortical tension (T_c), an intrinsic property of the cells, can be calculated from the threshold pressure obtained (P_D) and the geometry of the constrictions used (Equation 1.4 & 1.5). The equation is derived from a model in which the RBCs are considered as liquid drops [34].

Figure 1.9 (A) Overview of the Transit pressure device developed by Guo *et al.* [28] (B) Magnified view of the row of constrictions.

$$T_c = \frac{P_D}{\frac{2}{W_0} - \frac{1}{R_b}} \quad 1.4$$

$$R_b = \sqrt{\frac{\frac{\pi R_0^2 h_0}{H_0} + \frac{W_0^2 \cos\theta}{4 \sin\theta} - \frac{\pi W_0^2}{8}}{\frac{\pi}{2} + \frac{\cos\theta}{\sin\theta}}} \quad 1.5$$

As shown in Figure 1.9A, the row of constrictions is connected in parallel with a short microchannel (section c and d). This microchannel is connected in series with a long microchannel (section a and b). This network allows the attenuation of the applied pressure between the ports a and b.

To perform the measurements, the RBCs need to be individually captured. This process is achieved using valves located on a second PDMS layer [35]. The RBCs are initially introduced into the cell inlet. When a RBC is close to the row of constrictions, valves v1 and v2 are closed to orientate the cell towards the constrictions. Right after the cell enters the row of constrictions, valves v3 and v4 are closed while valves v1 and v2 are simultaneously opened. The pressure drop in the row of constrictions is then controlled by the pressure applied between ports a and b.

This technique is performed by skilled technicians. In addition, specialized equipment is necessary to control the valves. Also, the device fabrication is arduous since it requires a two-layer alignment. Due to the high sensitivity of this technique and the potential for improvement, the work presented in this thesis is based on the work of Guo *et al* [28].

1.1.3.3 Transit time through microscale constrictions

Another way of measuring RBC deformability using microscale constrictions is to measure the transit time of individual RBCs [29], [30]. Different designs of constrictions exist: arrays of triangle-shaped pillars (Figure 1.10A) [29] and a capillary network (Figure 1.10B) [30]. For both

designs, the constrictions are smaller than the diameter of the RBCs. Consequently, RBCs with different deformability will traverse the constrictions with different velocity. In the first device, also called deformability-based flow cytometer, each RBC travels through a row of constrictions (Figure 1.10A) [29]. The deformability is defined by the velocity of the cells. In the second case, the capillary network consists of microchannels that bifurcate into smaller microchannels [30]. The deformability is characterized by the time required for the RBCs to travel between two regions of interest (ROIs). These ROIs are located before and after the smallest microchannels (Figure 1.10B).

Figure 1.10 RBCs have different locations after a period of time since they transit at a different speed through (A) array of triangle-shaped pillars and (B) a capillary network.

Transit time measurements depend on the relaxation of the RBC membrane in response to bending [29]. This process is less sensitive than transit pressure, but has a much higher throughput since the bending time constant for RBC membranes is typically $\ll 1$ s [36]. However, transit time experiments are confounded by clogging, since they rely on pushing many RBCs through a single microscale constriction.

1.2 Thesis Goals

The recent advances presented above have significantly improved the capabilities for the measurement of RBC deformability, but are not suitable to evaluate multiple single RBCs to

analyze heterogeneous populations. To address this issue, this thesis presents the development of a new microfluidic mechanism to perform multiplexed measurements of RBC deformability. This mechanism is designed to be highly sensitive, have sufficient throughput to detect subpopulations of RBCs, and be simple to use and fabricate.

Specifically, the goals of this thesis will be realized through the following steps:

1. Develop the multiplexed fluidic plunger mechanism.
2. Verify the ability of the mechanism to consistently measure RBCs while overcoming potential errors associated with constriction occupancy.
3. Compare the sensitivity of the device to other techniques by measuring various concentration of glutaraldehyde(GTA)-treated RBCs.
4. Determine the RBCs deformability profile of blood samples parasitized by *P. falciparum*.
5. Assess the potential of the device to detect rare subpopulations (typically <10%) present in RBCs samples parasitized by *P. falciparum*.

Chapter 2 Multiplexed Fluidic Plunger Mechanism

This chapter introduces the Multiplexed Fluidic Plunger (MFP) mechanism. Section 2.1 describes the measurement principle of the original fluidic plunger mechanism that uses pressure to push a cell through a micrometer constriction. Section 2.2 explains the design of the MFP. Section 2.3 introduces the multiplexing error, which is a key challenge of the MFP mechanism. Section 2.4 and 2.5 describes the characteristics of the deformation, loading, and bypass microchannels. Section 2.6 presents the pressure attenuator design that allows the application of small pressure on the RBCs. Finally, Section 2.7 introduces the instrumentation and throughput considerations.

2.1 Fluidic Plunger Mechanism

The original fluidic plunger mechanism, developed by Quan Guo from our group, measures single cell deformability using the threshold pressure required to squeeze each cell through a micrometer constriction smaller than the diameter of the cell. This process is analogous to Haine's jump, which describes the transit of droplets through orifices known [37]. In the original fluidic plunger mechanism, the single cell deformability measurement process involves infusing a single cell into a microchannel containing a constriction. Before the cell reaches the constriction, the applied pressure is distributed across the microchannel. Once the cell flows into the constriction, it blocks the flow of liquid and the applied pressure focuses across the cell, effectively acting as a fluidic plunger to remotely push on the cell (Figure 2.1). Varying the applied pressure while observing the position of the cell enables the measurement of the threshold transit pressure. The threshold pressure is typically defined as the pressure recorded at the moment just before the front of the cells start to expand, as shown in Figure 2.2.

Figure 2.1 Fluidic plunger mechanism (A) When a cell is not trapped in a constriction, the applied pressure is distributed across the microchannel. (B) When a cell is trapped in a constriction, the applied pressure focuses across the cell.

Figure 2.2 A RBC transiting through a constriction for different applied pressure ($P_5 > P_4 > P_3 > P_2 > P_1$).

2.2 Multiplexed Fluidic Plunger

The MFP mechanism multiplexes and automates the remote plunger process by using a parallel array of deformation microchannels (Figure 2.3A) and a saw-tooth pressure waveform as shown in Figure 2.3B. Each deformation microchannel contains a constriction for deforming RBCs. The deformation microchannels are connected together on each extremity by the loading microchannels (Figure 2.4C). Bypass microchannels are also located on each side of the deformation microchannel array. These microchannels are connected with the loading microchannels to form a rectangular detour surrounding the array of deformation microchannels (Figure 2.4C). This rectangular detour is connected at two opposing corners to a long pressure attenuator microchannel (Figure 2.4B). The pressure is applied at each extremity of the pressure attenuator microchannel to the inlet and outlet.

Figure 2.3 Graphic representation of the RBC loading process and threshold pressure measurement process (A) Cells located in the loading microchannel are loading into the deformation microchannels. (B) Applied pressure waveform. (C) Configuration of streamlines in the loading microchannel when all the constrictions are occupied with cells and its associated hydrodynamic circuit. (D) Configuration of streamlines in the loading microchannel when only one constriction is unoccupied and its equivalent hydrodynamic circuit.

At the start of the measurement process, single RBCs are loaded into the mouth of each constriction at a low pressure that is insufficient for them to transit. Once the majority of the constrictions are loaded with cells, a saw-tooth pressure waveform is applied while a video simultaneously records the position of the cells. The threshold transit pressure can then be determined by relating the time of transit with the pressure-time data of the saw-tooth waveform.

Figure 2.4 (A) Equivalent hydrodynamic circuit of the Multiplexed Fluidic Plunger (MFP) Device. (B) Overview of the MFP device. (C) Magnified view of the deformation, loading and bypass microchannels. (D) Pressure in the two loading microchannels (P_L) as a function of position. The difference between these pressures is the pressure across the deformation microchannel (P_D), which remains constant. (E) 3D model of the loading and deformation microchannels. (F) Schematic representation of the front and side view of a loaded constriction. (G) Micrographs of deformation microchannels near the opening of the constrictions.

2.3 Multiplexing Error

A key challenge in obtaining a consistent threshold pressure measurement is that the pressure applied across the deformation microchannels (P_D) varies with the number of funnel constrictions occupied with cells. This phenomenon could be understood by considering fluid flow in the following two situations: 1.) When the constrictions contain no cells, streamlines in the loading microchannels are evenly distributed across the deformation microchannels, as shown in Figure 2.3C along with the equivalent hydrodynamic resistance network. 2.) When one or more of the funnel constrictions are occupied with cells that block fluid flow in that channel, streamlines in the loading microchannel are skewed to feed fluid into the remaining unblocked deformation microchannels (Figure 2.3D). The difference between these two situations causes an inconsistency in the flow inside the deformation microchannels, and consequently, the resulting deformation pressure, P_D .

To estimate the potential error in the magnitude of P_D for a device with N deformation microchannels, the worst-case pressure difference is considered, which occurs when the deformation microchannels are occupied with a single cell and when the deformation microchannels are completely occupied with cells. The pressure measured across the deformation microchannels in these two situations can be estimated as follows:

Deformation microchannels occupied with a single cell:

$$P_{D,1} \approx P_{CD} \left(\frac{R_D}{R_D + (N - 1)R_L} \right) \quad 2.1$$

Deformation microchannels completely occupied:

$$P_{D,N} = P_{CD} \quad 2.2$$

where N is the number of parallel deformation microchannels, R_D and R_L is the hydrodynamic resistance of the deformation microchannels and loading microchannel, respectively. The resulting multiplexing error can be estimated as,

$$\text{Multiplexing error} = \frac{P_{D,N}}{P_{D,1}} - 1 = (N - 1) \frac{R_L}{R_D} \quad 2.3$$

Since natural variability of most biological systems are greater than 0.03, it is sufficient to ensure a multiplexing error of <0.03 (Table 2.1). This error can be minimized by maximizing the ratio of R_D/R_L based on a target N . Therefore, the hydrodynamic resistance of the deformation (R_D) and loading microchannels (R_L) must follow the condition of Equation 2.4. The number of deformation microchannels of our design range between 34 and 48. A MFP

prototype having 90 deformation microchannels was also fabricated, which consists of two 45 deformation microchannels designs in series.

$$\frac{R_D}{(N-1)} \gg R_L \quad 2.4$$

Table 2.1 Example of natural variability (NV) in different biological systems.

Biological system	State studied	Technique	NV	Reference
Sickle-cell disease	Steady vs Crisis	Filtration	19-33%	Kenny <i>et al.</i> [7]
Thalassemia	Healthy vs patients	AFM	54%	Dulińska <i>et al.</i> [24]
Iron deficiency	Healthy vs patients	Ektacytometry	11%	Yip <i>et al.</i> [8]
Iron deficiency	Healthy vs patients	Ektacytometry	17%	Vayá <i>et al.</i> [9]
Malaria	uRBCs vs ring stage	Transit Pressure	31%	Guo <i>et al.</i> [28]
Malaria	Healthy vs infected	Rheoscopy	18%	Dobbe <i>et al.</i> [38]
Malaria	Healthy vs uRBCs	MPA	14%	Glenister <i>et al.</i> [12]
Diabete	Healthy vs patients	MPA	50%	McMillan <i>et al.</i> [39]

To validate the theoretical multiplexing error of the designs, Comsol simulations were performed. Both worst-case pressure differences were determined by simulating the flow in the device in the two following situation: 1.) the design contains all the microchannels (estimate of the deformation microchannels occupied with a single cell), and 2.) the design doesn't contain any deformation microchannel (deformation microchannels completely occupied). A pressure of 1 Pa was applied between the inlet and outlet, and the deformation pressure corresponding to the difference of both extremity of each deformation microchannel was determined for both situations. The theoretical multiplexing error was then calculated with Equation 2.3.

