Microfluidic Device for Measuring the Deformability of Single Cells

by

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Abstract

The deformability of single cells can be used as a biomarker to evaluate the status of many diseases including cancer, malaria, and arthritis. Traditional techniques for measuring single cell deformability, such as micropipette aspiration, optical tweezers, and atomic force microscopy, involve delicate experiments performed by highly-skilled technicians using specialized equipment. This thesis presents a new mechanism for measuring the deformability of single cell using the pressure required to deform single cells through a micro-scale constriction. This technique is in principle similar to the micropipette aspiration, but involves considerably simpler operation, is less prone to errors, and requires less specialized equipment and technical skill. The ability of this mechanism to measure single cell deformability is initially verified by testing neutrophils, which demonstrated similar results and measurement precision as micropipette aspiration. Subsequently, this device was used to study the deformability of human red blood cells and specifically, the decrease in deformability of red blood cells parasitized by *Plasmodium falciparum*, the most common species of the parasite that causes malaria. Finally, this device was used to measure the directional asymmetry associated with the deformation of single cells along the direction of the funnel taper and against the direction of the funnel taper. This asymmetry was used to create a microfluidic ratchet to enable unidirectional transport of cells from a fluctuating fluid flow.

Preface

A version of Chapter 7 has been published in *Q. Guo, S.M. McFaul, H. Ma, Deterministic Microfluidic Ratchet Based on the Deformation of Individual Cells, Physical Review E, 83, 051910, 2011.*

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A version of Sections 1.1.2, 2.1 - 2.3, 3.3.1, 3.3.3, and 4.2, as well as Chapter 5 (except Section 5.4) have been submitted as a paper titled, *Microfluidic Micropipette Aspiration for Measuring the Deformability of Single Cells*. This paper has been accepted for publication at *Lab on a Chip* with minor corrections on March 28^{th} , 2012.

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List of Abbreviations

MPA = Micropipette Aspiration AFM = Atomic Force Microscopy PDMS = Polydimethylsiloxane BSA = Bovine Serum Albumin MTC = Magnetic Twisting Cytometry TFM = Traction Force Microscopy CD = Cytoindentation RBCs = Red Blood Cells PCR = Polymer Chain Reaction MLC = Mouse Lymphoma Cell PBMCs = Peripheral Blood Mononuclear Cells PBS = Phosphate Buffered Saline CV = Coefficient of Variance

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Dedication

I dedicate this book to my family because of all the wonderful things they do for me and supporting me all the way.

In memory of my Grandpa

Chapter 1: Introduction

The pathology of many human diseases can be studied using the biomechanical properties of single cells. For example, red blood cells infected by the parasite that causes malaria are known to lose their intrinsic deformability, which prevent them from carrying out their normal physiological function to transport oxygen and carbon dioxide to and from tissues in the body [1-9]. The pathology of cancer development and metastasis can also be studied through the changes of the biomechanical properties of individual cells [4, 8, 10, 11]. Changes in cell morphology, including size, adhesion, and deformability can be observed during the transformation from normal cells into malignant cells. Therefore, mechanical properties, such as deformability, of diseased cells can potentially be used as a biomarker for the assessment of disease progression and the efficacy of treatment.

Existing techniques for studying the mechanical properties of cells measure these properties at the cell population, single cell, and subcellular level. At the *cell population* level, shear flow techniques, such as rheoscope or viscometer, have been used to study changes in the viscosity of blood containing malaria infected red cells [12]. Another technique, known as substrate stretcher, applies static and cyclic forces on populations of chondrocytes (cartilage cells) [13], melanocytes (skin cells) [14], and neurons [15] cells adherent on the substrate to study their biological response to deformation. At the single cell level, cell deformability has been studied using micropipette aspiration (MPA) [16-19] and optical tweezers [20-22]. These techniques have been used to study the deformability of malaria parasite infected RBCs [6, 8, 22-26] as well as the changes in deformability and internal viscosity of malignant cells from non-malignant cells [21, 27, 28]. At the *subcellular* level, a portion of the cell membrane is deformed to study changes in the internal structure of the cell. These techniques include traction force microscopy (TFM) [29, 30], magnetic twisting cytometry (MTC) [31-34], atomic force microscopy (AFM) [10, 35-39] and cytoindentation (CD) [40]. In these techniques, forces on the order of $pN \sim \mu N$ are generated using either beads embedded in the substrate (TFM), engulfed by cells (MTC), or sharp tips at the free end of the cantilever (AFM), or cylindrical-shaped probe (CD).

Microfabrication and microfluidics technologies present capabilities to fabricate structures at the length scale of single cells and to precisely control the flow of minute volumes of liquid. This thesis aims to leverage these capabilities to develop a microfluidic device for measuring the deformability of single cells. Specifically, our goal is to develop a technique that can achieve similar accuracy, precision and sensitivity to the traditional techniques such as micropipette aspiration and optical deformation, but with improved ease of operation and reduced instrumentation requirements. A secondary part of this work is a study of the physical asymmetry in the force required to deform single cells through a tapered constriction both along the direction of the taper and against the direction of the taper. This property enables the creation of a microfluidic ratchet mechanism to deterministically transport and sort cells.

In this chapter, Section 1.1 presents the principle, operation, and drawbacks of traditional cell deformability measurement techniques, including micropipette aspiration and optical tweezers, as well as recently developed microfluidic techniques. Section 1.2 presents the background and motivation for the study of the physical asymmetry associated with deformation through a tapered constriction. Finally, Section 1.3 presents the design concept of our microfluidic device to study single cell deformability and summarizes the research objectives of the thesis.

1.1 Techniques to Study Single Cell Deformability

Previous studies of the deformability of individual cells using traditional techniques, including AFM, MPA, optical tweezers, and optical stretcher, are summarized in Table 1. Each of these studies begins with a measurement of displacement, force, or pressure. These measured results combined with the geometry of the cell are then converted to an intrinsic deformability based on a model of the deformation process. The models used in these studies include the liquid drop model[16, 17, 41, 42] and the solid model (linear elastic model) [10, 36, 43]. A detailed description of the modeling work associated with our device will be presented in the Chapter 3. Descriptions of optical deformation, micropipette aspiration, and microfluidic techniques to study single cell deformability are presented as follows.

Techniques	Deformability	Model	Cell Types	Measurement Parameter Values	References	
MPA	T_c and η	Liquid	Neutrophils	35 pN/um (T _c); 210 ± 100 Pa s (η)	[17]	
MPA	η	Liquid	Neutrophils	135 ± 54 Pa s (η)	[41]	
MPA	T _c	Liquid	Neutrophils	24 ± 3 pN/um (T _c)	[42]	
MPA	T _c	Liquid	Neutrophils	151.7 ± 39.8 Pa s (η)	[44]	
MPA	T _c	Liquid	Neutrophils	27 pN/um (T _c)	[45]	
MPA	T _c	Liquid	Macrophage(J774)	140 pN/um (T _c)	[46]	
MPA	T _c	Liquid	Mouse Fibroblast(L929)	413.6±15.2 pN/um (T _c)	[47]	
AFM	T _c	Liquid	HL60 (Myeloid cells)	155±81 pN/um (T _c)	[36]	
AFM	T _c	Liquid	Jurkat (Lymphoid cells)	21±13 pN/um (T _c)	[36]	
MPA	T _c	Liquid	Lymphocyte	35 pN/um (T _c)	[48]	
MPA	T _c	Liquid	Chicken embryo fibroblasts	~300 pN/um (T _c)	[49]	
AFM	E	Solid (linear elastic model)	Neutrophils	156±87 Pa (E)	[36]	
			HL60 (Myeloid cells)	855±670 Pa (E)	[36]	
			Jurkat (Lymphoid cells)	48±35 Pa (E)	[36]	
AFM	E	Solid (linear elastic model)	MCF-7(breast cancer cells)	N/A	[10]	
AFM	E	Solid (linear elastic model)	A549(lung cancer cells)	260 kPa (E)	[50]	

Table 1 Traditional techniques utilized to study single cell deformability coupled with liquid drop model and solid models
(Cortical Tension T_c ; Young's Modulus E; Viscosity η ; Shear Modulus μ)

Table 1.1 Continued

Techniques	Deformability	Model	Cell Types	Measurement Values	Refs
	Parameter				
AFM	E	Solid (linear elastic model)	HEFC(human embryonic	185 kPa (E)	[50]
			fibroblasts cells)		
AFM	E	Solid (linear elastic model)	Bladder epithelial cells(non-	10~12.9 kPa (E)	[43]
			malignant)		
AFM	E	Solid (linear elastic model)	Bladder cancer cells	0.4 ~ 1.4 kPa (E)	[43]
Optical	μ	Solid (linear elastic model)	Malaria infected red cells	Health RBCs (~8 uN/m);	[22]
Tweezer				Uninfected (~10 uN/m); ring (~18	
				uN/m); Trophozoite (~20 uN/m);	
				Schizont stage RBCs (~55 uN/m)	
MPA	E	Solid (Viscoelastic model)	Regular and malignant mouse	N/A	[27]
			fibroblast		
MPA	μ/recovery	Solid (linear elastic model)	Malaria infected red cells	See paper	[25]
	time/entry				
	time				
MPA	μ	Solid (linear elastic model)	Malaria infected red cells	See paper	[2]
Optical	E	Solid (linear elastic model)	Sickle cell disease	See paper	[51]
Tweezer					
MPA	μ	Solid (linear elastic model)	RBCs and PMS treated RBCs	See paper	[52]
MPA	μ	Solid (linear elastic model)	RBCs and sickle RBCs	See paper	[53]
AFM	μ	Solid (linear elastic model)	Human alveolar (A549) and	Shear modulus under different	[38]
			bronchial (BEAS-	loading forces and frequency	
			2B) epithelial cells		

1.1.1 Optical Deformation

Figure 1.1 shows the operation of the optical deformation techniques, including optical tweezer and optical stretcher. In the case of the optical tweezer [22, 51, 54], dielectric beads are placed on both sides of each cell. One of these beads is attached to glass slide, while the other is controlled by a laser beam. The photon density gradient created by the refraction of a laser beam that enters and exits the bead exerts a restoring force on the bead. Forces applied to the beads are on the order of a few pN up to a few hundred pN with resolution better than 1 pN, which is sufficient to induce deformation of single cells. In the case of optical stretcher [21, 55], instead of using the auxiliary beads attached to the cell surface, two lasers are shone directly on diametrically opposite portions of the cell, which generate forces to deform single cells.



Figure 1.1 Schematic illustration of the principle of optical stretcher (A) and tweezer (B) techniques

Both of these techniques utilize forces generated from focused laser beam without direct contact of cell. However, there are some specific restrictions associated with this type of technique. First of all, not all cell types can be tested. This technique requires that the refractive indices of the trapped objects (either dielectric beads or cells) and surrounding environment to be uniform, and that the biological objects be sufficiently compliant that the laser power required to deform it will not cause damage of radiation. Therefore, cells with anisotropic shape or internal structure, including adherent cells on substrates, are not easy to

analyze via this method. Additionally, in the case of optical tweezer, the attachment of the beads to the cell requires surface treatment of the cell, which may affect the deformability of the cell. Furthermore, for both optical tweezer and optical stretcher, the alignment of the focused laser beam with the cell or the bead is a technically demanding task that not only requires significant skills but is also time consuming. Lastly, the cost of the equipment associated with the experimentation makes this procedure infeasible for routine clinical use.