2.4 Deformation and Loading Microchannels

The deformation microchannels are a parallel array of long, thin microchannels each containing an anterior funnel-shaped constriction (Figure 2.4C). The thickness of the deformation microchannels is selected to be similar to the thickness of the RBCs to orientate the cells into the planar configuration as they deform through the constrictions. Indeed, if the deformation

microchannel is too thick, the RBCs would rotate to a perpendicular orientation to the plane of the microchannel and could transit through the funnel constriction without creating a temporary seal required for the fluidic plunger effect. Different deformation microchannels thickness (H_0) may be useful for different types of RBCs. For example, normal human RBCs is ideally tested using a microchannel thickness of $\sim 3.0 \mu\text{m}$, while normal murine RBCs is ideally tested using a microchannel thickness of $\sim 2.6 \mu\text{m}$ [1]. When RBC membranes are altered and contain irregular bulges [40], such as during infection with *P. falciparum*, a device with deformation microchannels thickness of $\sim 3.7 \mu\text{m}$ is more appropriate.

To minimize the multiplexing error, the hydrodynamic resistance of the loading microchannels must be small relative to the deformation microchannels (Equation 2.4). Based on Equation 2.3, the hydrodynamic resistance of the loading microchannels (R_L) should be $\leq 0.09\%$ and $\leq 0.06\%$ the hydrodynamic resistance of the deformation microchannels (R_D) to obtain a multiplexing error of $< 3\%$, for the prototypes having 34 and 48 constrictions respectively. Since the thickness of the deformation microchannels (H_0) are set by the geometry of target RBCs, the hydrodynamic resistance of the deformation microchannels can be increased by reducing the width (w) of the microchannels, while increasing the length (L) of the microchannels, as shown in Equation 2.5 where μ is the viscosity of the fluid. The minimum width of the deformation microchannels that can be designed is limited by the RBCs diameter, which is $7.80 \pm 0.62 \mu\text{m}$ [1]. Therefore, a width of $10 \mu\text{m}$ was selected since it is slightly larger than the RBC diameter and the effect on the fluidity of the RBCs in the channels is thus limited. The length of the deformation microchannels was selected to obtain a compromise between the maximization of the hydrodynamic resistance and the time required to load the RBCs into the constrictions. Indeed, the flow of the RBCs inside the deformation microchannels decreases for greater hydrodynamic resistance. In consequence, the loading time increases with the length of the deformation microchannels. For design purposes, a thickness of $4 \mu\text{m}$ is used for the deformation microchannels in order to underestimate their hydrodynamic resistance, and therefore overestimate the associated multiplexing error.

$$R \approx \frac{12\mu L}{wH_0^3(1 - 0.630h/w)} \quad 2.5$$

The loading microchannels are the channels that connect the deformation microchannels together at each extremity, as shown in Figure 2.4C. To minimize the hydrodynamic resistance of these microchannels, they must be designed to have a greater thickness and width (Figure 2.4E). However, the loading microchannels also cannot be too thick since RBCs flowing in the center of the microchannel would have difficulty to enter the deformation microchannels located at the bottom. Also, the aspect ratio of the microchannel thickness to the width must not exceed ~1:10 to prevent microchannel collapse in microfluidic devices fabricated using soft lithography of PDMS (Section 3.2).

To obtain a multiplexing error of <0.03, a loading microchannel having width and thickness of 250 μm and 25 μm and a deformation microchannels having a length of 220 μm was found to be optimal. With these characteristics, the ratio of the hydrodynamic resistance of the loading (R_L) and deformation microchannels (R_D) is of $R_L/R_D=0.04\%$. Therefore, the theoretical multiplexing error calculated from Equation 2.3 is of 0.015 for a design having N=34 deformation microchannels and of 0.021 for a design having N=48 deformation microchannels, which are both < 0.03. For the prototypes having 90 deformation microchannels with two designs having each N=45, the width of the loading microchannel was reduced to 150 μm for the visualization of both arrays of deformation microchannels. The multiplexing error is then 0.034 for this prototype, which is slightly superior to 0.03.

These results are similar to the simulation results obtain using Comsol (Section 2.3). For a prototype having 34 and 48 deformation microchannels, the theoretical multiplexing errors are 0.015 ± 0.002 and 0.025 ± 0.002 respectively. For the prototype having 90 deformation microchannels, the theoretical multiplexing error is 0.028 ± 0.004 , which is less than the target error of 0.03. The uncertainty of the error was calculated from the standard deviation (SD) of the deformation pressure of the different deformation microchannel.

2.5 Bypass Microchannel

The pressure drop across the loading and deformation microchannels (P_{CD}) is controlled by the pressure drop across two bypass microchannels located on each side of the deformation microchannel array (Figure 2.4C). The overall resistance of the bypass microchannels (R_B) is designed to be <1% the overall hydrodynamic resistance of the deformation microchannels. With this requirement, the impact of the constriction occupancy on the equivalent hydrodynamic resistance (R_{eq}) of the bypass and deformation microchannels is limited. With this design, the error on the applied pressure P_{CD} is then estimated by considering the following worst-case pressure difference, where the hydrodynamic resistance of the loading microchannel (R_L) is ignored since it is negligible (<3% of R_D/N , as mentioned in Section 2.4):

Deformation microchannels occupied with a single cell:

$$R_{eq,1} = \frac{R_D R_B}{R_D + (N - 1)R_B} \quad 2.6$$

Deformation microchannels completely occupied ($N \geq 34$):

$$R_{eq,N} = R_B \quad 2.7$$

The error on the deformation pressure P_{CD} (E) is defined by Equation 2.8. For minimal error of <1%, which is much smaller than the natural variability of most of the biological systems, the hydrodynamic resistance of the bypass microchannels (R_L) must be of <1% times the overall hydrodynamic resistance of the deformation microchannels (R_D/N).

$$E = \frac{R_{eq,N}}{R_{eq,1}} - 1 = \frac{(N - 1)R_B}{R_D} \quad 2.8$$

With bypass microchannels having the same thickness and width than the loading microchannels, their combined hydrodynamic resistance (R_B) is only of 0.4%, 0.6% and 0.95% times the overall hydrodynamic resistance of the deformation microchannels (R_D/N) for prototypes having 34, 48 and 90 constrictions respectively. The applied pressure error (E) across the loading and deformation microchannels is then of $\leq 0.95\%$, which is negligible.

2.6 Pressure Attenuator

Typical pressures required to deform single RBCs through a 1.5 to 2 μm funnel-shaped constriction range between 1 to 25 Pa. Such small pressures are extremely difficult to generate reliably using external instrumentation and therefore require additional fluidic infrastructure to produce the necessary pressure on-chip. The pressure divider fluidic circuit, used previously by others [41], produces an attenuated pressure from an external source by attaching a branched microchannel network across a small segment of a long microchannel. The pressure applied across the long microchannel is attenuated by a factor equal to the length ratio of the segment and the long microchannel.

The application of the standard pressure divider fluidic circuit to generate precision pressures across multiple deformation microchannels in parallel is challenged in two key ways. First, the branch microchannel delivering the attenuated pressure to the deformation microchannels is equivalent to the loading microchannel shown in Figure 2.4C, and therefore its hydrodynamic resistance must be minimized to limit the P_D error. Second, since fluid in the loading microchannels must flow into multiple deformation microchannels, different deformation microchannels may experience different pressures based on their spatial position.

These two challenges are addressed using the pressure attenuator structure shown in Figure 2.4B where a fluid stream in a long pressure attenuator microchannel is bifurcated around a rectangular structure containing the parallel deformation microchannels. The two microchannels perpendicular to the deformation microchannels act as the loading microchannels (Figure 2.4C). Whereas the two microchannels parallel to the deformation

microchannels are the bypass microchannels that set the pressure across the deformation microchannels. Importantly, the bifurcation structure compensates for the different positions of the deformation microchannels by providing identical pressure drops in the loading microchannels across the starting and ending locations of the deformation microchannels (Figure 2.4D). Furthermore, the pressure divider ratio is set by the ratio (α) of the hydrodynamic resistance of the bypass microchannels (R_B) and the long microchannel (R_A) (Equation 2.9).

$$\alpha = \frac{R_A}{R_B} + 1 \cong \frac{R_A}{R_B} \quad 2.9$$

To ensure the pressure divider ratio is unaffected by the presence of cells in the deformation microchannels, as previously mentioned the hydrodynamic resistance of the bypass microchannels (R_B) are designed to be <1% of the combined hydrodynamic resistance of the parallel deformation microchannels (R_D/N). With this design, the error on the applied pressure P_{CD} (ϵ) is estimated by considering the following worst-case pressure difference:

Deformation microchannels occupied with a single cell:

$$P_{CD,1} = \frac{P_{AB}}{1 + (E + 1)(\alpha - 1)} \quad 2.10$$

Deformation microchannels completely occupied ($N \geq 34$):

$$P_{CD,N} = \frac{P_{AB}}{\alpha} \quad 2.11$$

For attenuation factor $\alpha \geq 100$, the error on the applied pressure P_{CD} (ϵ), as expressed by Equation 2.12, is then equivalent to the error E (Equation 2.8). Therefore, the error ϵ is $\leq 0.95\%$, which is much smaller than the natural variation of most of the biological systems (Table 2.1).

The hydrodynamic resistance of the deformation (R_D), bypass (R_B) and pressure attenuator microchannels (R_A) must follow the condition of Equation 2.13.

$$\varepsilon = \frac{P_{CD,N}}{P_{CD,1}} - 1 = \frac{E(\alpha - 1)}{\alpha} \cong E \quad 2.12$$

$$0.01 \frac{R_D}{N} \geq R_B \leq 100R_A \quad 2.13$$

2.7 Instrumentation and Throughput Considerations

The instrumentation required for MFP deformability measurements consists of a pressure controller and a microscope and camera. The pressure controller allows the application of a variable pressure difference on each side of individual RBCs. The microscope allows the visualisation of the position of the cell in the different constrictions. For the measurement process, the external applied pressure is incrementally increased while a video is simultaneously captured. A video analysis software was developed by Richard Ang to record the cell position and pressure-time data, and then identify the moment at which each RBC begins to transit through each constriction.

The quantity of deformation microchannels, *i.e.* the number of individual RBC that can be simultaneously measured, was limited by the field of view of the 20X objective of our microscope. Different fabrication attempts were made with different gap sizes between the deformation microchannels. As a result, devices with 34 and 48 channels were successfully fabricated.

Once the proof of concept was established for this design (Section 5.1), a device having two inverted designs, each containing 45 deformation microchannels connected in parallel was designed and fabricated (Figure 2.5A). This new device now has 90 deformation microchannels. Therefore, the throughput of the devices is now nearly doubled.

Figure 2.5 (A) Overview of the fourth device generation. (B) Magnified view of the two inverted device designs with the alignment marks.

For both the single and double designs, dots aligned with the constrictions were added on both sides of the deformation microchannels, as markers for the image recognition video analysis software (Figure 2.5B). The two dots are also aligned with a row of equally spaced dots. For the double designs prototype, more markers are necessary to detect and locate both arrays of constrictions. Thus, circles having known position are present between the two designs. With the help of the dots and circles, the software had the ability to detect the position of the extremity of the different constrictions. With the pixels intensity, the presence of the RBCs at the extremity of the constrictions was identified, and therefore allowing the determination of the moment of transit of each RBC. On the software interface (Figure 2.6), each column represents a different constriction while the vertical axis represents the time. The time is associated with the corresponding pressure of the waveform (Figure 2.3B). Black bands represent the moment where the RBCs start to cross the extremity of the constrictions, while white bands represent the moment where the RBCs exit the constrictions. Therefore, the threshold pressure of each individual RBC can be easily determined by moving the cursor at the corresponding black bands. Only the first RBC that transit in each deformation microchannels is measured.

Figure 2.6 Interface of the video analysis software developed to identify the threshold pressures of each individual cell.

2.8 Summary

In summary, the original fluidic plunger mechanism enabled the remote application of a precisely controlled pressure difference across single cells in a constriction. The MFP enables simultaneous measurement of multiple individual RBCs using a parallel array of funnel constrictions and a saw-tooth pressure waveform. A key challenge of the MFP mechanism is the impact of the constriction occupancy on the deformation pressure, defined as the multiplexing error. The geometry of the deformation and loading microchannels can be selected to minimize multiplexing error to a desired target value. In addition, the specific geometry of the bypass microchannels allows the generation of a precisely controlled pressure on-chip using a pressure attenuator fluidic circuit. Therefore, the threshold pressure of individual RBCs can be measured with precision. The determination of the threshold pressures is possible with the simultaneous capture of a video during the measurement process, and the video analysis software developed by Richard Ang.

Chapter 3 Materials and Methods

This chapter describes the fabrication proofs of the microscale channels required for the MFP device. To fabricate such small features, photolithography (section 3.1) and soft lithography (section 3.2) are carried out, as illustrated in Figure 3.1. Once the devices are fabricated, the different biological samples are prepared, as described in section 3.3. Section 3.4 introduces the experimental apparatus and Section 3.5 defines the experimental procedures. Finally, section 3.6 describes the different statistical analysis performed on the obtained data.

Figure 3.1. Schematic representation of the fabrication process for a positive photoresist.

3.1 Microfabrication

The molds were fabricated on silicon wafers using the photolithography technique (Figure 3.1A). This process consists of printing a pattern on a photoresist with UV light. A photoresist is a light sensitive material that changes of solubility when exposed to UV light. SU-8 (MicroChem, Newton, MA, USA), a negative photoresist, was used to fabricate the MFP device. A negative photoresist becomes insoluble once it is exposed to UV light so that the unexposed part can be dissolved in the corresponding developer, and a positive photoresist reacts in an opposite manner. The desired printed features are then obtained on the photoresist using a mask that reveals the features. A mask may be either an opaque sheet with transparent pattern, or a transparent sheet with an opaque pattern, depending on the type of photoresist used.

The features on the wafer consisted of two layers of different thickness. To fabricate the channels containing the funnels, SU-8 3005 photoresist (MicroChem, Newton, MA, USA) thinned with cyclopentanone was used at a ratio of 0.94:1 or 2:1 by volume. Indeed, the 3000 series have more adherences, which is necessary to successfully fabricate the smallest part of the constrictions. The remaining layer was fabricated in two steps. First, SU-8 2010 or 2015 was used to set new alignment marks since the thin features of the first layer can only be visualized through a second layer with a thickness of less than 20 μm . Second, SU-8 3025 was used to obtain the features. The designs of all three masks were drawn using DraftSight.

The first layer was fabricated on a cleaned 100 mm silicon wafer. The wafer was dehydrated on a hotplate at 200 $^{\circ}\text{C}$ for 5 minutes. The thinned SU-8 3005 was spin-coated at 500 rpm for 10 s followed by another step for 30 s. The speed of this second step is adjusted depending on the layer thickness desired as defined in Table 3.1.

Table 3.1 Resulting thickness obtained with the different photoresists and spin speeds.

Device	Photoresist	Spin Speed (rpm)	Thickness (μm)
Gen 1, v1	SU-8 3005 and Thinner at 0.94:1 ratio	700	3.0 ± 0.1
	SU-8 2015	3000	16 ± 1
	SPR 220-7.0	600	30 ± 1
Gen 1, v2 Gen 2	SU-8 3005 and Thinner at 0.94:1 ratio	800	3.1 ± 0.1
	SPR 220-7.0	500	40 ± 1
Prototype #1	SU-8 3005 and Thinner at 0.94:1 ratio	1400	2.6 ± 0.1
	SU-8 3025	3000	29 ± 1
Prototype #2	SU-8 3005 and Thinner at 2:1 ratio	1400	3.0 ± 0.1
	SU-8 3025	3000	21 ± 1
Prototype #3	SU-8 3005 and Thinner at 2:1 ratio	750	3.0 ± 0.1
	SU-8 3025	3000	22 ± 1
Prototype #4	SU-8 3005 and Thinner at 2:1 ratio	1200	3.7 ± 0.1
	SU-8 3025	3000	23 ± 1
Prototype #5	SU-8 3005 and Thinner at 2:1 ratio	600	4.0 ± 0.1
Prototype #6	SU-8 3025	3000	26 ± 1
Prototype #7	SU-8 3005 and Thinner at 2:1 ratio	600	3.7 ± 0.1
	SU-8 3025	3000	26 ± 1

Next, the wafer was soft-baked on the hot plate at 95 °C for 2 min and exposed to UV light in a mask aligner for 33 s. The exposed wafer was placed on the hotplate for the post-exposure bake in the sequence of 65 °C for 1 min, 95 °C for 1.75 min and 65 °C for 1 min and developed using the SU-8 developer (MicroChem). To stabilize the features on the wafer, it was hard baked on a hotplate by gradually ramping the temperature from 40 °C to 200 °C for 2h40. This final temperature was applied for an hour and the wafer was gradually cooled back to 40 °C.

The second layer of SU-8 was fabricated using a similar process. For the first step, the SU-8 2015 was spin-coated at 500 rpm for 10s followed by 3000 rpm for 30 sec. The sequence of 65 °C for 1 min, 95 °C for 2 min and 65 °C for 1 min was used for the soft-bake. The wafer was then exposed to UV for 55 s, followed by a post-exposure bake with the same sequence as the soft-bake. After the development, the third layer was proceeded right away. SU-8 3025 was spin-coated as before to obtain a layer thickness of $\sim 25 \mu\text{m}$. The sequence of 65 °C for 1 min, 95 °C for 10 min and 65 °C for 1 min was used for the soft-bake. The wafer was then exposed

for 60 s, followed by a post-exposure bake with the sequence of 65 °C for 1 min, 95 °C for 4 min and 65 °C for 1 min. After the development, the wafer was hard baked using the same process as the first layer.

The valve layer requires a rounded shape channel [35]. For this reason, positive SPR 220-7.0 photoresist was selected to fabricate this layer since the top of the resulting channels reflows and forms a round profile during the post exposure bake.

The different photolithography steps for the SPR series are the following:

- 1) Spin-coat photoresist 50 seconds on the silicon wafer with the speed indicated in Table 3.1;
- 2) Allow the wafer to rest for 3 minutes;
- 3) Manually remove the edge bead with a cleanroom wipe;
- 4) Softbake the wafer on a hotplate for 1 minute at 65°C, 3 minutes at 95°C and 1 minute at 65°C;
- 5) Allow the wafer to rest for 3 hours;
- 6) Align the mask with the previous features on the wafer;
- 7) Place the photoresist in contact with the mask;
- 8) Expose the mask with UV light for 2 minutes, in sequences of 30 seconds exposure and 30 seconds pause;
- 9) Allow the wafer to rest for 30 minutes;
- 10) Develop the photoresist for 4-5 minutes with MF 319 developer;
- 11) Post exposure bake the wafer on a hotplate for 1 minute at 65°C, 5 minutes at 95°C and 1 minute at 65°C.

The different photolithography steps for the SU-8 series are the following:

- 1) Clean an 100 mm circular silicon wafer with Acetone, Methanol and Isopropanol;
- 2) Bake the wafer for 5 minutes on a 200°C hotplate to dehydrate it;
- 3) Spin-coat photoresist for 30 seconds on the silicon wafer with the speed indicated in Table 3.1;

- 4) Softbake the wafer for 2 minutes on a 95°C hotplate for SU-8 3005, or for 1 minute at 65°C, 2 minutes at 95°C and 1 minute at 65°C for SU-8 2015;
- 5) When applicable, align the mask with the previous features on the wafer;
- 6) Place the photoresist in contact with the mask;
- 7) Expose the mask with UV light for 45 seconds for SU-8 3005, and 55 seconds for SU-8 2015;
- 8) Post exposure bake the wafer on a hotplate for 1 minute at 65°C, 1.75 minutes at 95°C and 1 minute at 65°C for SU-8 3005, or for 1 minute at 65°C, 2 minutes at 95°C and 1 minute at 65°C for SU-8 2015;
- 9) Develop the photoresist for 1-2 minutes for SU-8 3005 or for 4-5 minutes for SU-8 2015 with SU-8 developer;
- 10) Hard bake the wafer for one hour on a 200°C hotplate (The hotplate temperature must be increased from 40°C to 200°C by 90°C/h, and decreased by 300°C/h)

Once the pattern was fabricated on the silicon wafer, the deformation channel thickness was measured on different positions on the wafer using a profilometer (Alpha Step 200). The thickness is the average of the measurements, while the uncertainty is the SD of the measurements (Table 3.1).

3.2 Soft-lithography

Replicas of the silicon wafer were made using a polyurethane-based plastic (Smooth-Cast ONYX, Smooth-On) as described by Desai et al. [42] First, a PDMS master was fabricated by pouring Sylgard-184 PDMS (Ellsworth Adhesives Canada), at a ratio of 10: 1 (w/w) base to hardener directly onto the silicon wafer (Figure 3.1B). Next, holes were punched for the fluidic reservoirs using a 6 mm hole punch (Technical Innovations, Angleton, TX, USA). The plastic molds were fabricated by mixing both bases of the polyurethane, in equal volumes, and by pouring the mixture over the PDMS master attached to a silicon mold. The mold was allowed to rest at room temperature for an hour, after which, it was baked at 65 °C for 3 hours.

Sylgard-184 PDMS at the same ratio was poured into the molds to fabricate the replica devices. The pre-cured PDMS was degassed in a vacuum dessicator for 15 minutes prior to baking for 2 hours at 65 °C.

To prevent the RBCs from sticking to the glass slide, the device was bonded onto a thin PDMS surface, made by spin-coating RTV615 PDMS (Momentive Performance Material) at a ratio of 10: 1 (w/w) base to hardener, onto a blank or recycled wafer at 1700 rpm for 1 minute. The layer was baked at 65 °C for 1 hour. The device and the PDMS coated wafer were then exposed to oxygen plasma (Model PDC-001, Harrick Plasma) for 75 s and then joined to create a permanent covalent bond between them. To strengthen the bond, the device was further baked for 15 minutes at 65 °C, after which, the resulting device was peeled off and bonded onto a standard microscope slide (Fisher Scientific) using the same process.

3.3 Sample Preparation

3.3.1 Healthy RBCs

Whole blood was collected into 6ml BD EDTA vacutainer tubes from healthy donors after informed consent was obtained. In some cases, a droplet of whole blood was collected using a finger-prick lancet (Unistik 3, Owen Mumford, Fisher). The blood was diluted to 30% in a buffer solution, which consisted of Phosphate Buffered Saline (PBS, Gibco) with 0.2% Pluronic F127 solution (Sigma).

3.3.2 Glutaraldehyde (GTA)-treated RBCs

For device sensitivity experiments, Glutaraldehyde (GTA)-treated RBCs were used. GTA is a common fixative agent, which, in low concentrations, mildly induces cross-linking and stabilization of the proteins in the cell membrane of RBCs [43], [44]. Whole blood was diluted to 5% in PBS and GTA at final concentrations of 0.0005, 0.001, 0.002 and 0.003% was added. The same device was used for each concentration for one experiment. The experiments were repeated three times with different devices.

3.3.3 Culture of *Plasmodium falciparum*

In vitro cultures of *Plasmodium falciparum* were prepared by Xiaoyan Deng as described by Kim, *et al.* [45] Briefly, RBCs were washed, infected with *P. falciparum* (from frozen stock) and incubated in a hypoxic incubator (5% O₂ and 6% CO₂) at 37 °C. The culture was maintained by alternating the daily addition of culture media or fresh RBC samples obtained from CBS NetCAD. The infected samples were then added to the buffer solution (at 30% as described above) for the deformability measurements.