1.1.2 Micropipette Aspiration (MPA)

Micropipette aspiration (MPA) is a classical technique for measuring the stiffness of individual cells by complete or partial suction into the orifice of a glass micropipette using a small negative pressure [16-19, 45, 56]. The concept of this technique involves drawing a single cell a glass pipette, the inner diameter of which is chosen to induce sufficient deformation of the cell, via stepwise application of negative suction pressure. Compared with optical deformation technique, there is no requirement on the cell shape or symmetry. Using MPA, the intrinsic mechanical properties of aspirated cells can be derived using various models based on the measured relationship between the suction pressure, the diameter of the pipette orifice, cell size, and the protrusion length of the cell in the pipette [17, 19, 45]. Initially, polymorphonuclear leukocytes (also known as granulocytes and mostly composed of neutrophils) were tested with micropipette to determine their biophysical properties such as cortical tension, internal viscosity and so on [16, 17]. Figure 1.2 (A-B) shows the schematic illustration and diagram of micropipette aspiration setup.

Although MPA is conceptually straightforward, it usually involves complicated and specialized equipment and delicate procedures performed by highly skilled technicians. As a result, MPA is mostly viewed as a technique for fundamental biophysical studies and not usually considered as method to assay biological phenomena. The equipment for MPA experiments consist of a precision negative pressure generator, a 3-axis micro-manipulator, and a microscopy imaging system [19, 57, 58]. The measurement process involves initially installing a pipette, the tip of which is aligned with the focal plane of the microscopy system. Each target cell is then located, aligned to the pipette tip, and finally aspirated using a variable negative pressure while observed by microscopy.



Figure 1.2 (A) Schematic illustration of MPA setup; (B) Schematic diagram of the MPA system

A number of technical challenges make MPA experiments both difficult and timeconsuming. First, the aspiration of single cells requires extremely small and precise pressures, typically less than 100 Pa with an adjustment resolution of 0.1 Pa. At this pressure scale, conventional instruments are easily compromised as a result of mechanical vibration, temperature and humidity fluctuation, as well as electronic noise and offsets. Second, the alignment of the pipette tip with the focal plane of the imaging system and the surface of the target cells is a manual process that requires considerable skills and experience. To reduce the mechanical difficulty, the target cell is usually stabilized using another pipette on the other end. Third, MPA experiments are performed in open-air conditions where the sample liquid is continuously lost due to evaporation. This slow volume change results in a drifting baseline of the aspiration pressure that must be corrected by periodic recalibration [19], which, as a result, constrains the time available to aspirate each target cell, as well as the minimum sample volume. Finally, each target cell can only be measured using a single pipette in conventional MPA. As a result, the correct pipette diameter must be selected prior to the experiment and during the experiments.

1.1.3 Microfluidics Techniques

Advances in microfabrication technologies have enabled the fabrication of structures at the length scale of individual cells. Recently, a number of micro-scale structures have been developed on the microfluidic platform to study the deformability of single cells. Table 2 below summarizes the various customized micro-fabricated structures developed to measure single cell deformability, along with the parameters used to characterize intrinsic cell deformability.

As shown in Figure 1.3 (A-D), microstructures such as tapered or wedged long channel [3, 59], funnel filters [60], gap shape constriction [61-63] are created to mimic the in vivo situations of spleen filtration of red cells and red cell circulating in capillaries. In all these cases, they employed either customized parameters, including cell transit time through constrictions[62-64], minimum cylindrical diameter [3] (calculated from measurement of cell surface area and volume) or observed behaviors of the cell under flow shear stress [61] to characterize cell deformability. Using these techniques, blood cells such as erythrocyte and leukocytes deformability are investigated in both normal and diseased states. The diseases include malaria (red cells parasitized by *Plasmodium falciparum*) [3, 60], sepsis[65], and leukostasis [63]. Additionally, similar structures have also been found in the study of cancer cell rigidity, using parameters such as transit/passaging time[66], entry time (time taken for the cell to squeeze through the micro-channel)[64] and transit velocity [66].



Figure 1.3 Schematic illustrations of various micro-scale constructions to cause deformation of single suspended cells either through direct contact of cells with microconstrictions (A-D) or via converging streamlines where the cells are in the center of the flows (E,F)

Another way to deform cells using microfluidics involves the creation of converging streamlines where the cell at target is in the center of the two streamlines instead of direct contact between cell and microstructure (Figure 1.3 E, F). Specifically, hyperbolic shape [65] and cross-road shape structures [67] were developed to create extensional flow that causes cell deformation. In these designs, deformation index defined as the ratio of both axis of cross-sectional area of a deformed cell was used to represent and compare the deformability of single cells.

Table 2 Microfluidic devices designed to study single cell deformability

Microfluidic	Parameters Measured	Cell type analyzed	References
Structure			
Wedge shaped	Area, volume, Hemoglobin	Reticulocytes (immature red cells)	[59]
micro-channel			
Capillary	Transit time	Red cell and neutrophils extracted	[63]
structure		from Sepsis and leukostasis disease	
		patients	
Wedge shape	Minimum cylindrical diameter (cell surface area and volume	Red cell parasitized by Plasmodium	[3]
capillary		falciparum	
Hyperbolic	Deformation Index (extensional flow induced deformation)	Red blood cell	[65]
shaped micro-			
channel			
long pinched	Percentage of cell tumbling, stretching and recoiling when passing	Red cells and chemically treated red	[61]
constriction	through the gap	cells with diamide and glutaraldehyde	
Capillary	transit time	Human Leukocyte	[62]
structure			
Array of funnel	velocity of cells travelling through the funnel obstacles	Red cell parasitized by Plasmodium	[60]
filters		falciparum	
Vertical gap	Young's model calculated using elastic solid model	MC-3T3 osteoblast cells	[68]
constriction			
Adjustable	Transit time (passaging time)	Breast cancer cells (MCF-7) and	[64]
vertical gap		human mesenchymal stem cells	
		(hMSCs)	
Long pinched	entry time (time taken for the cell to squeeze into the microchannel);	Breast cancer cells	[66]
constriction	transit velocity (speed of the cell flowing through the microchannel)		
Cross shape	Deformation Index	Patient-derived melanoma cells line	[67]
micro-channel			

The microfluidic devices reviewed above have several advantages over traditional methods. First, these techniques simplify the experimentation by incorporating functional parts such as cell introduction and capture on a single microfluidic chip. Since the cell and the microstructure are encapsulated onto the same channel layer, the step of aligning of the cell with the structures is eliminated, rendering it much simpler to operate compared with optical deformation and MPA techniques. However, the output measurement results produced using these microfluidic devices, such as transit time, velocity and deformation index, suffer from poor sensitivity due to limitation by the optics. In other words, they require the presence of microscope and camera to record the location and behavior of cells, and to simultaneously measure the displacement and/or shape changes of individual cells. The regular microscope can measure minimum length step of approximately $0.1 \sim 0.3 \mu m/pixel$, and comparing to the normal size of a single cell with diameter of $\sim 10 \mu m$, this resolution is not fine enough to achieve results with high sensitivity. Therefore, it is necessary to design a new microfluidic technique with an output measurement that doesn't rely on optics and has better resolution.

1.2 Ratchet Mechanism

A secondary goal of this work is to study the asymmetry in the force required to deform single cells through a tapered constriction both along the direction of taper and against the direction of taper. If an asymmetry exists, then this mechanism could be used to create a ratchet that unidirectionally transport biological objects in a fluctuating flow. Ratchet mechanisms are important in low-Reynolds number flow as a means to overcome kinematic reversibility in order to transport small particles against the motion of its carrier fluid [69]. These mechanisms are found ubiquitously in biological systems such as sodium-potassium ion pumps [70, 71], actin-myosin protein motors [72], and flagella propulsion of single cell organisms [69, 73]. Recently, using microfabrication and microfluidics technologies, researchers have been able to emulate this principle to create engineered ratchet mechanisms are a periodic spatial inversion asymmetry and a fluctuating excitation [71, 74]. The former presents an asymmetrical resistance to particle motion that can be described using repeating saw-tooth potential wells. The latter energizes the particles to periodically bifurcate their flow paths into neighboring potential wells. Thus, such a mechanism would transport the

particles along the direction dictated by the asymmetry. Previous studies have demonstrated ratchet mechanisms that take advantage of physical asymmetries based on electrical potential [75], dielectrophoresis [76, 77], optical traps [78], contact forces between microparticles and obstacles [79, 80], and bacteria and cell motility [81-83]. Table 3 summarizes some engineered ratchets mechanism that utilized man-made periodic asymmetry of certain nature coupled with unbiased fluctuation excitation.

particles						
Periodic Asymmetry		Fluctuation	Results	References		
Nature	Source	Excitation				
Dielectric potential	Interdigitated electrodes	Diffusion	Particles drifting in one direction	[77]		
Optical potential	Focusing an infrared laser beam to form an optical tweezer	Diffusion	1.5 um diameter particle moving	[78]		
Electric potential	Interdigitated electrodes	Diffusion	Charged DNA transported in one direction	[75]		
Dielectrophoretic	Saw tooth canal	Diffusion	Latex beads	[76]		
Structural	Heart shape structure	Diffusion	E.Coli moving in one direction	[82]		
Structural	Funnel Barriers	Diffusion	Swimming bacteria moving unidirectionally	[81]		
Structural	Series of Christmas- tree shaped pores	unbiased periodic pressure profile	Net motion of a population of particles	[80]		

 Table 3 Summary of engineered ratchet mechanism to induce unidirectional flow of (bio)

 particles

These engineered ratchets provide a potentially new way to separate particles and cells. For example, the transport of DNA uni-directionally [75] provides an alternate way to separate DNA instead of complex and time-consuming gel-based separation. Currently, the potential of using ratchets to separation particles based on size has been shown, but no method exists to separate cells based on deformability [80].

1.3 Device Concept and Research Goals

To study single cell deformability and ratchet mechanism, we design funnel-shaped microscale constriction as shown in Figure 1.4. The funnel opening, or pore size (W_0) , is sufficiently small to require the application of force, or equivalently, pressure, to transport a single cell through the constriction. Single cell deformability is measured from this threshold transport pressure. The funnel shape is not critical for the measurement, but this design reduces the uncertainty associated with the fabrication of sharp corners and provides the test cells smooth transition to deform through. Furthermore, a series of funnels with a variety of pore sizes allow the study of cells under a range of deformations. This measurement approach is similar to traditional MPA, but by integrating the critical functions into a microfluidic platform enables this device to offer simplified operation, improved ease-of-use, and improved robustness to errors.