The parasitemia, i.e. the proportion of iRBCs in an infected sample, was measured using Giemsa staining (Sigma-Aldrich) [46]. In summary, the infected samples were spread onto a microscope slide, then fixed with Methanol and washed with Deionized (DI) water. Giemsa staining and PBS were mixed in a 1:5 volume ratio and applied on the RBCs for 20 minutes. The stain was removed and the slide was washed with DI water. The parasitemia was determined by counting ~1000 cells using a 100X oil immersion objective (Nikon).

For purified parasitized RBCs (iRBCs), the *P. falciparum* cultures were first washed with culture media and then added to a LS column (Miltenyl Biotec) surrounded by Neodymium Super Magnets (Applied Magnets) [45]. The sample was collected beneath the column by gravity. The late-stage iRBCs, i.e. the late-trophozoites and schizonts, were held in the column due to the presence of haemozoin (iron-containing by-product of the hemoglobin ingested by the parasite [47]). Next, the column was removed from the magnets and the remaining sample removed from the column by syringe. The iRBCs were added to the buffer solution for the deformability measurements.

3.4 Experimental Apparatus

The MFP device contains sample reservoirs on the chip. Female Luer Lock Connectors (Qosino) were inserted into the reservoirs, forming a water and airtight seal. The Female Luer Lock Connectors were then connected to a pneumatic pressure control system via a Male Luer Lock to Barb Connector (Qosino) and 0.5 mm ID flexible Tygon tubing (Cole-Parmer). The pneumatic control system used was the MFCS-2C (Fluigent SA, Paris, France), which applies a precise

pressure using a closed-loop control at a resolution of 0.25 mbar (25 Pa), with a range of 1000 mbar. The device was mounted on a Nikon microscope and viewed through a 20X objective using a CCD camera (QImaging).

3.5 Experimental Protocol

Before performing an experiment, the size of 10-15 constrictions (W_0 , Figure 2.4F) for each device was measured using software developed in-house by Richard Ang (Figure 3.2). The constrictions were visualized under fluorescence with a 60X objective for better contrast and resolution. The software detected the edges of a constriction and returned the average measured size of 10 frames. The average constriction size of 21 devices was measured twice and the variation was found to be $\sim 0.9\%$. For each prototype of the MFP devices, the average constriction size of the different fabricated devices was determined. Table 3.2 presents the different prototypes and their corresponding characteristics. In this table, the uncertainty of the constriction size corresponds to the SD of the different measured devices.

Figure 3.2 Interface of the constriction size measurement software developed by Richard Ang.

Table 3.2 Different prototypes and their characteristics.

Prototype	Quantity of deformation microchannels	α	H_0 (μm)	W_0 (μm)
1	34	100	2.6 ± 0.1	1.61 ± 0.04
2	48	100	3.0 ± 0.1	1.8 ± 0.1
3	48	1000	3.0 ± 0.1	1.33 ± 0.08
4	34	300	3.7 ± 0.1	2.0 ± 0.1
5	48	1000	4.0 ± 0.1	1.5 ± 0.1
6	34	500	4.0 ± 0.1	1.5 ± 0.1
7	90	600	3.7 ± 0.1	1.77 ± 0.07

To begin an experiment, buffer solution was loaded into the cell outlet reservoir (Figure 2.4B). A pressure (P_{AB}) high enough to fill the entire channel network was applied. Once the network was filled, the RBC sample was placed into the inlet reservoir. Next, the device was calibrated for the threshold pressure determination. To calibrate the device, the external applied pressure (P_{AB}) corresponding to a null flow rate is first determined. P_{AB} is not always equal to zero since a difference in the volume fluid of the reservoirs could affect the applied pressure. To identify this pressure, the inlet pressure is adjusted until the flow ceases while the outlet pressure remains constant. The corresponding inlet pressure is recorded as the calibration pressure and is identified for each set of measurements. During the measurement process, the outlet pressure remains similarly constant while a saw-tooth pressure waveform is applied to the inlet (Figure 2.3B). The measured threshold pressure is then identified as the difference between the inlet pressure and calibration pressure.

Loading more than 80% of the constrictions is accomplished if a sufficient RBC concentration is used. If the concentration is too low, the probability that a RBC is in the correct streamline for entering a constriction is significantly reduced. A 30% dilution of whole blood (~12-15% hematocrit) was found to be sufficient to obtain a loading phase of 2 to 4 minutes. To load the RBCs into the constrictions, it was determined that a low pressure was required; otherwise the RBCs were not in the right streamline to enter the deformation microchannels. Indeed, only a few streamlines are oriented toward the deformation microchannels since they are located at

the bottom of the loading microchannels. The probability is thus low for the RBCs to enter the deformation microchannels since they tend to flow into the center of the microchannels [48]. By reducing the flow velocity into the loading microchannels, the RBCs settle down and form part of the bottom streamlines in order to reach the deformation microchannels. This phenomenon is possible since RBCs have a fast sedimentation rate [49].

When only a small volume of blood is available, 1 μl of the sample is introduced into the outlet reservoir and a reverse pressure is applied to bring the RBCs towards the Inlet channel. Once a fair amount of RBCs reach this latter channel, the measurement procedure was initialized. For a new data set, the outlet reservoir is first emptied and then filled with a new portion of the sample under study.

A video analysis software was developed by Richard Ang to enable efficient and accurate deformability measurements. As shown in Figure 2.6, each deformation channel is represented in a column. A dark strip indicates the beginning of a RBC transit event through a constriction, and a white strip represents the full passage of a RBC through the constriction. Each individual RBC transit can be selected and visualized using the cursor and the corresponding threshold pressure can be determined.

3.6 Statistical Analysis

The constriction size of each device was determined by the mean of the 10-15 measurements. For each set of pressure measurements, the median and interquartile range was calculated. An unpaired t-test was used to compare two groups. When more than two groups of samples were investigated, an ANOVA test was performed. All statistical analysis was done using GraphPad Prism software.

Chapter 4 Previous Generations of the MFP Device

To obtain the final prototypes described in Chapter 2, different designs were tested. The previous designs that lead to the final versions are elaborated in this chapter. A design was considered as being successful when the single and multiple cell measurements were not significantly different, since the multiplexing error would be negligible. Section 4.1 describes the first two-layer design that was based on the successful device developed by Guo, *et al.* [28] Section 4.2 introduces the second generation device that consists of a single-layer design.

4.1 First Generation: Two-layer Device with 8 Constrictions

4.1.1 First version

The goal was to first assess the feasibility of the device regarding the multiplexing error. To assess the theoretical multiplexing error of the different designs, Comsol simulations were performed (Section 2.4). To determine the experimental multiplexing error, single cell measurement (constriction array nearly empty) and multiple cell measurement were performed (constriction array nearly full). The single cell measurements were performed with one cell occupying the constrictions, while the multiple cell measurements were carried out with none or one empty constriction.

A device with 8 channels was initially designed. The first generation design is similar to the Transit pressure device developed by Guo, *et al.*, [28] as shown in Figure 4.1. It has an identical design of the pressure divider and consists of a two layered device. To remain as close as possible as the Transit pressure device (Section 1.1.3.2), the deformation microchannels have the same length. In addition, the width of the pressure attenuator microchannel is 50 μm , and the width of the remaining parts is 200 μm .

Figure 4.1 Overview of the first version of the first generation device.

The flow layer is comprised of three layers of different thickness. When possible, negative SU-8 photoresist was selected since it is easier to use and gives better results. The deformation microchannels layer was fabricated using SU-8 3005 and thinner, and the resulting thickness was $3.0 \pm 0.1 \mu\text{m}$. In order to obtain a multiplexing error less than 0.05, the Pressure Attenuator and the loading microchannels had to have a thickness of $\sim 25 \mu\text{m}$. After an unsuccessful attempt to fabricate this layer, it was discovered that the desired thickness couldn't be achieved since the thin deformation microchannels are not visible underneath the layer. Therefore, the alignment between the two layers is impossible. Instead, SU-8 2015 was used and the resulting thickness of $16 \pm 1 \mu\text{m}$ was reached. Finally, the cell inlet and outlet were made of SPR 220-7.0, and the thickness was $30 \pm 1 \mu\text{m}$.

For the first generation, the device was designed to have 8 channels only. Because of some alignment issues, a 9th channel is connected. Indeed, the mask containing the deformation microchannels pattern was designed with more channels since it was intended to be used for the following versions of the device. With these current parameters, the theoretical multiplexing error obtained by COMSOL simulation was 0.069 ± 0.003 .

To carry out the experiments, the buffer solution is first introduced into the inlets A and B as well as the cell outlet, while the blood sample is added into the cell inlet. Once the RBCs reach the loading microchannels, the valves V1 and V2 are closed. The pressure is then only controlled by the inlets A and B. Next, a high pressure (~300 mbar) is applied to bring the RBCs close to the deformation microchannels. This step is necessary because the velocity of RBCs is very low. Because the cells need to reach the constrictions without passing them, the pressure needs to be decreased to a value below the threshold. With such a low pressure, the loading phase is very slow. To make the multiple cell measurements process faster, a high blood concentration is necessary so the cells can reach the constrictions simultaneously. The blood concentration also has to be low enough so single cell measurements can be performed. A blood concentration of 2% hematocrit was found to be adequate. With this concentration, the constriction loading process took about 5 to 20 minutes.

Seven different blood samples were measured with different devices (Exp1-7, Figure 4.2). The single and multiple cell measurements are significantly different for four of the seven experiments ($p < 0.05$), meaning that the multiplexing error is not negligible (Figure 4.2).

Figure 4.2 Bar graph of the measured threshold pressure for single and multiple cell measurements of the first version of the first generation. *The values are normalized to the median of the single cell measurements.* $p < 0.05$, $n \geq 27$ for Single and $n \geq 26$ for Multiple.

Since the multiplexing error is not negligible, a design with loading microchannels having lower hydrodynamic resistance is next assessed. Therefore, the length of the loading microchannels was reduced and their thickness was increased.

4.1.2 Second version

For the second version, the flow layer was comprised of only two layers of photoresist. Indeed, the Pressure Attenuator and the loading microchannels were now made of SPR 220-7.0 to allow for the fabrication of a thicker layer measuring $40 \pm 1 \mu\text{m}$. In addition, the deformation microchannels, with a thickness of $3.1 \pm 0.1 \mu\text{m}$, are located closer to the Pressure Attenuator (Figure 4.3). Consequently, the length of the portion CD of the Pressure Attenuator is longer. To ensure the hydrodynamic resistance of this portion is low enough compared to the deformation microchannels, the Pressure Attenuator is now $200 \mu\text{m}$ wide. With these parameters, the theoretical multiplexing error obtained by the COMSOL simulation is 0.02 ± 0.01 .

Figure 4.3 Overview of the second device version of the first generation.

The same measurement process was performed for this version as for the first version of the device. Four different blood samples were measured with different devices (Exp1-4, Figure 4.4).

Figure 4.4 Bar-graph showing no distinction between single and multiple cell measurements of the second version of the first generation device. *The values are normalized to the median of the single cell measurements.* $p > 0.7$, $n \geq 35$ for Single and $n \geq 30$ for Multiple.

As shown in Figure 4.4, the single and multiple cell measurements are not significantly different for each sample ($p > 0.7$). This design is then considered to be successful. However, the cell loading process remained very slow, and the multilayer soft-lithography and experimental manipulations require several steps that could be avoided. Consequently, the valves were removed in the next device generation. Therefore, only one inlet and outlet are present and bypass microchannels are introduced.

4.2 Second Generation: Single-layer Device with 8 Constrictions

For this generation, the valves were removed and bypass microchannels on both sides of the rectangular structure containing the constrictions are introduced. The thickness of the deformation microchannels layer and the second layer are $3.1 \pm 0.1 \mu\text{m}$ and $40 \pm 1 \mu\text{m}$ respectively. To increase the speed of the cell loading process, the hydrodynamic resistance of deformation microchannels are reduced. Hence, these channels are designed with a shorter length of $220 \mu\text{m}$. With this design, the theoretical multiplexing error obtained by the COMSOL simulation was 0.014 ± 0.008 .

Four different blood samples were measured with different devices (Exp1-4, Figure 4.5). As shown in Figure 4.5, the single and multiple cell measurements were not significantly different

for each sample ($p>0.1$). The multiplexing error can be considered negligible. Thus, this design demonstrates the validity of the mechanism to avoid the multiple cell error. Consequently, the different prototypes having 34 (prototypes #1, #4 and #6), 48 (prototypes #2, #3 and #5) and 90 (prototype #7) deformation microchannels were designed, fabricated and tested.

Figure 4.5 Bar graph showing no distinction between single and multiple cell measurements of the second version of the second generation. *The values are normalized to the median of the single cell measurements.*

$p>0.1$, $n\geq 23$ for Single and $n\geq 24$ for Multiple.

Chapter 5 Design validation

This chapter reports the validation of the multiplexed fluidic plunger mechanism. Experiments were performed using the prototypes of the devices described in Chapter 2. Section 5.1 introduces the validation of the mechanism by the assessment of the multiplexing error. Sections 5.2 through 5.4 report on the device variability, sensitivity, and throughput respectively.

5.1 Multiplexing Error

5.1.1 Error arising from simultaneous measurements in parallel

One of the key advantages of the MFP mechanism is its ability to simultaneously measure the deformation pressure of multiple individual RBCs. However, as discussed in Section 2.2, the measured threshold pressure can be affected by the constriction occupancy, which is referred to here as the multiplexing error. Based on the design discussed in Section 2.4 and 2.6, this error could be minimized by specifying the hydrodynamic resistances of the deformation, bypass, and loading microchannels. To confirm the effectiveness of this design, the threshold deformation pressure (P_D) was measured at the conditions where the maximum multiplexing error could be observed, *i.e.* with the funnel array nearly empty (single cell measurements) and nearly full (multiple cell measurements). Specifically, the single cell measurements were performed with $\leq 3/34$ or $\leq 3/48$ constrictions loaded with RBCs, and the multiple cell measurements were carried out with $\geq 27/34$ and $\geq 38/48$ constrictions loaded for the prototype #1 and #2 respectively (Table 3.2). Both measurements were performed for the same RBC sample and device, and were repeated 2 times for each prototype. For each repetition, different paired samples and devices were used. This test was carried out on prototypes #1 (Exp1 & 2, Figure 5.1) and #2 (Exp3 & 4, Figure 5.1), which have 34 and 48 deformation microchannels respectively. Similar results were obtained for both. Since the theoretical multiplexing error obtained by Somsol simulations of the prototype #7 is similar to

the prototype having 48 deformation microchannels (Section 2.4), with 0.028 ± 0.004 compared to 0.025 ± 0.002 , no further tests were carried out.

As shown in Figure 5.1, the distributions of results from empty and full funnel arrays can be considered statistically identical ($p=0.39$), thus confirming the validity of our approach to minimize inconsistency between different funnel occupancy states.

Figure 5.1 Graphs showing no distinction between single and multiple cell measurements for (A) 4 different paired experiments ($p \geq 0.29$, $n \geq 27$ for Single and $n \geq 91$ for Multiple) and (B) the combination of the 4 different experiments ($p=0.39$, $n=114$ for Single and $n=472$ for Multiple). *The values are normalized to the median of the single cell measurements.*

5.1.2 Error arising from multiple measurements in series

Since the measurements are performed continuously with the application of the saw-tooth pressure waveform (Figure 2.3B), each deformation microchannel can contain more than one RBC at the same time (Figure 5.2). A potential issue is the impact on the pressure distribution across the deformation microchannel when a measurement on a cell is performed (Figure 2.1B). In theory, the deformation pressure shouldn't be affected, since the other RBCs don't block the fluid and are free to move inside the channel (Figure 2.1). This phenomenon was assessed for deformation microchannels each containing different number of cells during the threshold pressure measurements. In total, 12 different samples were

measured using different devices of the prototype #4. The results for each sample were grouped with the corresponding number of RBCs present in the deformation microchannels. To combine the results from the different samples, the measurements are normalized with the median of the results from the presence of two RBCs per channel (Figure 5.3).

Figure 5.2 Micrographs of the deformation microchannels showing the different quantities of RBCs present in each microchannels.

Figure 5.3 Scatter plot showing similar normalized threshold pressures for deformation microchannels containing different number of RBCs (combination of 12 different experiments). *The values are normalized to the median of 2 RBCs per channel.* $p=0.26$, $n \geq 132$.

As shown in Figure 5.3, the measured threshold pressure was not significantly different for different numbers of RBCs present in each deformation microchannels ($p=0.26$). It can be concluded that the presence of other cells in each deformation microchannels has no effect on the threshold deformation pressure.

5.2 Variability

5.2.1 Variability arising from threshold pressure determination

The determination of threshold deformation pressure for each cell is subject to variability and experimental errors resulting from the definition of cell transit, quantization error in the pressure controller, and pressure offsets within the device.

The deformation of single cells through the funnel-shaped constrictions is determined from manual analysis of the deformation videos. Therefore, a random variation is present for the threshold pressure determination of each RBC. This variation was assessed by evaluating the threshold pressure of the same cells for 10 repetitions. The SD of the variation was found to be of 1%.

Another source of variation for the threshold pressure determination is related to the quantization error in the pneumatic control system that provides the external applied pressure. Threshold pressures with a difference of less than 0.25 mbar cannot be detected. The associated random variation is then 0.12 mbar. For example, the average measured threshold pressure is of 35 mbar for the prototypes #3 of 2.3 mbar for the prototype #4. Therefore, the pressure variation caused by the quantization error in the pneumatic control system corresponds to ~0.4% and ~5% respectively.

Finally, a systematic variation arises from pressure offsets due to the measurements calibration. Indeed, the calibration pressure needs to be identified for each set of measurements to obtain the values of the threshold pressures (Section 3.5). This pressure is sometime difficult to identify since the flow ceases for a range of about 0.5 mbar. Therefore,

the systematic variation due to the pressure offsets is estimated as 0.25 mbar. This variation corresponds to ~0.8% and ~11% for the prototypes #3 and #4 respectively.

5.2.2 Variability arising from device geometry

The soft-lithography fabrication process may produce variability in device geometry. From observations, the magnitude of these variations is usually smaller than 1 μm . Such small changes in the loading microchannels width or thickness will not change the hydrodynamic resistance of the deformation microchannels sufficiently to affect the multiplexing error. However, this variability will affect the geometry of the constriction and therefore the threshold deformation pressure. To estimate this variation, the same healthy RBCs sample was first measured using 21 devices of prototype #3 (Figure 5.4).

Figure 5.4 Scatter plot showing measured threshold pressures of the same healthy RBC sample using devices of the prototype #3 having different constriction size. $n \geq 44$.

The measured threshold pressures (P) obtained with these devices correlate with the constriction size, as shown by Equation 5.1 ($R^2=0.5396$).

$$P = AW_0 + B = -39.688W_0 + 87.253 \quad 5.1$$

The slope of the curve (A) of Figure 5.4 corresponds to Equation 5.2. Therefore with this slope, the pressure variation (ΔP) for a given constriction variation (ΔW_0) can be determined. For the devices of the prototype #3, the SD of the variation of the constriction size is of 0.08 μm . Consequently, based on Equation 5.2, the pressure variation for these devices is of 3 mbar. Compared to the average measured threshold pressure obtained with all the 21 devices, which is of 34 mbar, this variation corresponds to 9%.

$$A = \frac{\Delta P}{\Delta W_0} \quad 5.2$$

The pressure variation must be less than 3% to allow the distinction of most biological systems (Table 2.1). Since the measured threshold pressure variation obtained from the different devices is higher than the minimum accepted variation of 3%, it is important to only select the devices with similar geometry to reduce the pressure variation. Therefore, the acceptable constriction variation to obtain negligible pressure changes can be derived from Equation 5.3. For example, for devices having an average constriction size of 1.3 μm , the acceptable variation ΔW_0 is of 0.03 μm .

$$\Delta W_0 = -\frac{0.03(AW_0 + B)}{A} \quad 5.3$$

The measured threshold pressure varies differently in function of the constriction size for different prototypes. Therefore, the same analysis was performed in 25 devices of the prototype #4 (Figure 5.5). For these devices, the average and SD of the constriction size were of 2.0 and 0.1 μm respectively, and the average measured threshold pressure was of 2.3 μm . With these characteristics, the curve of the measured threshold pressure in function of the constriction size had the parameters $A=-2.3382 \text{ mbar}/\mu\text{m}$ and $B=6.9972 \text{ mbar}$ ($R^2=0.3779$). Consequently, the pressure variation for all the devices is of 10%. Again, when the use of different devices is necessary for the measurements and comparison of different samples, the geometry of the devices must be selected carefully to minimize the pressure variation. When small deformability changes are expected for different cell populations, the same device should be used for the different experiments. If the devices are carefully washed with the buffer solution, a new sample can be introduced. This method is possible for samples measured within a short period of time so as to prevent contamination as well as damage to the device.

Figure 5.5 Scatter plot showing measured threshold pressures of the same healthy RBC sample using devices of the prototype #4 having different constriction size. $n \geq 31$.

The microfabrication processes also introduces variation on constriction size. For the same device, this variation represents only $\sim 3\%$ of the average constriction size. With different devices, the impact of this random error is of 4 and 7% for prototypes #3 and #4 respectively.

In summary, the measured threshold pressure is subjected to variation due to different sources. First, the variation due to the definition of the threshold pressure is random and is estimated as being 1%. The pressure also has a variation of 0.12 and 0.25 mbar due to the quantization error in the pressure controller and the pressure offsets within the device respectively. Finally, the measured threshold pressure varies for devices having different constriction size. The variation of constriction size from device to devices generates a pressure variation of 9 and 10% for the prototypes #3 and #4, respectively. Furthermore, the constriction geometry within the same device also has a variation, with an associated pressure variation of 4 % for the prototype #3 and 7% for the prototypes #4. Table 5.1 Summarize the different uncertainty and their corresponding impact of the measured threshold pressures.

Table 5.1 Summary of the different sources of variability and the corresponding magnitude.

Source	Error type	Magnitude
Threshold pressure identification	Random	1%
Quantization error in the pressure controller	Random	0.12mbar (~0.4% for Prototype #3) (~5% for Prototype #4)
Pressure offsets	Systematic	0.25 mbar (~0.8% for Prototype #3) (~11% for Prototype #4)
Constriction size inter-device	Systematic	~9% (Prototype #3) ~10% (Prototype #4)
Constriction size intra-device	Random	~4% (Prototype #3) ~7% (Prototype #4)

5.3 Sensitivity

The sensitivity of the MFP device was established by measuring the deformability profile of RBC samples treated with small amounts of glutaraldehyde (GTA). GTA is a common fixative agent, which induces cross-linking and stabilization of the proteins in the cell membrane of RBCs [43], [44]. The prototype #4 was selected since the threshold pressure measurements have low values and are therefore subject to the pressure offsets systematic error. The control and GTA

treated-RBCs were measured using the same device. In total, 3 different samples (Exp1-3, Figure 5.6) were tested using different devices, and at least 3 sets of measurements were taken per sample ($N \geq 98$). As shown in Figure 5.6, the MFP device obtained distinguishable deformability profiles of RBCs treated with 0.0005% GTA relative to a control RBC sample ($p < 0.005$), which is similar to or better than ektacytometry and other microfluidic methods [15], [29], [50]. Considering the high sensitivity of the device, it can be concluded that the pressure offsets error as well as the random errors don't affect the overall quality of the results (Table 5.1).