The research goals of this thesis are as follows:

- 1) To design a microfluidic device for measuring the threshold transport pressures required to squeeze single cells through a range of funnel pores;
- 2) To develop a model of the deformation of single cells in a funnel-shaped constriction;
- Validate device using neutrophils and compare the results with those derived using micropipette aspiration;
- To measure the change in deformability of red blood cells parasitized by *Plasmodium falciparum* in order to distinguish different stages of infection stages;
- 5) To measure the directional asymmetry of associated with the deformation of single cells along and against the direction of taper, and to demonstrate a microfluidic ratchet mechanism.



Figure 1.4 Tapered funnel constriction designed to deform single cells

Chapter 2: Device Principle, Design, and Operation

This chapter describes the principle, design, and operation of the microfluidic device to measure single cell deformability. Section 2.1 illustrates the overall design of the device and specifically, cell inlet/outlet, pressure attenuator and funnel chain structure are discussed in details in Section 2.2. Section 2.2 also describes the pressure measurement principle of cell deforming through the funnel; followed by the device operation in Section 2.3 and experimental setup in Section 2.4.

2.1 Device Overview

The microfluidic device designed to deform single cells using precisely controlled pressure is a two-layer silicone (PDMS) structure fabricated using multi-layer soft lithography [84]. The overall 2D design of the device is shown in Figure 2.1.

The device contains two layers named flow and control layer. The flow layer is situated above the control layer and is infused with the fluid containing the sample cells. The control layer is infused with pure water and is used to direct fluid flow in the flow layer. The intersection membrane between the two layers forms *push-up valve* [85], which uses the deflection of the intersecting membrane to blocks the fluid flow in the flow layer.

The flow layer consists of three networks including (i) chain funnel constrictions, (ii) pressure attenuator, and (iii) cell inlet/outlet. These three micro-channel networks are isolated from each other using membrane micro-valves, valve 1 through valve 4.



Figure 2.1 2D Schematics of the two-layer microfluidic device

2.2 Device Networks

2.2.1 Cell Inlet and Outlet

Test cells are introduced through the cell inlet and extracted through the outlet if the collecting procedure is necessary. With valves 1 and 2 closed, the inlet and outlet are connected by the funnel chain. Cell Inlet/outlet and pressure attenuator are separated, branching out from the funnel chain on the right and left side of it, as shown in Figure 2.1. The width of the inlet/outlet channel is 200 μ m and height of the channel is 20 μ m, approximately.

2.2.2 Pressure Attenuator

Typically, the pressure required to aspire a single cell through a small pore, in the case of micropipette, is on the order of a few Pa to hundreds of Pa [19]. The minimum pressure the available pressure regulator (Fluigent) can provide is around 0.3 mbar (30 Pa). In order to achieve a reasonably small pressure, the pressure is further stepped down through the on-chip attenuator by a ratio of 100:1. Figure 2.2 below shows the pressure attenuator principle.



Figure 2.2 Electric circuit analogy to the fluidic circuitry illustrating the principle of pressure attenuation

A long channel (a) - (b) with a side branch intercepting at point (c) and (b) constitutes the pressure attenuator network. The pressure attenuator reduces an external pressure (P_{ab}) and applies it across the funnel chain network (P_{ch}). This capability is achieved using the hydrodynamic equivalent of a resistive divider electronic circuit.

As shown in Figure 2.2, an external pressure is applied across a long channel between points (a) and (b). A segment of this micro-channel between the points (c) and (d), is spaced at 1/100 of its total length between (a) and (b). The chain of funnel constrictions is attached as an alternate branch between (c) and (d). The hydrodynamic resistance for a rectangular cross-section is defined as

$$R_h \approx \frac{12\mu L}{wh^3 (1 - 0.630 \frac{h}{w})} , \qquad (2.1)$$

where μ is the viscosity of the water, *L*, *h* and *w* are the length, height and width of the micro-channel, respectively. The hydrodynamic resistance of R₁, R₂ and R_{branch} of the device has been calculated in Table 4. As can be seen from the table, there are generally two types

of devices, classified by the size of the tested cells, which are cancer cell device and blood cell device. The height of the devices is customized for specific cell types.

	Cancer Cell Device		Blood Cell Device			
Funnel Height	12 µm	17 µm	3 µm	3.7 µm	7 μm	8.5 μm
(H ₀)	(MLCs)	(Bladder	(RBCs)	(RBCs,	(PBMCs)	(Neutrophil)
		Cancer)		malaria)		
R_2 (Pa s/m ³)	1.16E+15	1.16E+15	1.16E+15	1.16E+15	1.16E+15	1.16E+15
R_1 (Pa s/m ³)	1.16E+13	1.16E+13	1.16E+13	1.16E+13	1.16E+13	1.16E+13
R _{branch} (Pa s/m ³)	2.50E+15	8.79E+14	6.40E+17	3.41E+17	5.03E+16	2.81E+16
Attenuation Ratio	0.009954	0.009871	0.01000	0.01000	0.009998	0.009996

 Table 4 Hydrodynamic resistance (HR) calculated using Equation 2.1

The pressure attenuation ratio can be calculated using the equation:

$$\frac{P_{ch}}{P_{ab}} = \frac{R_1 / R_{branch}}{R_1 / R_{branch} + R_2}$$
(2.2)

Since the hydrodynamic resistance of this branch micro-channel network (R_{branch}) is significantly greater than the hydrodynamic resistance between (c) and (d) (R_1), therefore, the pressure difference across the funnel chain is defined approximately by the ratio of $R_1/(R_1+R_2)$, which is 1/100. Using an external pressure controller (Fluigent, Paris, France) with a resolution of 30 Pa allows the microfluidic device to operate with a resolution of 0.3 Pa. Smaller pressures can be easily achieved using a pressure divider with greater ratio or a more precise pressure controller.

2.2.3 Funnel Chain

Funnels are essential part of the device where a single cell is brought in contact with. Chain of funnels is connected in series with funnel pore size (W_0) from either ends decreasing to the center. This symmetric arrangement eliminates the potential of anisotropic flow due to structure asymmetry [86] and make the inlet and outlet interchangeable giving more flexibility to the device operation. Figure 2.3 illustrates the details of the funnel design in three different cross-sectional views.



Figure 2.3 (A) Microscopic image of the cell deforming through the funnel; (B, C, D) Frontal, midsagittal and transverse cross sectional views of the cell-funnel structure

Three parameters constitute a basic funnel constriction:

1) Funnel taper angle (θ)

The device is originally designed with different θ values, including 0°, 10°,15°, as well as parabolic shape. We choose 15° funnel since it survived the mold fabrication while other funnel shapes did not turn out well due to the steeper angle leading to more fragile structure. 2) Funnel pore size (W₀)

The pore size of the funnel determines how much the cell can be deformed. Typically, the device has a chain of funnel with W_0 ranging from 10 to 3 μ m for cancer cell device and 8 to 1.5 μ m for red blood cells device. This arrangement allows each cell to be tested using several constrictions and allows us to select a range of deformation that achieves optimal cell compression.

3) Funnel Thickness (H₀)

The thickness of the funnel is important to ensure precise measurement of pressure required to squeeze single cells through the funnel. If H_0 is much bigger than the diameter of the tested cell (Φ), the cell can't deform to fill the whole pore and therefore, there will be fluid leakage around the cell which leads to imprecise measurement of the pressure and in the case of H_0 is much smaller than Φ , the cell will experience much resistance of movement due to friction. Therefore, H_0 was customized to each cell type to make $H_0 \approx \Phi$. Besides, the case of $H_0 \approx \Phi$ makes it simple to model the cell with liquid drop model as discussed in Chapter 4:.

2.2.4 Pressure Measurement Principle

The output of the deformability testing device is the pressure required to completely squeeze the cell through each funnel constriction. The measurement principle is illustrated in Figure 2.4.



Figure 2.4 Pressure measurement principle (A,B) and potential error due to the leaked fluid around the cell (C)

Initially, the individual test cell in a carrier fluid is introduced into the funnel chain and carried towards the constriction by the fluid as shown in Figure 2.4 (A). At this point, the cell is hydrodynamically indistinguishable from the fluid, which means the cell won't cause any significant changes in the pressure distribution across the funnel chain.

However, as the cell arrives at the constriction, it blocks the flow of the fluid, and dramatically increases the hydrodynamic resistance of the constriction (R_{funnel}) relative to that of the rest of channel (R_{ch1} and R_{ch2}). As a result, the pressure difference applied across the channel (P_{ch}) is concentrated across the funnel constriction (P_{funnel}), such that $P_{funnel} \approx P_{ch}$, as shown in Figure 2.4 (B). Therefore, varying the pressure applied across the channel during this period enables various deformation pressures to be applied to the targeted cell. Furthermore, as discussed previously, $P_{ch} \approx 1/100 P_{ab}$, the pressure required to squeeze the cell through the funnel can be approximated by $P_{funnel} \approx P_{ab}/100$. P_{ab} is the applied pressure from the pressure regulator (Fluigent).

The importance of micro-channel thickness has been mentioned previously in order to have a precise pressure measurement. Figure 2.4 (C) explains the error in measurement due to fluid leakage around the cell as it deforms through the funnel with $H_0 > \Phi$. Because of the leakage, R_{funnel} is not significant higher than the rest of the funnel chain ($R_{ch1} + R_{ch2}$), leading to the mismatch between P_{funnel} and P_{ch} . In other words, to deform the same cell through the funnels of two different heights, it would require higher P_{ch} , and therefore, higher P_{ab} in order to get the same P_{funnel} to squeeze the cell through using device with $H_0 > W_0$ than with $H_0 \approx W_0$.

2.3 Operation of the Microfluidic Device

The pressure measurement with cells already in the funnel has just been discussed in detail and here, the operation of the device to introduce the cells into the funnel area was shown in Figure 2.5. The initial step of the experiment before any operation is to infuse the channel with medium in order to get rid of the air that originally fills the channel. Specifically, the flow channels in the microfluidic device are prepared by *dead-end filling* using PBS solution. The device was then incubated for 10 minutes to remove all the air bubbles. The control layer channels in the device were filled with DI H_2O .