Figure 5.6 (A) Scatter plot of the combination of 3 experiments showing the measured threshold pressure for the control and varying GTA-treated cultured RBCs ($p < 0.0001$, $n \geq 367$), and (B) bar graph of the results of each experiment for the control and 0.0005% GTA treatment ($p < 0.005$, $n \geq 98$). *The values are normalized to the median of the control.*

5.4 Throughput

In a typical experiment, on average 94% of the deformation microchannels are initially loaded with RBCs. The time required to load the constrictions is approximately of 2 to 4 minutes. This loading time is similar for the different prototypes and for devices having different characteristics.

The threshold deformability measurement process requires 1 to 4 minutes, depending on the deformability of the cells. The video acquisition must encompass all the cells that are loaded into the constrictions. Therefore, the least deformable cells require a longer measurement time. Once again, the total measurement time is similar for different prototypes. The rate at which the external applied pressure (P_{AB}) is increased, *i.e.* the slope of the saw-tooth pressure waveform (Figure 2.3B), is adjusted for each device to minimize the loading time without compromising the identification of the transit threshold pressure of the RBCs. Specifically, if the applied pressure is increased too quickly, the individual RBCs wouldn't have the time to reach the extremity of the constriction and thereby introduces an ambiguous threshold transit pressure. The throughput of the different devices is shown in Table 5.2, where the minimum throughput represents the case of a decreased deformability distribution, and the maximum throughput is calculated based on a normal deformability distribution.

Table 5.2 Throughput of the different devices.

Prototype	Quantity of deformation microchannels	Min Throughput (RBCs/hour)	Max Throughput (RBCs/hour)
1, 4, 6	34	275	480
2, 3, 5	48	385	675
7	90	730	1275

Chapter 6 Results: RBC Samples Parasitized by *P. Falciparum*

This chapter evaluates the potential of the MFP device to be used in studies of RBC parasitized by *Plasmodium falciparum*, the primary parasite species responsible for malaria. Section 6.1 discusses device selection. Section 6.2 presents the deformability profile of control unexposed and purified infected RBCs. Section 6.3 introduces a complete analysis of the deformability profile of infected samples at various parasitemia levels.

6.1 Prototype Selection

The sensitivity and the throughput of the MFP device is significant to detect and analyze rare subpopulations of cells in the RBC samples, such as those RBC samples parasitized by *P. falciparum* [13], [28]. The challenge is to select a constriction geometry that allows the measurement of both uRBCs and iRBCs. The values of the measured threshold pressure of the iRBCs must be of less than 450 mbar. Indeed, it is not recommended to apply an external pressure (P_{AB}) greater than 450 mbar since expansion and potential delamination of the PDMS microfluidic devices has been observed. Also, the threshold pressures must not be too low for the uRBCs, otherwise the impact of the systematic pressure offsets error could be significant, and thus the sensitivity of the prototypes could be affected. The characteristics of the designs that could affect the values of the measured threshold pressures are the pressure divider ratio (α), the thickness of the deformation microchannels (H_0) and the constriction size (W_0). The measured threshold pressure is increased by a greater α and smaller H_0 and W_0 . Since the iRBC membranes are altered and contain irregular bulges [40], a deformation microchannels thickness of $\sim 3.7 \mu\text{m}$ was selected.

The prototypes #4 and #7 were selected for the measurements of RBC parasitized by *P. falciparum*. The characteristics of these prototypes are summarized in Table 6.1. For each prototype, experiments were performed where control RBCs and samples infected by *P. falciparum* were separately measured using the same device. Different samples were measured using different devices and the quantity of RBCs too rigid to be measured, *i.e.* having

threshold pressure >450 mbar, was recorded. These RBCs have an attributed threshold pressure value of 600 mbar to be included in the median calculation.

Table 6.1 Characteristics of the prototypes used for the RBC sample parasitized by *P. falciparum*.

Prototype	Quantity of deformation microchannels	α	H_0 (μm)	W_0 (μm)	Threshold pressure (mbar)
4	34	300	3.7 ± 0.1	2.0 ± 0.1	3.0 ± 0.5
7	90	600	3.7 ± 0.1	1.77 ± 0.07	13 ± 6

For the prototype #4, the proportion of RBCs of the infected samples too rigid to be measured represent <10% of the parasitemia (Figure 6.1). With a proportion of <50%, the median of the iRBCs is not affected by the values of the rigid cells. The combination $\alpha=300$ and $W_0=2.0 \pm 0.1 \mu\text{m}$ is thus adequate for the measurements of both uRBCs and iRBCs.

Figure 6.1 Bar graphs showing the proportion of RBCs of the infected samples too rigid to be measured with their corresponding parasitemia for the prototype #4. $n \geq 100$ for control and $n \geq 198$ for infected samples.

The prototype #7 has the combination $\alpha=600$ and $W_0=1.77 \pm 0.07 \mu\text{m}$. With this prototype, the proportion of RBCs too rigid to be measured was >50% of the parasitemia for one of the five infected samples (Figure 6.2). In this situation, the median of the threshold pressures of the iRBCs corresponds now to the cells that were unsuccessfully measured. Consequently, this

prototype is suitable for the detection of iRBCs within an infected sample, but improvement is needed to identify the iRBCs deformability. The prototype #4 is then more suited to obtain the deformability profile of the iRBCs.

Figure 6.2 Bar graphs showing the proportion of RBCs of the infected samples too rigid to be measured with their corresponding parasitemia for the prototype #7. $n \geq 345$ for control and $n \geq 872$ for infected samples.

6.2 Purified Parasitized RBCs

It is well known that *P. falciparum* parasitism reduces the deformability of infected RBCs [13], [16], [28], [38], [51]. However, the deformability of infected RBC is difficult to measure in patient infected samples due to low parasitemia levels. Indeed, the majority of the symptomatic patients have a parasitemia of less than 1% [52]. One of the key advantages of the MFP device is its ability to measure the deformability of a large number of individual cells to establish a deformability profile of the overall cell population. To determine the deformability profile of an infected sample, purified iRBCs and control RBCs were first individually measured using the prototype #4 (Figure 6.3). The control RBCs are uninfected RBCs that have been incubated in the same media as the infected RBC for the same period of time. The purified iRBCs sample consists of only the trophozoites and schizonts (*i.e.* late) stage iRBCs, but not the ring (*i.e.* early) stage iRBCs.

Figure 6.3 Deformability difference between control and purified parasitized RBCs. Bar graph showing a decrease of deformability of the RBCs in the late-stages of *P. falciparum* infection (late trophozoite and schizont) for the combination of 3 experiments. The values are normalized to the median of the control. $p < 0.0001$, $n=177$ for control and $n=300$ for Purified.

As expected, the iRBCs in the late-stages are significantly less deformable (Figure 6.3). Consequently, the presence of a small proportion of this subpopulation in an infected blood sample could be easily detected by our device.

6.3 Deformability Profile of RBC Samples Parasitized by *P. falciparum*

To prove that the MFP device can detect the presence of iRBCs, the same device of the prototype #4 was used to measure *P. falciparum* infected samples compared to unexposed control RBCs. The measurements were first performed using the prototype #4 with 5 different samples with a parasitemia of $11 \pm 2\%$. Figure 6.4 shows the results of the combination of all the data for both the control and infected samples, where the values are normalized to the median of the control sample. As discussed previously, RBCs that were too rigid to be measured, *i.e.* having threshold pressure >450 mbar, were recorded and assigned threshold pressure value of 600 mbar (corresponding to a normalized pressure of ~ 200 in Figure 6.4).

Figure 6.4 (A) Histogram, (B) Box plot and (C) Cumulative histogram for the control and infected sample. The values are normalized to the median of the control. $p < 0.0001$, $n = 622$ for control and $n = 1609$ for infected sample, combination of 5 paired experiments, parasitemia = $11 \pm 2\%$.

In summary, the infected sample was found to have a statistically significant smaller mean RBC deformability compared to the control samples ($p < 0.0001$). A shift in the median measured threshold pressure can be observed in the Figure 6.4A and B. This shift could not be due to the iRBCs only, as pointed out in the cumulative histogram of Figure 6.4C where 50% of the control cells have a normalized threshold pressure of less than 1.0, while only $\sim 20\%$ for the iRBC are in this range. The difference in these proportions is greater than the parasitemia, which indicate that a shift in the uninfected RBCs exposed (uRBCs) to the *P. falciparum* parasitized population exists as well. Therefore, the deformability of the uRBCs, which represent $88 \pm 2\%$ of the total amount of RBC sample, is also affected by the presence of the parasites. This effect had already been observed by others [16], [51], [53], [54]. The release of heme when the schizonts

rupture may explain this reduction in deformability [53]. Indeed, heme has an oxidative effect on the membrane of RBCs. Another possible reason for the alteration of the uRBCs deformability is the release of neoantigens by the parasite [16]. Further investigation is needed to better understand this phenomenon. Infected samples having different parasitemia were measured and compared to the corresponding control sample (Figure 6.5). The measurements were normalized to the median of their respective control. As shown in Figure 6.5, the rigidification of the uRBCs doesn't correlate with the parasitemia of the infected samples ($R^2=0.0299$). Therefore, the rigidified RBCs of the infected samples contain both the iRBCs and the uRBCs. Consequently, the direct comparison of the infected samples to the control samples doesn't give information on the presence of the iRBCs since most of the rigidified RBCs consist of uRBCs in an unpredictable amount.

Figure 6.5 Scatter plot showing the median of the control RBCs and infected samples having different parasitemia. *The values are normalized to the median of the control.* $n \geq 102$ for control and $n = 251$ for infected sample

To identify the presence of the subpopulation of iRBCs in the infected sample, the shift in the uninfected RBCs must be removed to not confound some uninfected RBCs with parasitized ones. The impact of the shift can be partly removed by normalizing the deformability of the infected sample to its own median rather than the control (Figure 6.6). In other words, the

infected samples are now normalized to the uRBCs instead of the control. Consequently, the median of both control and infected samples are now equal to one.

Figure 6.6 (A) Histogram and (B) Box plot for the control and infected samples. *The values are normalized to their respective median.* $p=0.08$, $n=622$ for control and $n=1609$ for infected samples, combination of 5 different experiments, parasitemia= $11 \pm 2\%$.

As shown in Figure 6.6, the deformability profiles of the control and infected samples are now similar. Since only a small proportion of the cells are parasitized, their existence can be potentially detected by looking at the less deformable cells only. Consequently, all the RBCs having a threshold pressure above a certain cut-off were selected for each sample (Figure 6.7). Then, the median of these subsets of measurements was determined. The difference between these medians was defined as the deformability score (DS).

Figure 6.7 (A) Histogram and (B) Box plot for subsets of RBCs having normalized pressure above the cut-off of 1.196 for the control and infected samples. *The values are normalized to the respective median of the corresponding samples.* $p=0.07$, $n=193$ for control and $n=558$ for infected samples, combination of 5 different experiments, parasitemia= $11 \pm 2\%$.

The proportion of iRBCs present in the subsets of RBCs is now increased compared to the initial parasitemia. An optimal cut-off would be as close as possible to the iRBCs deformability profile. With our device, the deformability profile of only the late stages of infection was determined (Figure 6.3). It was observed that the parasitemia of the infected samples was composed of mostly ($\sim 70\%$) ring stages. Therefore, the subset of RBCs having normalized pressures above the cut-off must include the ring-iRBCs. It was not possible to obtain a ring-stage purified sample to measure their deformability profile. However, it has been shown by other studies that the ring-stage parasitized cells are about 1.4 or 3 times less deformable than the uninfected exposed RBCs [28], [51]. Therefore, the cut-off is selected to be between 1.4 and 3. To determine the optimal cut-off, infected samples having parasitemia ranging from 1 to 16% were measured using different devices.