Figure 2.5 Operation of the microfluidic device (A)Infuse a single red cell into the funnel area with valves 1 and 2 shut, valves 3 and 4 open; (B)shut valves 3 and 4, open valves 1 and 2; apply the pressure difference between a – b to carry the cell in the fluid through the funnel pores; (C) Remove the cell by flushing

The general operation of the device is as follows:

- 1) Firstly, a single cell is introduced through the inlet with only valves 1 and 2 activated (block the flow). The pressure differential applied (P_{intro}) between inlet and outlet controls the flow rate of the sample introduction. Typically, 50 mbar pressure difference is applied until test cells approach the entrance of the funnel chain, then P_{intro} is reduced to ~ 20 mbar. Test cells are typically suspended at a concentration of ~10⁶ /ml in PBS medium to ensure cells being introduced into the device without excessive waiting time (too few cells) or with multiple cells (too many cells) appearing in the funnel chain simultaneously.
- Once a single cell enters the funnel chain area, valve 3 and 4 are activated and immediately after that, valve 1 and 2 are deactivated. From this point on, the cell is manipulated by the pressure difference across the funnel chain (P_{ch}) attenuated from (P_{ab}).
- 3) The applied pressure is gradually increased to measure the *threshold deformation pressure* required to squeeze a single cell through the funnel pore. Initially, a relatively low pressure (~10 Pa after attenuation) is applied to move the cell to the mouth of the funnel. Once the cell arrives at the funnel, the pressure is then gradually increased until the cell is rapidly ejected through the funnel pore. This threshold pressure is recorded and used to calculate cortical tension discussed later in Chapter 4:. After ejecting through the pore, the cell is given a few seconds to relax to its original shape before going through another pore. Measured pressure values are deemed valid as long as the cell shows no

visible signs of damage, permanent change in shape, or activation in the case of neutrophils.

2.4 Experimental Setup

Figure 2.6 illustrates the setup of the experiments. It is composed of three parts with interfacial connectors and tubes. They are pressure controllers, fluid reservoirs and microfluidic device. Sample and medium fluids were contained in the 15 ml conical tubes (Fisher Scientific), sealed with custom-designed black caps. They act as pressurized reservoirs and feed fluids into the microfluidic device through 0.5 mm ID (inner diameter) flexible Tygon tubing (Cole-Parmer). At the microfluidic device end, 19 mm long 23-gauge stainless steel tubing (New England Small Tube, Litchfield, NH, USA) connects the Tygon tubing coming from the reservoir with the device through the custom punched holes in the chip. The connections provide an elastic, watertight seal on both ends.

Two pneumatic pressure control systems were used to pressurize the reservoirs in order to introduce fluids into the device. The first system is a custom pressure controller designed to supply pressure from 0 to 4 bar using manual pressure regulators (Omega). On/off pressure control is enabled using solenoid valves (Pneumadyne) activated by MOSFET switches that are controlled using a MSP430 microcontroller (Texas Instruments) integrated on a printed circuit board. The microcontroller is controlled from a Visual Basic user interface program on a PC. The second pressure control system is the MFCS-4C system (Fluigent SA, Paris, France). This system supplies precise pressure with a resolution of 30 mbar (30 Pa) and a range of 1000 mbar using closed-loop control.



Figure 2.6 (A) Schematics of the experimental setup composed of pressure controllers, fluidic reservoir and microfluidic device; (B)image of the three components

2.5 Threshold Deformation Pressure and Cell Deformability

The direct outcome of our device is *threshold deformation pressure* (P_{th}). It is defined as the maximum or critical pressure required to completely squeeze the cell through the funnel pore. There are many benefits of using pressure as the direct outcome of the device. As discussed earlier, pressure, in our system, has a resolution of 0.3 Pa or even smaller if we make higher attenuation ratio.

However, pressure is not an ideal parameter to characterize the cell deformability since it is size dependent. For example, cancer cells, due to their progressive changes in internal structure, suffer from inconsistency in cell sizes. Bigger cells will lead to higher P_{th} while smaller cells correspond to lower P_{th}, provided that they go through the same funnel gap. Intuitively, the way to solve the issue is to customize the dimension of constriction deformation based on the cell sizes, which was done by [64] using active feedback control of micro-channel valve constriction. The simpler method would be to develop a model that can yield a size-independent parameter. In our case, we choose liquid drop model and derive the cortical tension value of individual cells. The detailed modeling is introduced in Chapter 4:.

Chapter 3: Material and Methods

The microfluidic device is fabricated using standard techniques of photolithography and multilayer soft lithography. Silicon master molds were first fabricated using photolithography in a standard clean room, which were then replicated on demand in a regular laboratory setting, using PDMS via multilayer soft lithography technique. The device is finalized by bonding it to the glass slides. This chapter describes the exact details of these processes as well as the experimental procedures for preparing cells and device.

3.1 Fabrication of Silicon Masters

To fabricate the double layer microfluidic device, features for each layer were first created on the silicon wafer. The mold of the flow layer was defined by two consecutive applications of photoresist; first the funnel chain part was made using SU-8 series photoresist (MicroChem, Newton, MA, USA). The pressure attenuator and cell inlet/outlet were then added to the mold using SPR 220-7.0 photoresist. The mold for the control layer was made with a single application of SPR 220-7.0 photoresist. The fabrication processes were as follows:

- 1) A 100 mm silicon wafer is cleaned using acetone, methanol, and isopropanol;
- The cleaned wafer is baked on a hotplate at 200°C for ~5 minutes to ensure complete dehydration;
- 3) Hexamethyldisilazane (HMDS) is applied to the wafer in a fume hood;
- Several SU-8 varieties and spin speeds were used to produce the desired flow channel thicknesses required for different cell types. Refer to Table 5 for details;
- 5) The wafer with SU-8 photoresist is then soft baked at 95°C on hotplate for 20 min;
- 6) This wafer is then exposed in a mask aligner (Canon) for 30 to 40 seconds;
- The exposed wafer is given a post exposure bake on hotplates set at 65°C for 1 minute, 95°C for 1.5 minutes, and then 65°C for 1 minute;
- 8) The wafer is then developed using SU-8 developer (MicroChem);
- 9) The geometry of the SU-8 photoresist is stabilized by further baking on a hotplate. The hotplate temperature is gradually ramped from 40°C to 200°C, held at 200°C for one hour, and then gradually cooled to 40°C.
| Channel Thickness | Tested Cells | SU-8 series | Spin Speed (rpm) | |
|-------------------|------------------|---------------------|------------------|--|
| (µm) | | | | |
| 3 | RBCs | SU-8 3005 + Thinner | 3000 | |
| | | at 1:1 ratio | | |
| 3.7 | Parasitized RBCs | SU-8 3005 | 5000 | |
| 7 | Lymphocyte | SU-8 3005 | 3000 | |
| 8.3 | Neutrophil | SU-8 2007 | 2000 | |
| 12.8 | MLCs (L1210) | SU-8 3010 | 1500 | |
| 17 | Bladder Cancer | SU-8 2025 | 4000 | |
| | (RT4) | | | |

Table 5 Choices of SU-8 series and spin speeds to achieve the desired flow layer thickness

Following fabrication of the SU-8 micro-structure, a layer of SPR 220-7.0 photoresist was added to the existing silicon wafer to complete the flow layer master mold.

- The silicon wafer containing the SU-8 microstructures is spin-coated with SPR 220-7.0 (MicroChem) at 100 rpm for 10 seconds, then at 600 rpm for 50 seconds, and finally at 3000 rpm for 2 seconds;
- The wafer with photoresist is soft baked on hotplates set at 65°C for 1 minute, 95°C for 2 minutes, and then 65°C for 1 minute;
- The mask for the SPR pattern is aligned with the SU-8 pattern and exposed for 4 minutes in intervals of 30 seconds;
- 4) After waiting for approximately 30 minutes, the wafer is developed in MF-319 (MicroChem);
- 5) Finally, the developed wafer is annealed for 30 minutes in a 90°C oven to create a rounded channel profile.

The control layer was fabricated using SPR in the same procedures as described above without the alignment requirement. And the thickness for all SPR layer is approximately 20 µm.

3.2 Fabrication of PDMS Devices

After the fabrication of the silicon masters for both flow and control layer, the flow layer silicon molds, which has precise and delicate geometries, were replicated in a polyurethanebased plastic (Smooth-Cast 310, Smooth-On) via the process described by Desai et al. [87]. In contrast, since the control layer master mold provided a cast for valve membranes and contained large, simple structures, which slowed down degradation and made it easy to replace, the control layer PDMS was fabricated directly from the master mold.

Using the polyurethane mold for the flow layer and the master silicon wafer for the control layer, the two layers were fabricated simultaneously. There are two ways to fabricate the microfluidic device depending on how the two layer bonds to each other. One way is through diffusion bonding between two layers. RTV-615 PDMS (Momentive Performance Material) was poured together in a cup at a base/hardnener ratio of 5:1 for flow layer and 20:1 for control layer and evenly mixed (2.5 minutes) and defoamed (1.5 minutes) in the spinner (THINKY ARE-250). The PDMS (5:1 ratio) was first poured into the flow layer mold and degassed in the desiccator for 10 minutes. In the meantime, 20:1 ratio mixture was poured onto the control layer mold wafers and spun at 500 and 1800 rpm for 15 and 60 seconds respectively. Both layers were then cured at 95° C for 60 minutes. After the curing, the two layers were aligned together and bonded via second baking for at least three hours. The other method to form the two-layer device was through plasma bonding. The procedures are similar except that both layers of PDMS are made using 10:1 ratio. After the 1 hour baking, the two layers are both placed in the plasma generator (Model PDC-001, Harrick Plasma). With careful attention to alignment, the flow and control layer were brought together to form instant, irreversible covalent bonds.

After the bonding of the two layers via either of the two methods, the device was carefully removed from the wafer, 0.5 mm holes were punched into it using a manual hole puncher

(Technical Innovations, Angleton, TX) to form the fluidic introduction parts. 19 mm long 23gauge stainless steel tube would be inserted into the holes as mentioned earlier to form watertight seal as mentioned earlier in Chapter 2. Finally, the double layer PDMS devices were bonded to a standard glass slide cleaned with ethanol following air plasma activation (Harrick Plasma, Ithaca, NY, USA).

3.3 Cell Culture

The cells tested using the device include Mouse Lymphoma (L1210), Bladder cancer (RT4), red blood cells (RBCs) and *P. falciparum* parasitized RBCs, neutrophils and lymphocytes. The cell culture and preparation are introduced as follows.

3.3.1 Cancer Cells

L1210 Mouse Lymphoma cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. RT4 bladder cancer cells were cultured in MEM with 10% (v/v) fetal bovine serum (FBS), 1% L-glutamine, 1% MEM Non-Essential Amino Acids, 1% Sodium Pyruvate (Invitrogen) under the same condition as L1210 Mouse Lymphoma cells.

3.3.2 Red Blood Cells and Malaria

Fresh RBCs: Whole blood was collected with informed consent from healthy donors into 6 ml tubes containing sodium heparin or EDTA. Tubes were centrifuged at 1000 g (Clay Adams Serofuge) and washed 3 times with saline. The hematocrit of packed red blood cells was determined using Autocrit Ultra 3 and RBCs were resuspended in Hanks Balances Salt Solution with glucose at 20% or 40% hematocrit as required. Prior to testing with the developed microfluidic device, 5 μ l of the final RBCs sample was diluted in 3 ml of Phosphate Buffer Solution (PBS) with 5% (150 μ l) Bovine Serum Albumin (BSA).

P. falciparum Culture: The FCB strain of P. falciparum parasites was cultured under standard *in vitro* conditions with modifications [88]. Briefly, cultures were maintained at 5% hematocrit in malaria culture medium (1640 RPMI with HEPES; 0.2% sodium bicarbonate; 100 μM hypoxanthine; 10% heat-inactivated human serum, 1 mg/ml gentamicin). Parasites

were incubated in an atmosphere of 5% CO₂, 3% O₂ and 92% N₂ at 37 °C and 95% humidity. Prior to the experiments, blood smears of the cell cultures were prepared. The specimens were air-dried, fixed in methanol and stained with 10% Giemsa to evaluate the stages of the infected cells to be tested. Asynchronous cultures were used to obtain measurements of different parasite stages that were kept under the same conditions. The parasitemia in culture was determined to be 5 to 12%. Prior to the experiments, parasite cultures were centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and 4 μ l of the pellet was resuspended in 3 ml Ringer's solution (122.5 mM NaCl; 5.4 mM KCl; 1.2 mM CaCl₂:2H₂O; 0.8 mM MgCl₂:6H₂O; 5.5 mM D-Glucose; 10 mM HEPES; 1 mM NaH₂PO₄:H₂O; pH 7.4) supplemented with 5% BSA.

3.3.3 Neutrophils and Lymphocyte Extraction

Whole blood was collected from healthy donors with informed consent into 6 ml tubes containing EDTA as anti-coagulant. Neutrophils were isolated from whole blood by gradient density centrifugation using Histopaque solutions 1077 and 1119 (Sigma-Aldrich) [89]. Specifically, 3 ml of Histopaque 1077 was carefully layered on the top of 3 ml of Histopaque 1199 in a 15 ml centrifuge tube. Next, 6 ml of whole blood was added on top of the layered Histopaque 1077 and 1199. The tube was then centrifuged at 700 g for 30 minutes without braking at 24 °C. Two layers of cells, consisting of lymphocytes and neutrophils, were transfered into separate 15 ml centrifuge tubes containing 10ml of HBSS without Ca²⁺ and Mg²⁺ solution (Invitrogen). The collected cell suspensions were centrifuged at 300 g for 10 minutes. After removing the supernatant, the pelleted cells were washed twice using HBSS.

Chapter 4: Modeling of Cell Deformability

4.1 Overview of Cell Modeling

4.1.1 Different Types of Mechanical Models

The measurement of an intrinsic property, such as deformability, is a combination of the determination of a measurable parameter with a model of the deformation process. Generally, there are two established models of single cell deformability include the liquid drop model and the solid (linear elastic) model. The solid model assumes the whole cell as homogenous without the distinct cortical layer while the liquid drop model considers the cell with a constant cortical membrane surrounding a liquid interior.

4.1.2 Micropipette Aspiration and Liquid Drop Model

The setup of MPA and experimental procedures has been dealt with in details in Chapter 2:. Liquid drop model was developed first to account for the behavior of neutrophils in MPA [17, 56]. White blood cells (Leukocytes) behave like liquid droplet and remain spherical shape when suspended due to a constant surface tension. As shown in Figure 4.1, it has been found that they can deform continuously into a micropipette with much smaller diameters when the suction pressure exceeds a certain *threshold pressure*. This threshold pressure is also referred to as critical pressure, which is defined as when a static hemispherical projection of the cell is formed inside the pipette ($R_{pro} = R_{pip}$), as shown in Figure 4.1 (A). An excess pressure beyond this point will cause the cell to flow completely and continuously into the pipette. Figure 4.1 (B) is the cross sectional area of the glass pipette, which shows a circular pore shape.



Figure 4.1 (A) Process of the cell deforming through circular pore in the MPA experiment; (B) cross sectional view of the glass pipette; (C) Laplace Law developed in the case of circular pore size

The critical pressure can be determined using Laplace law, which relates cortical tension (T_c) of the cell membrane to the pressure difference between inside and outside of the cell by the radius of curvature. As shown in Figure 4.1 (C), in the case of the cell deforming through a circular pore entrance,

$$\left(P_c - P_2\right)\pi R_{out}^2 = T_c(2\pi R_{out})$$
(4.1)

And therefore,

$$P_c - P_2 = \frac{2T_c}{R_{out}}$$
(4.2)

Similarly,

$$P_{c} - P_{1} = \frac{2T_{c}}{R_{pro}} = \frac{2T_{c}}{R_{pip}}$$
(4.3)

where R_{out} is the radius of the cell outside the pipette and R_{pro} is the protrusion length of the cell into the pipette and R_{pip} is the radius of the pipette. From equation 4.2 and 4.3, the critical pressure can be determined by,

$$P_{crit} = P_2 - P_1 = 2T_c \left(\frac{1}{R_{pip}} - \frac{1}{R_{out}} \right)$$
(4.4)

To analyze what would happen after the critical moment where the cell is further aspirated into the pipette, P_2 - P_1 is increased in order to draw the cell further into the pipette. While R_{pip} and T_c is constant and R_{out} decreases as the cell is drawn into the pipette. Thus, the right hand

side decreases, but the left hand side increases. This means the cell is no longer in equilibrium and it will be drawn completely into the pipette, which is what has been observed in MPA experiments [19], indicating the passive behaves of white blood cells like liquid drop.

4.2 Calculation of Cortical Tension

4.2.1 Laplace Law of Spherical Cell Going Through Rectangular Shape Constriction

Figure 4.2 shows the geometry of the cells going through the funnel in two different crosssections. As shown in the figure, the internal pressure of the cell is denoted P_c while pressures P_1 and P_2 are the pressures acting on the leading and trailing membranes of the cell respectively. We can examine the balance of forces acting on these membranes separately as illustrated in Figure 4.2. The membrane on the each edge has two radii of curvature R_a , R_b and R_{c1} , R_{c2} . R_a and R_b is constrained by the geometry of the funnel, while R_{c1} and R_{c2} is also constrained by the floor and ceiling of the funnel since the height of the channel was customized to be roughly the same of the diameter of the target cell as mentioned in Chapter 3.

In the case of the leading edge as shown in Figure 4.2 (C), the normal components of the membrane tension forces, F_{Tc1} and F_{Ta} , are equal to the force from the difference in pressure on either side of the membrane as follows:

$$(P_1 - P_c)W_0 H_0 = F_{Tc1} \cos \theta_1 + F_{Ta} \cos \varphi_1$$
(4.5)



Figure 4.2 Top view (A) and side view (B) of cell deforming through the funnel pore; derivation of the relationship between threshold deformation pressure by looking at the geometry of leading (C) and trailing (D) membrane

Next, the membrane tension forces are defined as the cortical tension multiplied by the length of the membrane edges over which they act, such that:

$$F_{Tc1} = T_C(2W_0)$$
(4.6)

$$F_{Ta} = T_C (2H_0) \tag{4.7}$$

Substituting these tension force terms into Equation 4.5 yields:

$$(P_1 - P_c)W_0 H_0 = 2T_C(W_0 \cos \theta_1 + H_0 \cos \phi_1)$$
(4.8)

Furthermore, by geometry:

$$\cos\theta_1 = \frac{H_0}{2R_{c1}} \tag{4.9}$$

$$\cos\phi_1 = \frac{W_0}{2R_a} \tag{4.10}$$

Substituting Equations 4.9 and 4.10 into 4.8:

$$P_{1} - P_{c} = T_{C} \left(\frac{1}{R_{c1}} + \frac{1}{R_{a}} \right)$$
(4.11)

Following the same procedure for the trailing edge membrane yields:

$$P_2 - P_c = T_C \left(\frac{1}{R_{c2}} + \frac{1}{R_b} \right)$$
(4.12)

To find and expression for the applied pressure on the entire cell, we can subtract Equation 4.11 from Equation 4.12:

$$P_2 - P_1 = T_C \left(\frac{1}{R_a} + \frac{1}{R_{c1}} - \frac{1}{R_b} - \frac{1}{R_{c2}} \right)$$
(4.13)

 R_{c1} and R_{c2} are both constrained by the funnel height; therefore these two radii are approximated as being equal. This reduces Equation 4.13 to the following final form:

$$P_2 - P_1 = T_C \left(\frac{1}{R_a} - \frac{1}{R_b}\right)$$
(4.14)

Notice the difference between 4.4 and 4.14 by a factor of 2.

4.2.2 Determination of Cortical Tension

From Equation 4.14, the cortical tension can be determined based on the pressure measurement at the critical moment. Similar to whole cell MPA experiments, we measured the *threshold deformation pressure* by slowly increasing the applied pressure difference until each test cell is rapidly pulled across the constriction. In the case of MPA, the critical moment occurs when the leading edge radius of the cell equates to pipette radius [19]. Similarly, for the cell deforming through rectangular shape funnel gap, the threshold pressure also occurs as $R_a=W_0/2$. Based on Equation 4.14, the only unknown parameter is R_b , which can be approximated using volume conservation of the cell.

Specifically, we model the geometry of the cell at the critical point of deformation in 3 parts as shown in Figure 4.3. V_1 and V_3 are modeled as half-ellipsoids with in-plane radii R_a and R_b , as well as out-of-plane radii $H_0/2$. The volume of the middle section, V_2 , can be estimated by assuming the cell conforms entirely to fill a section of the funnel. This assumption is reasonable as long as the test cells undergo significant deformation and thereby conforms to

the shape of the funnel constriction. The statement of volume conservation for determining R_b can be expressed as

$$V_0 = V_1 + V_3 + V_2$$
 (4.15)
in which,

$$\frac{4}{3}\pi R_0^3 = \frac{H_0}{3}\pi R_a^2 + \frac{H_0}{3}\pi R_b^2 + H_0(R_a + R_b)L$$
(4.16)

$$L = \frac{1}{\tan \theta} (R_b - R_a) \tag{4.17}$$

where R_0 is the undeformed cell radius.

From the equations above, R_b can be determined to be;

$$R_{b} = \sqrt{\frac{\frac{4R_{0}^{3}}{3H_{0}} + \left(\frac{L}{\pi \tan \theta} - \frac{1}{3}\right)R_{a}^{2}}{\frac{1}{3} + \frac{L}{\pi \tan \theta}}}$$
(4.18)

The cortical tension for each cell can then be calculated using Equation 4.14.



Figure 4.3 Dissection of cell going through the funnel at critical moment

Chapter 5: Device Validation

This chapter presents results on the validation of the microfluidic device to measure the *threshold deformation pressure* to measure the deformability single cells. The cortical tension is derived from the measured pressure and cell diameter using the liquid drop model presented in Chapter 4. In Section 5.1, neutrophil extracted from human whole blood was tested with the funnel structures and cortical tension value was determined. In Section 5.2, a heuristic is developed to determine the range of deformation that provide a valid measure of cell deformability. Furthermore, the accuracy and precision of the results are studied and compared results from MPA experiment. In Section 5.3, the deformability of human lymphocytes, as well as cells from two cancer cell lines are measured and analyzed.

5.1 Neutrophils

Neutrophils are often used as a standard test cell in classical MPA experiments [13-15]. We tested neutrophils extracted from whole blood using devices with a channel thickness (H₀) of 8.3 μ m. Rigid micro-beads of various sizes were introduced through the funnel chain area and the thickness was determined by measuring minimum diameter of the beads that were trapped at the entrance of the funnel chain.