The DS of each infected samples was determined for different cut-off. It was found that for cut-off between 1.15 and 1.25, the DS increases with the parasitemia. This correlation is optimal for

a cut-off of 1.196 (Figure 6.8). Some measured samples were discarded from the analysis since it was found that their sample size (n) was too small. Indeed, to be able to detect the iRBCs, it was observed that the equivalent of at least 25 iRBCs must be measured. Therefore, the required sample size is defined by Equation 6.1. For example, at least 250 RBCs must be measured for an infected sample having a parasitemia of 10%. For the results of Figure 6.8, the sample size were of $n \geq 300$ for samples having parasitemia ranging from 10 to 16%, of $n = 527$ for the sample having 6.4% parasitemia, and $n \geq 2000$ for samples having parasitemia ranging from 1 to 5%. The infected samples having parasitemia ranging from 1 to 5% were measured using the prototype #7. The results of the prototypes #4 and #7 can be compared since all the values of the threshold pressure are compared to a control. In addition, the sample size of the control samples must be greater than 250 RBCs to obtain a sufficient amount of RBCs above the cut-off (≥ 40). For most of the measurements, the sample sizes of the control and infected samples were similar.

Figure 6.8 Scatter plot and logarithmic trendline of the deformability score in function of the parasitemia of the infected samples.

$$n \geq \frac{25}{\text{parasitemia}} \quad 6.1$$

As shown in Figure 6.8, a logarithmic trendline fits the DS and parasitemia data points, with a correlation coefficient of 0.8073, compared to a correlation coefficient of 0.5349 for a linear trendline. To assess the validity of the trendline, Matlab simulations were performed. The objective was first to identify the distribution of the control and iRBCs, and then to combine them at different amounts corresponding to the parasitemia to obtain the mixture distribution of the infected samples. From the control and the mixture distributions, the simulated DS can be derived for different parasitemia. The correlation of the simulated DS versus the parasitemia was then compared to the experimental curve of Figure 6.8.

First, the distributions of the control and infected samples were determined. It was observed that their distribution can be approximated as lognormal. To obtain the parameters of the lognormal distribution, the logarithmic of each value was calculated. From this new distribution, the mean and SD were obtained, which correspond to the parameters μ and σ of the lognormal distribution of Equation 6.2. The resulting lognormal distribution for the control and infected samples having a parasitemia of 6.4% are shown in Figure 6.9.

$$f_x = \frac{1}{x\sigma\sqrt{2\pi}} e^{-\frac{(\ln x - \mu)^2}{2\sigma^2}} \quad 6.2$$

Figure 6.9 Histograms and lognormal fit for (A) the control and (B) the infected samples having a parasitemia of 6.4%.

As previously mentioned, the overall distribution of the iRBCs is unknown since only the deformability profile of the late trophozoite and schizont stages was obtained. To estimate the iRBCs distribution, the distribution of the control sample was subtracted from the infected samples. The resulting distribution was next normalized to obtain an area under the curve of one, as shown in Figure 6.10. The iRBCs of Figure 6.10 contains two peaks, one for the less deformable RBCs and another one for the more deformable RBCs. The latter is possibly due to some control RBCs that were not rigidified by the presence of the parasite. Therefore, they appear to be more deformable than the control since they are now normalized to the uRBCs, which are rigidified.

Figure 6.10 Lognormal fit of the control and infected samples having a parasitemia of 6.4% and the resulting iRBCs distribution.

Next, the parts of the distribution above the optimal cut-off of 1.196 were considered for further analysis. The control and iRBCs distributions were then combined in different proportion based on the parasitemia to obtain the different mixture distributions representing the infected samples. For example, Figure 6.11 presents the mixture distribution of a parasitemia of 10%.

Figure 6.11 Simulated mixture distribution for a parasitemia of 10% and the corresponding control and iRBCs contributions.

From the control distribution and the mixture distributions obtained for each parasitemia, the DS were determined for each parasitemia, as shown in Figure 6.12. The simulated trendline is then linear ($R^2=0.9962$). The same analysis was repeated for the infected samples having parasitemia greater than 5% and their corresponding control sample. Similar curves having a linear trendline were obtained. Therefore, the simulations don't support the logarithmic trendline experimentally obtained (Figure 6.8). However, the iRBCs distributions obtained by simulation are possibly not representative of the real distributions since the expected number of iRBCs in the infected samples is of only 30 to 90. Also, the peak of the more deformable iRBCs is probably not present in the real iRBCs distribution. The distribution obtained is not complete. Therefore, the simulated trendline could be different for different distributions. On the other side, the number of infected samples measured might not be sufficient to observe the linear curve. Indeed, the DS obtained from the same parasitemia could be variable for the RBCs of different donors. Since the measurements were performed with different RBCs samples, a larger amount of samples might be necessary to conclude on the trendline. In summary, no conclusion can be made on the trendline of the DS versus the parasitemia.

Figure 6.12 Simulated DS vs parasitemia obtained from the control and infected samples distribution for a parasitemia of 6.4%.

Based on Figure 6.8, it can still be affirmed that the DS have the tendency to increase with the parasitemia. Therefore, the MFP device can potentially detect the presence of iRBCs for samples with a parasitemia as low as 1.8%. The DS analysis could also be used to study the effect of some drugs on the iRBCs. In this case, the control blood sample could be the untreated infected sample instead of the healthy sample. If the drugs only affect the late-stage iRBCs, the selection of a higher cut-off might be necessary.

Chapter 7 Summary and Conclusions

7.1 Summary of Thesis

This thesis describes the multiplexed fluidic plunger (MFP) mechanism and its application to the measurement of RBC deformability, particularly in the context of RBC parasitized by *P. falciparum* parasite. The device uses a parallel array of micropore constrictions to perform high-throughput assessment of RBC deformability, based on the threshold pressure required to transit the micropores. The RBCs are first loaded into the mouth of the different constrictions. Next, the applied pressure is incrementally increased until all the RBCs transit. A video is simultaneously recorded. The deformability of each RBC can then be determined via video analysis. The deformability obtained from the threshold pressure is consistent even in the presence of other cells in the surrounding constrictions.

The design of the MFP device greatly facilitates its application in a clinical context. The MFP device consists of a single layer of PDMS and therefore does not require valves or alignments with subsequent layers. Also, the arrayed design of microconstrictions in parallel allows for the simultaneous measurement of 90 cells and greatly improves the sample throughput. Furthermore, while other filtration-based strategies are prone to clogging, the use of the surfactant additive minimizes the incidence of clogging and the multiplexed parallel design of the device ensures that a clogged microchannel does not significantly influence the pressure experienced by RBCs in the other microchannels. Based on this simple and efficient design, this microfluidic analysis can be performed without the need for specialized reagents, laboratory equipment or technical expertise.

The MFP device was evaluated by treating RBCs by mild fixation over a range of glutaraldehyde (GTA) concentrations and was found to be sufficiently sensitive to detect changes in RBC deformability, following incubation in GTA concentrations as low as 0.0005%. This high degree of measurement sensitivity prompted the application of this device in the analysis of blood that has been parasitized by the malaria (*P. falciparum*) parasite. RBCs that are parasitized exhibit a significant change in cellular deformability and our MFP technology was able to discriminate

parasitize-exposed from unexposed blood, at a parasitemia as low as 1.8%, which permits detection of malaria parasitism within the biologically relevant range.

Together, the sensitivity and efficacy of this microfluidic technology as well as its elegant design make it highly amenable to a clinical diagnostics. The simple design and minimal requirement for sample processing, specialized equipment and reagents, and technical expertise are all critical factors for the utility of this technology in malaria diagnostics.

7.2 Statement of Impact

Malaria is a severe disease responsible for about one million deaths annually. It is predominantly present in resource poor regions with limited access to medical care. While effective treatments are available, they are often not applied properly, and consequently contributing to the development of drug resistance. The spread of malaria as well as the development of drug-resistant strains can be addressed by rapid diagnosis and effective treatment of the disease. Current diagnostic techniques, such as microscopic analysis of blood, have been proposed but they are expensive and require sensitive biological reagents. RBC deformability has been proposed as a biomarker for malaria parasitism but no one technology has yet demonstrated sufficient sensitivity, simplicity and cost-effectiveness to be effectively applied in these resource-poor settings.

The MFP satisfies all of these requirements and represents a potentially valuable tool for malaria diagnosis. We have demonstrated that it can perform high-throughput and sensitive measurement of RBC deformability. The simple design ensures that the only equipment that is needed is a standard microscope and pressure control system. Since the MFP measures a biomechanical property of the cell and can sample unprocessed blood, there is no need for expensive and sensitive reagents. The low cost of microfluidic chip production makes it ideally suited for malaria diagnosis. Together, application of MFP technology represents a significant advancement over conventional malaria diagnostic strategies. The ability to rapidly diagnose malaria and to monitor the effectiveness of drug treatment can ensure that these outbreaks

remain contained and may potentially reduce the significant morbidity and mortality associated with this disease.

7.3 Future Work

The MFP device is currently being used for a broad range of studies. For example, the effect of anti-malarial drugs on the deformability of iRBCs is currently under investigation. In addition, the oxidative stress effect of hemin on healthy RBC deformability is also being assessed as it represents a potential factor responsible for the change in deformability of the late stages of iRBCs. Furthermore, the throughput and sensitivity of this device is highly suitable for other applications. The MFP device is also being used to monitor RBC deformability changes during prolonged storage in blood bags for use in transfusion medicine. Furthermore, this device will continue to be useful in characterizing various hemoglobinopathies, such as thalassemia's and sickle cell disease.

References

- [1] J. M. Kwan, Q. Guo, D. L. Klyuk-Price, H. Ma, and M. D. Scott, "Microfluidic analysis of cellular deformability of normal and oxidatively damaged red blood cells," *Am. J. Hematol.*, vol. 88, no. 8, pp. 682–689, Aug. 2013.
- [2] S. Yedgar, A. Koshkaryev, and G. Alexander, "The red blood cell in vascular occlusion," *Pathophysiol. Haemost. Thromb.*, vol. 32, no. 5–6, pp. 263–268, 2002.
- [3] B. M. Cooke, N. Mohandas, and R. L. Coppel, "Malaria and the red blood cell membrane," *Semin. Hematol.*, vol. 41, no. 2, pp. 173–188, 2004.
- [4] F. C. Mokken, M. Kedaria, C. P. Henny, M. R. Hardeman, and A. W. Gelb, "The clinical importance of erythrocyte deformability, a hemorrheological parameter," *Ann. Hematol.*, vol. 64, no. 3, pp. 113–122, Mar. 1992.
- [5] G. Y. H. Lee and C. T. Lim, "Biomechanics approaches to studying human diseases," *TRENDS Biotechnol.*, vol. 25, no. 3, pp. 111–118, 2007.
- [6] J. Stuart and G. B. Nash, "Red Cell Deformability and Haematological Disorders," *Blood Rev.*, vol. 4, no. 3, pp. 141–147, 1990.
- [7] M. W. Kenny, M. Meakin, D. J. Worthington, and J. Stuart, "Erythrocyte Deformability in Sickle-Cell Crisis," *Br. J. Haematol.*, vol. 49, no. 1, pp. 103 – 109, 1981.
- [8] R. Yip, M. R. Clark, S. Jain, S. B. Shohet, and P. R. Dallman, "Red cell membrane stiffness in iron deficiency," *Blood*, vol. 62, no. 1, pp. 99–106, 1983.
- [9] A. Vayá, M. Simó, M. Santaolara, J. Todolí, and J. Aznar, "Red blood cell deformability in iron deficiency anaemia," *Clin. Hemorheol. Microcirc.*, vol. 33, no. 1, pp. 75 – 80, 2005.
- [10] R. W. Snow, C. A. Guerra, A. M. Noor, H. Y. Myint, and S. I. Hay, "The global distribution of clinical episodes of *Plasmodium falciparum* malaria," *Nature*, vol. 434, no. 7030, pp. 214–217, Mar. 2005.
- [11] Z. Bozdech, M. Llinás, B. L. Pulliam, E. D. Wong, J. Zhu, and J. L. DeRisi, "The Transcriptome of the Intraerythrocytic Developmental Cycle of *Plasmodium falciparum*," *PLoS Biol.*, vol. 1, no. 1, p. e5, Aug. 2003.
- [12] F. K. Glenister, R. L. Coppel, A. F. Cowman, N. Mohandas, and B. M. Cooke, "Contribution of parasite proteins to altered mechanical properties of malaria-infected red blood cells," *Blood*, vol. 99, no. 3, pp. 1060–1063, 2002.
- [13] G. B. Nash, E. O'Brien, E. C. Gordon-Smith, and J. A. Dormandy, "Abnormalities in the mechanical properties of red blood cells caused by *Plasmodium falciparum*," *Blood*, vol. 74, no. 2, pp. 855–861, 1989.
- [14] Z. Wen, Z. Yan, L. Song, H. Dou, D. Sun, Z. Lü, Y. Shi, and H. Xiao, "Biophysical meanings of orientation and deformation of RBCs in shear flow field of low viscosity with new Ektacytometry," *Sci. China*, vol. 41, no. 2, pp. 195–202, 1998.
- [15] O. K. Baskurt, M. R. Hardeman, M. Uyuklu, P. Ulker, M. Cengiz, N. Nemeth, S. Shin, T. Alexy, and H. J. Meiselman, "Comparison of three commercially available ektacytometers with different shearing geometries," *Biorheology*, vol. 46, no. 3, pp. 251–264, 2009.
- [16] A. M. Dondorp, P. A. Kager, J. Vreeken, and N. J. White, "Abnormal blood flow and red blood cell deformability in severe malaria," *Parasitol. Today*, vol. 16, no. 6, pp. 228–232, 2000.