Figure 5.1 Images of the four stages of a single neutrophil going through the funnel: (A) initial approach, (B) deformation, (C) critical point, and (D) ejection through the funnel.

Threshold deformation pressures of neutrophils were measured using funnels with pore sizes of 4.1, 3.8, 3, 2.5, 2, 1.4, 1.2 μ m. Figure 5.1 shows example microscopy images from the deformation of a single neutrophil, including initial approach, deformation, critical moment, and ejection through the funnel. The measurement process is as follows. Firstly, a single neutrophil is typically infused into the funnel and stopped before coming in contact with a funnel surface to ensure the cell is deformed using a quasi-static process instead of rushing through by inertial. Following that, the applied pressure is increased slowly to push the cell through the funnel until a visible protrusion appears beyond the funnel opening.

Subsequently, the cell rapidly advances through the constriction as predicted by the liquiddrop model. After that, the cell is given a few seconds to return to its original shape before being deformed through a subsequent funnel. Throughout our testing, most tested neutrophils appeared to remain in a passive state and showed no morphological changes that are characteristic of being activated. If the cell did not return to its original shape, then it is removed from the microfluidic device by flushing. In total, we made 80 measurements on 12 neutrophils in one device. The measured deformation pressure as a function of $(1/R_{a1}-1/R_{b1})$ was plotted as shown in Figure 5.2. A linear regression line was fit through the scatterplot with slope representing the fitted cortical tension of neutrophils based on equation 4.14. The value (T_{fit}) is 39 pN/µm.



Figure 5.2 (A)Measured threshold pressure as a function $1/R_{a1}$ - $1/R_{b1}$.(B) Residue of the linear fit plotted as the ratio of the calculated cortical tension (T_c) and the fitted cortical tension (T_{fit}) as a function of the cell deformation index defined in equation 5.1.

5.2 Heuristics to Determine the Optimal Range of Deformation

Several outliers can be observed in Figure 5.2 (A), especially at smaller pore size (large $1/R_{a1}$ - $1/R_{b1}$). To study errors associated with our measurement process, cortical tension (T_c) was calculated from each of 80 measurements. *Residual plot* was drawn where the ratio of the calculated cortical tension from each measured neutrophil and the average value determined using the linear fit (T_c/T_{fit}) as a function of cell deformation index (DI) in Figure 5.2 (B). DI is defined as the ratio of the cross-sectional area of the cell and of the funnel opening where

$$DI = \frac{A_{cell}}{A_{window}} = \frac{\pi R_0^2}{H_0 W_0}$$
(5.1)

As indicated in Figure 5.2 (B), cortical tension values measured using a median deformation index (4.5 > DI > 2.2) were independent of deformation index, whereas measurements at both large and small deformation indices resulted in cortical tension values noticeably greater than the fitted mean value.

At large DI values (DI > 4.5), deviations from the model likely result from excessive deformation, where internal cellular structures, such as the nucleus and cytoskeleton, begin contributing to the measured cell deformability. Similar characteristics have been observed in previous MPA studies [42, 46, 90], where the measured cortical tension increases as cells are aspirated into smaller pipettes using greater pressures.

At small DI values (DI < 2.2), deviations from the model likely result from leaked fluid flow when the cell insufficiently conforms to the rectangular funnel constriction. Since our measurement technique relies on being able to create an effective seal as each cell is deformed into a funnel constriction, a leaky seal would require greater pressures to be applied to the funnel chain in order to induce cell deforming through the funnel. The pressure leakage issue was dealt with in Chapter 4. As a result, the calculated cortical tension based on these measurements over-estimates the actual value.

Based on the results in Figure 5.2 (B), we can develop a heuristic for qualifying cell deformability measurements made using our system. Specifically, measurements are considered valid when the deformation index is between 2.2 and 4.5. Analyzing neutrophil data in this range, we find the average neutrophil cortical tension to be $37.0 \pm 4.8 \text{ pN/}\mu\text{m}$. This result is consistent with values reported in previous MPA experiments as shown in Figure 5.3.



Figure 5.3 Comparison of the cortical tension values obtained using our device and with others measured using MPA.

5.3 Cancer Cell Deformability

To evaluate the utility of our device, we measured the deformability of four cell types with distinct deformability, including human lymphocytes, RT4 human bladder cancer cells, and L1210 mouse lymphoma cells. Since photolithographic processes are primarily suitable for fabricating planar microstructures, the height of the device used must be selected for each cell type. As mentioned earlier in Chapter 4, various versions of the microfluidic device were fabricated to customize the thickness of the funnel channel for each cell type. The optimal device geometry, including micro-channel thickness (H₀) and deformation index is shown in Table 5. The cell diameter was measured from at least 30 cells. The measured deformation range is based on the heuristic developed in Section 5.2.

Cell Type	Cell Diameter (µm)	Channel Thickness H $_0$ (μ m)	Deformation range
Neutrophils	8.2 ± 0.7	8.3	2.2 ~ 4.5
Lymphocytes	7.0 ± 0.8	7.0	2.5 ~ 3.5
Bladder Cancer (RT4)	18.0 ± 2.0	17.0	2.2 ~ 3.0
Mouse Lymphoma (L1210)	12.8 ± 0.5	12.0	2.5 ~ 4.0

Table 6 Microchannel thicknesses used to test different cell types

Example threshold deformation pressures from each of the four types are plotted as a function of DI in Figure 5.4 (A). The data shown are from example cells where the cell diameter is approximately the same as the mean diameter for the cell population. These results show that neutrophils require the least amount of deformation pressure, while mouse lymphoma require the most. Figure 5.4 (B) compares the cortical tensions between the four measured cell types. Each data point shows the mean and standard deviation of the result from around 20 cells. The L1210 mouse lymphoma and RT4 bladder cancer cells is approximately 5 times and 6.5 times more rigid than leukocytes.



Figure 5.4 (A) Example threshold pressures measured as a function of DI from each of the four tested cell types. (B) Comparison of measured cortical tension from four different cell types.

This result is not surprising given that the cancer cells have a larger nucleus and welldeveloped cytoskeleton. The greater rigidity of L1210 cells compared to RT4 cells is likely due to differences in the size of the nucleus. In order to determine the relative size of the nucleus, we measured the nucleus area as well as the cytoplasmic area of cells by dying the nucleus part of the cell. The ratio of nucleus/cytoplasm area (N/C) was measured to be 0.50 ± 0.17 for RT4 and 1.09 ± 0.43 for L1210. In other words, L1210 cells contain a nucleus that nearly fills half of the entire cytosol, whereas RT4 cells contain a nucleus that fills approximately one-third of the cytosol. Blood cells, which experience large shear stress while transiting through circulatory system, are physiologically and functionally different from epithelial cells and therefore, more deformable. Neutrophils and lymphocyte are both leukocytes. Neutrophils have multiple granular nuclei, while lymphocyte contains only one nucleus. The difference in nucleus configuration leads to the variability in deformability between two types of leukocytes.

5.4 Evaluation of Measurement Uncertainties

The sources of errors in our measurement process include errors associated with the measurement of pressure, errors associated with the optical measurement of device and cell geometry, and errors associated with the measurement of the thickness of the channel. Potential errors associated with the measurement of pressure include:

- 1) Resolution of the pressure generator is 30 mbar and 0.3 Pa after pressure attenuation;
- 2) The pressure attenuation ratio is not exactly 100:1 ratio as shown in table 4;
- The remolding of the cell internal structure due to constant exposure to stresses caused by compression

Potential errors associated with the optical measurements of cell and device geometry include:

- Measurement of the cell radius (R₀) is limited by the camera resolution, which is 0.3 µm/pixel;
- 2) Measurement of the pore size (W_0) ;

Potential errors associated with the measurement of device thickness are caused by the inherent resolution of the Dektak machine, which has 0.5 nm resolution. In order to evaluate the uncertainty due to the pressure measurements process, multiple measurements were done on a single mouse lymphoma (L1210) cell going through a sequence of funnel pores. $3 \sim 5$

measurements were done per cell per pore constriction. The relative standard uncertainty [91, 92] has been determined, which is defined as:

$$\frac{u(x)}{x_0} = \frac{sd}{\sqrt{n}} \cdot \frac{1}{x_0}$$
(5.2)

where u(x) is the standard uncertainty, and x_0 is the measurement mean; *sd* is the standard deviation of n measurements. Based on the results and equation 5.2, the relative standard uncertainty is ±8.4% for pressure measurement process of the cell funnel system.

Standard uncertainty due to the resolution of either pressure generator, optics or thickness measurement can be calculated by considering the distribution to be triangular or uniform and the standard uncertainty can be calculated as $\frac{a}{\sqrt{3}}$, where *a* is the semi-range (or halfwidth) between upper and lower limits. Table 7 summaries all the potential uncertainty in the developed technique and their nature. Based on equation 4.14 and 4.18, the combinatory standard relative uncertainty of the cortical tension calculation due to the uncertainty in pressure measurement (ΔP), and length measurements of R_a and R_b can be determined by:

$$\frac{u(T_c)}{T_c} = \sqrt{\left(\frac{u(\Delta P)}{\Delta P}\right)^2 + \left(\frac{u(R_a)}{R_a}\right)^2 + \left(\frac{u(R_b)}{R_b}\right)^2},$$
(5.3)

where,

$$\frac{u(R_a)}{R_a} = \frac{u(W_0)}{W_0}$$
(5.4)

$$\frac{u(R_b)}{R_b} = \sqrt{\left(\frac{1.5u(W_0)}{W_0}\right)^2 + \left(\frac{u(R_b)}{R_b}\right)^2}$$
(5.5)

Based on equations 5.3, 5.4 and 5.5, the combined relative standard uncertainty can therefore be calculated to be $\pm 11.7\%$. The system errors do not contribute to the sensitivity of the device to discriminate deformability of various cell types since they are equal in each calculation. The possible error to determine the cortical tension solely depends on the error caused by the uncertainty due to inherent threshold pressure measurement process, which is within 8.4%.

Source of	Value (±)	Probability	Divisor	Standard	Nature of
uncertainty		distribution		uncertainty	uncertainty
Relative	8.4%	Normal	1	8.4%	random
standard					
uncertainty					
of mean of					
3~ 5 repeated					
readings					
Pressure	0.3 Pa	Uniform	$\sqrt{3}$	0.1732 Pa	Systematic
resolution					
Funnel pore	0.3 µm	Uniform	$\sqrt{3}$	0.1732 µm	Systematic
size					
measurement					
Cell size	0.3 µm	Uniform	$\sqrt{3}$	0.1732 µm	Systematic
measurement					
Funnel	0.5 nm	Uniform	$\sqrt{3}$	0.289 nm	Systematic
thickness					
measurement					

 Table 7 The error budget of our measurement technique

Chapter 6: Application: P. falciparum parasitized RBC

This chapter reports the application of the developed device in studying red blood cells and malaria parasite (*P. falciparum*) invaded red blood cells. In Section 6.1, the healthy red cells from different donors were tested first using 2.5 µm thick funnel constrictions. In Section 6.2, parasitized (*P. falciparum*) red cells in different stages were identified and tested using 3.9 µm thick funnel constrictions. Using this method, parasitized cells from ring through Schizont stages were shown to be 1.5 to 200 times stiffer than uninfected red cells. The measured deformability values of uninfected and parasitized cells showed clearly distinct distributions, demonstrating the potential of using this technique to study the pathophysiology of this disease, and the effect of potential drugs.

6.1 Deformability of Healthy, Mature Red Blood Cells

The healthy red blood cells were measured first. The size of the red cells is $7.8 \pm 0.6 \mu m$ in diameter and $2.6 \pm 0.3 \mu m$ in thickness. 3 μm thickness devices were chosen, so that the cell would go through the funnel in a planar configuration. The chain of funnels has W₀ ranging from 8 μm down to 1.5 μm . Figure 6.1 shows the microscopic images of red cell squeezing through 1.5 μm funnel. The RBCs are so deformable that it can deform through funnels of pore sizes more than 3 μm with pressure less than 1 Pa. Therefore, we choose the minimum funnel gap 1.5 μm to measure the *threshold deformation pressures*.



Figure 6.1 Microscopic images of a single red cell going through the funnel

It has been discussed in Section 2.5 the necessity to use parameter such as cortical tension to indicate the deformability of the single cells rather than to use *threshold deformation pressures* due to the huge variance in cell sizes of a particular cell population. However, in the case of studying healthy red blood cells, *threshold deformation pressures* can be used as

the representative outcome because the size of the red cells is very consistent. RBCs that are much bigger or smaller than normal will be excluded.



Figure 6.2 Histogram of threshold deformation pressures of 100 healthy red cells from three different donors squeezing through 1.5 µm pore size

Figure 6.2 shows the histogram of deformation pressures for each donor. In total, 100 red cells from three different donors were tested. Individual red cells were squeezed through 1.5

µm funnel pore size and threshold deformation pressures are measured. The histogram follows approximate normal distribution.

6.2 P. falciparum Parasitized Red Blood Cells

6.2.1 Modeling of Red Cell Deforming Through Rectangular Gap

Red cells parasitized by *P. falciparum* were tested. Instead of using 3 µm height devices, 3.7 µm was selected to accommodate the bulging of the red cells due to the lodging of the parasite leading to the increase in cell thickness. The minimum gap of the funnel is measured to be 1 µm; and uninfected cells, cells at ring stage and Trophozoite stage can fit through 1 µm with measurable *threshold deformation pressures*. Infected red cells at the Schizont stages were observed to be too rigid to go through 1 µm gap. Due to these drastic morphological changes, *threshold deformation pressure* was not a proper indicator in this case and liquid drop model was used to derive the cortical tension as a parameter to compare the deformability of infected red cells at different stages.

Equation 4.14 was used to calculate the *effective cortical tension*. Threshold pressure occurred as the protrusion length of the red cell is $W_0/2$. R_b can be determined based on volume conservation by solving the following equation,

$$\pi R_{cell}^2 H_{cell} = \left(\frac{1}{2}\pi R_b^2 + \left(R_b^2 - R_a^2\right)\frac{\cos(\theta)}{\sin(\theta)} + \frac{1}{2}\pi R_a^2\right) H_0$$
(6.1)

where R_{cell} and H_{cell} is the undeformed diameter and thickness of the test cell. The value is set to be 8 µm for R_{cell} and 2.5 µm for H_{cell} . Once values for R_a and R_b are known, the *effective cortical tension* (T_{eff}) could then be calculated from the measured threshold pressure using Equation 4.14.

6.2.2 Rigidity of *P. falciparum* Infected RBCs

To evaluate the ability of our technique to discriminate uninfected from various stages of parasitized cells, we measured the deformability of *P. falciparum* infected RBCs from *in situ* cultures. Specifically, we infused asynchronous cell cultures, which include infected red cells at various stages of infection, into the device. The individual RBCs were subsequently identified through observation as uninfected, ring, early Trophozoite, mid-late Trophozoite,

and Schizont stages under standard criteria [93] and the threshold pressures are measured using one or several constrictions. Briefly, RBCs infected with ring stages contain a faint parasite moving rapidly inside the cell; early Trophozoite stages begin with the parasite lodged at a fixed location; mid-late Trophozoite stages begin to express parasite-derived adhesion molecules on the cell surface that are accumulated in knobs [94] and show the typical malaria pigment in their digestive vacuole [95]; finally, Schizont stages occupy most of the RBC cytosol. Micrographs of uninfected and infected cells from each stage deforming through constrictions are shown in Figure 6.3. At least 25 cells were measured from each stage.



Figure 6.3 Micrographs of uninfected red cells and parasitized cells in different stages

There are two sets of control cells: unexposed and uninfected red cells. Unexposed cells refer to the ones that are not exposed to the parasites and cultured separately from the infected cells. Uninfected red cells are those that are exposed to the parasites but not invaded by them. Figure 6.4 documents the threshold deformation pressures required to squeeze unexposed, uninfected cells, cells at ring stage, early Trophozoite, Mid-late Trophozoite and Schizont stage through 1.5 µm and 1 µm gap.



Figure 6.4 Threshold deformation pressures measured of control RBCs and infected RBCs at different stages

Threshold deformation pressures of Schizont stages cells were measured using 5, 4, and/or 3 μ m constrictions and they were typically obstructed by constrictions less than 3 μ m. At this stage, the presence of the parasite dramatically elevates the required deformation pressures. The variation of the measured threshold pressures is also increased, likely due to the rapid and inconsistent morphological changes associated with the replication of merozoites. In order to compare the stiffness of Schizont stage cells and cells at previous stages, cortical tension was calculated based on equation 4.14 and 6.1. Figure 6.4 illustrates the histogram and bar graph of cortical tension values at different stages. The results from the each stage (except Schizont stage) showed an approximately normal distribution with mean and standard deviation values of 3.22 ±0.64 (uninfected), 4.66 ±1.15 (rings), 8.26 ±2.84 (early trophozoites), and 21.38 ±5.81 (mid-late trophozoites) pN/ μ m. The measured cortical tension of Schizont stage cells range from 85 pN/ μ m to 1300 pN/ μ m, with an average value of 607 pN/ μ m.



Figure 6.5 (A)Histogram of cortical tension of a number of red cells at various stages of infection; (B) Bar graph of the cortical tension of uninfected and infected cells at progressive stages.

6.2.3 Comparison of Our Results with Previous Studies

From Figure 6.4 and Figure 6.5, the loss of RBC deformability associated with the presence and maturity of the parasite is clearly evident. The coefficient of variance (CV) values, defined as the ratio of standard deviation over mean, are 0.20 (uninfected), 0.25 (ring stage), 0.34 (early Trophozoite) and 0.27 (mid-late Trophozoite). The CV value indicates how much the distribution is dispersing and therefore, the precision of the developed microfluidic device.

Compared with previous biomechanical measurements of parasitized RBCs performed using other microfluidic techniques [3, 7, 60], as well as micropipette aspiration [6, 24, 25], our technique offers significantly enhanced signal due to the precise pressure measurement, along with narrower distributions (better CV) of the characteristic deformability values associated with each parasite growth stage.

The mean deformability of uninfected and ring stage parasites are more than two standard deviations apart from each other, which is important for the purpose of detecting malaria infection in peripheral blood where the majority of the infected cells will be in the ring and early Trophozoite stages [96]. The clear separation of the deformability between uninfected and various stages of infected cells validate the potential of using deformability

measurements to identify parasitized cells in order to determine parasitemia level, as well as the growth stage of the parasite.

Compared with the measurement data of parasitized RBCs performed using optical tweezers [8, 22], our technique indicated dramatically higher relative stiffness for Trophozoite and Schizont stage parasites, as well as reduced relative stiffness for ring stage parasites. Specifically, we found Trophozoite and Schizont stage parasites to be ~10 and ~200 times stiffer than uninfected cells, whereas optical tweezers studies found these cells to be ~4 and ~10 time stiffer respectively. Conversely, we found ring stage parasites to be ~2 times stiffer than uninfected cells, whereas optical tweezers studies found these cells to be \sim 3 times stiffer. These differences likely stem from the nature of the mechanical constraint associated with each measurement. Optical tweezers studies measure the resistance to tensile stretching applied to microbeads attached to two points on the cell surface, where the restoring force is primarily provided by cell membrane. Our microfluidic technique deforms the test cell in compression without tangential constraint, where the restoring force is primarily provided by the cytoplasm. In ring stage parasites, the dominant stiffening mechanism is the modification of the cell membrane caused by proteins exported by the parasite [26]. Therefore, a greater mechanical change is measured using optical tweezers. In Trophozoite and Schizont stage parasites, the dominant stiffening mechanism may be caused by the presence of the parasite bodies in the cytoplasm of the RBC. Consequently, a greater mechanical change is measured using our technique. For biophysical studies, our technique provides a measure of the stiffness of parasitized RBCs that more closely resembles the mechanics of circulation and blood vessel occlusion.

Furthermore, we did not observe a statistically significant difference between unexposed versus uninfected RBCs, which suggest that the presence of *P. falciparum* proteins in the cell culture medium does not change the stiffness of uninfected RBCs. Our finding contradicts results previously obtained using optical tweezers [8, 22]. Again, it can be explained by the difference in deformation of cells between in our device and under optical stretching mechanism.

Chapter 7: Deterministic Ratchet

This chapter studies the physical asymmetry associated of the deformation of single cells through a funnel constriction in the forward (along the tapered angle) and reverse (against the tapered angle) directions, and the potential to create a deterministic microfluidic ratchet. Section 7.1 presents results related to on the directional asymmetry of the deformation process. Section 7.2 examines the ratcheting behavior of cells traversing through a series of identical funnel constrictions.

7.1 Microfluidic Funnel Ratchet

In previous chapters, we demonstrated the ability of our microfluidic device to study single cell deformability by deforming single cells through the funnel along the taper direction (forward direction). In this chapter, we demonstrate and characterize the directional asymmetry associated with deforming single cells through such a funnel microstructure. Figure 7.1 illustrates the processes of cell deforming through the funnel along the taper angle (forward direction) and against the taper angle (reverse direction).

In theory, compressing cells through this constriction in the forward direction requires a smaller pressure than in the reverse direction because of equation 4.14 where R_b is smaller in the case of forward than the case of reverse direction due to the confinement of the funnel taper. Therefore, if an oscillatory pressure with amplitude between forward and reverse threshold pressures were to be applied, the cell would traverse through the funnel but could not squeeze back. A millimeter-scale version of this mechanism has been previously studied [97], where instead of using cells, these researchers deformed mercury droplet through a millimeter-scale funnel structure.