- [17] H. L. Reid, A. J. Barnes, P. J. Lock, J. A. Dormandy, and T. L. Dormandy, "A simple method for measuring erythrocyte deformability.," *J. Clin. Pathol.*, vol. 29, no. 9, pp. 855–858, Sep. 1976.
- [18] T. L. Berezina, S. B. Zaets, C. Morgan, C. R. Spillert, M. Kamiyama, Z. Spolarics, E. A. Deitch, and G. W. Machiedo, "Influence of Storage on Red Blood Cell Rheological Properties," *J. Surg. Res.*, vol. 102, no. 1, pp. 6–12, Jan. 2002.
- [19] E. Evans, N. Mohandas, and A. Leung, "Static and dynamic rigidities of normal and sickle erythrocytes. Major influence of cell hemoglobin concentration," *J. Clin. Invest.*, vol. 73, no. 2, pp. 477–488, Feb. 1984.
- [20] R. P. Hebbel, A. Leung, and N. Mohandas, "Oxidation-induced changes in microrheologic properties of the red blood cell membrane," *Blood*, vol. 76, no. 5, pp. 1015–1020, 1990.
- [21] J. P. Mills, L. Qie, M. Dao, C. T. Lim, and S. Suresh, "Nonlinear Elastic and Viscoelastic Deformation of the Human Red Blood Cell with Optical Tweezers," *Mech. Chem. Biosyst.*, vol. 1, no. 3, pp. 169–180, 2004.
- [22] M. Dao, C. T. Lim, and S. Suresh, "Mechanics of the human red blood cell deformed by optical tweezers," *J. Mech. Phys. Solids*, vol. 51, no. 11–12, pp. 2259–2280, Nov. 2003.
- [23] S. Hénon, G. Lenormand, A. Richert, and F. Gallet, "A New Determination of the Shear Modulus of the Human Erythrocyte Membrane Using Optical Tweezers," *Biophys. J.*, vol. 76, no. 2, pp. 1145–1151, Feb. 1999.
- [24] I. Dulińska, M. Targosz, W. Strojny, M. Lekka, P. Czuba, W. Balwierz, and M. Szymoński, "Stiffness of normal and pathological erythrocytes studied by means of atomic force microscopy," *J. Biochem. Biophys. Methods*, vol. 66, no. 1–3, pp. 1–11, 2006.
- [25] M. Lekka, M. Fornal, G. Pyka-Fościak, K. Lebed, B. Wizner, T. Grodzicki, and J. Styczeń, "Erythrocyte stiffness probed using atomic force microscope," *Biorheology*, vol. 42, no. 4, pp. 307–317, 2005.
- [26] S. C. Gifford, M. G. Frank, J. Derganc, C. Gabel, R. H. Austin, T. Yoshida, and M. W. Bitensky, "Parallel Microchannel-Based Measurements of Individual Erythrocyte Areas and Volumes," *Biophys. J.*, vol. 84, no. 1, pp. 623–633, 2003.
- [27] T. Herricks, M. Antia, and P. K. Rathod, "Deformability limits of Plasmodium falciparum-infected red blood cells," *Cell. Microbiol.*, vol. 11, no. 9, pp. 1340–1353, Sep. 2009.
- [28] Q. Guo, S. J. Reiling, P. Rohrbach, and H. Ma, "Microfluidic biomechanical assay for red blood cells parasitized by Plasmodium falciparum," *Lab. Chip*, vol. 12, no. 6, pp. 1143–1150, 2012.
- [29] H. Bow, I. V. Pivkin, M. Diez-Silva, S. J. Goldfless, M. Dao, J. C. Niles, S. Suresh, and J. Han, "A microfabricated deformability-based flow cytometer with application to malaria," *Lab. Chip*, vol. 11, no. 6, p. 1065, 2011.
- [30] M. J. Rosenbluth, W. A. Lam, and D. A. Fletcher, "Analyzing cell mechanics in hematologic diseases with microfluidic biophysical flow cytometry," *Lab. Chip*, vol. 8, no. 7, pp. 1062–1070, Jun. 2008.
- [31] J. Stuart, "Erythrocyte rheology.," *J. Clin. Pathol.*, vol. 38, no. 9, pp. 965–977, Sep. 1985.
- [32] P. C. Buchan, "Evaluation and modification of whole blood filtration in the measurement of erythrocyte deformability in pregnancy and the newborn," *Br. J. Haematol.*, vol. 45, no. 1, pp. 97–105, May 1980.

- [33] M. Musielak, "Red blood cell-deformability measurement: Review of techniques," *Clin. Hemorheol. Microcirc.*, vol. 42, no. 1, pp. 47–64, 2009.
- [34] R. M. Hochmuth, "Micropipette aspiration of living cells," *J. Biomech.*, vol. 33, no. 1, pp. 15–22, Jan. 2000.
- [35] M. A. Unger, H.-P. Chou, T. Thorsen, A. Scherer, and S. R. Quake, "Monolithic Microfabricated Valves and Pumps by Multilayer Soft Lithography," *Science*, vol. 288, no. 5463, pp. 113–116, Apr. 2000.
- [36] R. M. Hochmuth, P. R. Worthy, and E. A. Evans, "Red cell extensional recovery and the determination of membrane viscosity.," *Biophys. J.*, vol. 26, no. 1, pp. 101–114, Apr. 1979.
- [37] W. B. Haines, "Studies in the physical properties of soil. V. The hysteresis effect in capillary properties, and the modes of moisture distribution associated therewith," *J. Agric. Sci.*, vol. 20, no. 1, pp. 97–116, 1930.
- [38] J. G. G. Dobbe, M. R. Hardeman, G. J. Streekstra, J. Strackee, C. Ince, and C. A. Grimbergen, "Analyzing red blood cell-deformability distributions," *Blood Cells. Mol. Dis.*, vol. 28, no. 3, pp. 373–384, Jun. 2002.
- [39] D. E. McMillan, N. G. Utterback, and J. L. Puma, "Reduced Erythrocyte Deformability in," *Diabetes*, vol. 27, no. 9, pp. 895–901, Sep. 1978.
- [40] A. Esposito, J.-B. Choimet, J. N. Skepper, J. M. A. Mauritz, V. L. Lew, C. F. Kaminski, and T. Tiffert, "Quantitative imaging of human red blood cells infected with *Plasmodium falciparum*," *Biophys. J.*, vol. 99, no. 3, pp. 953–960, Aug. 2010.
- [41] Q. Guo, S. Park, and H. Ma, "Microfluidic micropipette aspiration for measuring the deformability of single cells," *Lab. Chip*, vol. 12, no. 15, pp. 2687–2695, Aug. 2012.
- [42] S. P. Desai, D. M. Freeman, and J. Voldman, "Plastic masters-rigid templates for soft lithography," *Lab. Chip*, vol. 9, no. 11, pp. 1631–1637, Jun. 2009.
- [43] M. Komorowska, M. Koter, G. Bartosz, and J. Gomułkiewicz, "The effects of glutaraldehyde and osmium tetroxide on the erythrocyte membrane. A spin label study," *Biochim. Biophys. Acta*, vol. 686, no. 1, pp. 94–98, Mar. 1982.
- [44] T. L. Steck, "Cross-linking the major proteins of the isolated erythrocyte membrane.," *J. Mol. Biol.*, vol. 66, no. 2, pp. 295–305, 1972.
- [45] C. C. Kim, E. B. Wilson, and J. L. DeRisi, "Improved methods for magnetic purification of malaria parasites and haemozoin," *Malar. J.*, vol. 9, pp. 17–21, 2010.
- [46] K. Moll, I. Ljungström, H. Perlmann, A. Scherf, and M. Wahlgren, *Methods in Malaria Research*, 5th ed. Manassas: MR4/ATCC, 2008.
- [47] S. Hackett, J. Hamzah, T. M. E. Davis, and T. G. St Pierre, "Magnetic susceptibility of iron in malaria-infected red blood cells," *Biochim. Biophys. Acta BBA - Mol. Basis Dis.*, vol. 1792, no. 2, pp. 93–99, Feb. 2009.
- [48] S. S. Shevkoplyas, T. Yoshida, L. L. Munn, and M. W. Bitensky, "Biomimetic autoseparation of leukocytes from whole blood in a microfluidic device," *Anal. Chem.*, vol. 77, no. 3, pp. 933–937, Feb. 2005.
- [49] X.-B. Zhang, Z.-Q. Wu, K. Wang, J. Zhu, J.-J. Xu, X.-H. Xia, and H.-Y. Chen, "Gravitational sedimentation induced blood delamination for continuous plasma separation on a microfluidics chip," *Anal. Chem.*, vol. 84, no. 8, pp. 3780–3786, Apr. 2012.

- [50] Q. Guo, S. P. Duffy, K. Matthews, A. T. Santoso, M. D. Scott, and H. Ma, "Microfluidic analysis of red blood cell deformability," *J. Biomech.*
- [51] J. P. Mills, L. Qie, M. Dao, K. S. W. Tan, C. T. Lim, and S. Suresh, "Continuous force-displacement relationships for the human red blood cell at different erythrocytic developmental stages of Plasmodium falciparum malaria parasite," *MRS Online Proc. Libr.*, vol. 844, pp. 179–184, 2004.
- [52] A. Moody, A. Hunt-Cooke, E. Gabbett, and P. Chiodini, "Performance of the OptiMAL malaria antigen capture dipstick for malaria diagnosis and treatment monitoring at the Hospital for Tropical Diseases, London," *Br. J. Haematol.*, vol. 109, no. 4, pp. 891–894, Jun. 2000.
- [53] F. Nuchsongsin, K. Chotivanich, P. Charunwatthana, O. S. Fausta, D. Taramelli, N. P. Day, N. J. White, and A. M. Dondorp, "Effects of malaria heme products on red blood cell deformability," *Am. J. Trop. Med. Hyg.*, vol. 77, no. 4, pp. 617–622, 2007.
- [54] F. Omodeo-Salè, A. Motti, N. Basilico, S. Parapini, P. Olliaro, and D. Taramelli, "Accelerated senescence of human erythrocytes cultured with Plasmodium falciparum," *Blood*, vol. 102, no. 2, pp. 705–711, Jul. 2003.