Figure 7.1 Schematic illustrations of cell deforming through the funnel constriction in the forward (A) and reverse (B) directions

An important distinction between this mechanism and previously reported micro-scale ratchet mechanisms is that previous mechanisms, with a few exceptions [76, 79, 80], are Brownian ratchets that rely on diffusion to provide the required fluctuating excitation. While these schemes are capable of transporting bacteria, micro-particles, and molecules with characteristic dimensions of approximately 1 μ m or less, they cannot be extended to the transport of mammalian cells, which typically have diameters greater than 10 μ m, and therefore have greatly reduced diffusivities. Furthermore, the ratcheting behavior reported here is thresholded by cell deformability, which is a physical parameter that can be associated with cell phenotype, morphology, and disease state [4, 98]. A mechanism that enables selective transport based on cell deformability could therefore be used to develop a wide range of new devices for cell separation and analysis.

7.2 Pressure Asymmetry

The measurements of *threshold deformation pressures* in both directions were the same as what was demonstrated previously. The pressure asymmetry was defined as the ratio of reverse threshold pressure versus forward threshold pressure. Here, we choose mouse lymphoma cells (L1210) and a single cell ($\Phi = 15.6 \mu m$) was tested to go through funnel

pore sizes of 6.8, 7.7, 8.7 and 9.6 µm with channel thickness of 13 µm. Figure 7.2 (A) shows the micrographs of single MLC traversing through funnel in both directions. Three to five measurements were made of a single cell going through each funnel in each direction and Figure 7.2 (B) illustrates the experimental results. Each data point on the graph shows the averaged threshold pressures the ranges of measured pressures. In order to minimize the residual stresses after the cell just fitted through the funnel, it is then given at least 30 seconds to recover its original shape before the pressure polarity is switched to squeeze the cell in the reverse direction.



Figure 7.2 (A) Micrographs of a single MLC being deformed through a funnel constriction in forward and reverse directions. (B) Measured forward and reverse threshold pressure required to deform a single MLC ($\Phi_{cell} = 15.6 \mu m$) through a 10 ° funnel constriction.

The pressure asymmetry results measured from the deformation of MLCs are also shown in a non-dimensional plot of the reverse-to-forward pressure ratio *versus* the cell-diameter-to-funnel-opening ratio (Φ_{cell}/W_0) in Figure 7.3 (B) for funnel constrictions tapered at 10°, 5°, and 0°. As expected, the measured asymmetry becomes greater at smaller funnel angles because of the more gradual taper leading to smaller R_b. The average asymmetry values for the 5° funnel and the 10° funnel are approximately 1.8 and 1.5 respectively. The 0° funnel is simply a 20 µm long rectangular slot constriction, as shown in Figure 8.3. Not surprisingly, deforming cells through this *control* constriction showed no pressure asymmetry.



Figure 7.3 (A) Micrographs of single MLC deforming through funnels of different shapes; (B) Pressure asymmetry for 10 , 5, and 0 funnel constrictions as a function of the cell diameter to pore size ratio (Φ_{cell}/W_0). The average asymmetry values for these microstructures are 1.5, 1.8, and 1.0 respectively.

7.3 Ratchet Behavior

To study the ratcheting behavior of cells in micro-scale funnel constrictions, we designed and fabricated a modified device consisting of 37 funnels with identical pore size ($W_0 = 6 \mu m$) arranged in series at a pitch of 60 μm , as shown in Figure 7.4. The chain of funnel microstructures is connected to the pressure attenuator and the cell inlet as before. The key difference is that the pressure attenuator is modified to generate a square-wave oscillatory pressure. This function is accomplished using a fluidic H-bridge, composed of four microvalves (Valve5 - Vavle8), which switches the polarity of the applied pressure differential (100 ΔP). The attenuation network then reduces this pressure differential to $\pm \Delta P$ across the funnel chain. The advantages of using fluidic H-bridge include the rapid reversal of the source pressure polarity and the elimination of the delay associated with inverting the pressure using an external source.



Figure 7.4 Design of the two-layer microfluidic device used to study rectified cell motion under unbiased oscillatory flow. ΔP is the desired pressure exerted across the funnel chain. $100\Delta P$ is applied across the inlet associated with Valve5 and Valve6, and in the opposite polarity between Valve7 and Valve8. Oscillatory pressure is generated by alternately sealing and opening Valve5 and Valve6 versus Valve7 and Valve8.

Single cells are introduced into this funnel chain through the inlet network. Once a cell reaches the funnel chain, an unbiased oscillatory pressure is applied, and the motion of an individual MLC through the chain of funnel constrictions is tracked by video analysis. The microscope stage is moved manually to follow the motion of the cell, while the funnel numbers are identified by markings on the side of the funnel chain (Figure 8.5 A). The displacement of single MLC in a funnel chain for several different amplitudes of the square-wave oscillatory pressure is shown in Figure 7.5 (B). The funnel pore size in this device is 6 µm, while the pressure oscillation frequency is 0.5 Hz. This graph also includes cell displacement data from a control experiment where the oscillatory pressure was applied to a MLC in an unconstrained micro-channel region prior to entering the funnel chain. The

sinusoidal cell displacement shown here confirms the reversible unbiased fluid flow in the funnel chain.



Figure 7.5 (A) Micrograph of the funnel chain; (B) The displacement of a single MLC $(\Phi_{cell} = 10.5 \mu m)$ in the funnel chain with a 0.5 Hz oscillation at various pressure amplitudes. The initial position of these curves has been offset such that the curves start at the same point. The no funnel curve is a control experiment that tracks the cell motion in an unconstrained microchannel prior to entering the funnel chain. (C) Frequency dependence of ratchet motion. Oscillatory pressure with a magnitude of 150 Pa is applied at frequencies of 1, 0.5, and 0.333 Hz.

Ratcheting behavior was observed when the pressure amplitude exceeds the threshold required to deform MLCs across a single funnel constriction along the direction of the taper. Specifically, at amplitude of 100 Pa, cells are confined to oscillate in the region between two funnels, but at 200 Pa and above, the cell begins to reliably ratchet forward in the funnel chain. Above 200 Pa, the cell travels both forward and backwards through several funnel

constrictions in each cycle. Ratcheting behavior is preserved at these higher oscillation pressure amplitudes since the asymmetrical threshold pressure enables these cells to transit through a greater number of funnels in the forward direction than the backward direction. The increased applied pressure also increases the net cell velocity. The average flow velocity for pressure amplitudes of 200 Pa, 250 Pa and 350 Pa are 15 μ m/s, 30 μ m/s, and 60 μ m/s respectively.

The ratcheting behavior is found to depend on oscillation frequency as shown in Figure 7.5 (C). At a frequency of 1 Hz and pressure amplitude of 150 Pa, the cell does not have sufficient time to traverse the region between the funnels (~50 μ m) and also deform across the funnel. Ratcheting transport is enabled at 0.5 Hz, and proceeds at an increased rate at 0.333 Hz, suggesting that ~0.5 s is required to complete the process to deform through the funnel constriction. Additionally, the ratcheting process is also dependent on the synchronization between the applied pressure and the location of the cell in a funnel constriction, as well as non-specific interactions between the surfaces of the cell and the microstructure.

Chapter 8: Summary and Conclusions

8.1 Summary of Thesis

In summary, we have developed a microfluidic device to test the single cell deformability using funnel-shaped micro-scale constrictions. *Threshold deformation pressures* require to deform individual cells through these constrictions are measured for funnel geometries with decreasing pore sizes and the results are converted to cortical tension, which is used to characterize intrinsic cell deformability.

To confirm the capability of the device to precisely measure the deformability of individual cells and to compare with traditional MPA, we used this device to measure the deformability of human neutrophils, which are the standard test cells in MPA. We measured a cortical tension of $37.0 \pm 4.8 \text{ pN/}\mu\text{m}$, which is consistent with previous studies. The CV value of the results from the microfluidic device is 0.13, which is in line with the best available results obtained using MPA. The deformability of other spherical cells, such as human lymphocytes, bladder cancer cells and mouse lymphoma cells, were also measured.

Subsequently, we used this device to measure the deformability of red cells parasitized by *P*. *falciparum*. Our results showed the expected progressive loss in red cell deformability as the parasite matures inside the host red cells. Moreover, the deformability of uninfected red blood cells can be distinguished from cells at the four stages of infection. The device can potentially provide a useful biomechanical assay to aid the development of new drugs and to study the mechanisms of drug resistance.

In the final chapter, we investigated the pressure asymmetry between forward and reverse deformation pressures. This directional asymmetry is then used to create a ratchet mechanism that enables unidirectional flow of cells through a series of funnel constrictions under unbiased oscillatory flow. The significance of the results includes the application of the ratchet mechanism in cell separation based on size and deformability. This work was initiated by McFaul [99] and continued by my colleagues Bill Lin and Emily Park.

8.2 Advantages over Micropipette Aspiration Experiment

The developed microfluidic device to measure cell deformability is similar to MPA in principle, but it leverages recent advances in microfluidics and microfabrication to improve ease-of-use while preserving measurement precision. The advantages of this microfluidic device over traditional MPA include the followings:

First, MPA experiment operates in an open environment where liquid from the sample is continuously evaporating. This fluid loss presents a drifting baseline pressure that must be periodically recalibrated in order to generate precise negative pressure. Our microfluidic device is a closed system impervious to evaporative losses, and thereby improves the accuracy of the applied pressure. The use of microfluidic networks to create a pressure attenuator further reduces the specifications required of external pressure controllers.

Secondly, MPA experiments require the use of a 3-axis micromanipulator to localize the pipette tip to the surface of a target cell. This process not only requires specialized equipment, but also considerable experimental skills, which further adds to the cost and difficulty of micropipette aspiration studies. Finally, MPA experiments typically use a single pipette to study a small population of cells from a sample. Since significant cell size variations exist within a sample or a phenotype, the diameter of the pipette orifice may not be optimal for each cell. Our device contains multiple constrictions that cover a range of sizes, thereby enabling the optimal constriction to be used for each cell and aspiration experiments using multiple constrictions.

8.3 Challenges Associated the Developed Microfluidic Device

The challenges associated with the application of the developed device are as follows. First, it is essential to ensure the matching of the device thickness with the cell type based on the size of the cell in order for proper pressure measurement. Cases of the funnel either thicker or thinner than the cell size will lead to either pressure leakage or too much friction and compression, both of which will cause incorrect pressure measurements. This limitation adds to the fabrication complexity.

Second, the device can only test the deformability of suspension cells with regular shape. Cells with irregular shape will complicate the pressure measurement since the directionality will affect the pressure required to squeeze the cell through the funnel. The device can't measure the cells that innately adhere to either PDMS or glass without any surface treatment.

Finally, the throughput of the device needs to be improved. The throughput of the device is approximately 25~30 measurements/hour, with at least 15 minutes device preparation time. This level of throughput is better than traditional MPA. However, in order for this device to be more useful in clinical situations, automated testing combined with greater throughput or reduced testing time would be more desirable.

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