Chromatographic Cell Separation based on Size and Rigidity using Dynamic Microstructures

by

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Abstract

The separation of cells by phenotype from heterogeneous mixtures, such as whole blood, is important in a wide range of fields in medicine and biology. Cell separation methods can be classified as either chemical or physical. Chemical separation methods are based on affinity capture and flow cytometry to label and select for specific target cell species. These techniques can be limited by the lack of specific chemistry that uniquely select for the target cell types, as well as the inability to extract viable cells for propagation in culture. When chemical separation methods cannot be applied, it is sometimes possible to discriminate cells based on their physical properties. As material systems, cells have an enormous range in size and rigidity and these differences can be exploited to achieve separation. Recent advances in microfabrication and microfluidic technologies have presented several innovative methods to approach mechanical cell separation.

Our research leverages key characteristics of microfluidic technologies to approach cell separation in a manner similar to liquid chromatography. In chromatography, target species are separated from a mixture by imparting different velocities based on interactions with the column. We apply this process to separate cells based on differences in their size and rigidity using a microfluidic channel with dynamic geometry. This channel is formed between a static surface, containing a series of traps, and a flexible membrane. The device is fabricated using standard microfabrication methods, including photolithography and multi-layer soft lithography.

As the cell mixture is flowed through the channel, the height of the channel is varied repeatedly causing periodic entrapment of the larger and more rigid cells, which impart a reduced average velocity to these cells compared to smaller and more deformable cells. Using this technique, we demonstrated chromatographic separation of L5178Y mouse lymphoma cells, representing larger and more rigid species, from human red blood cells, representing smaller and less rigid species. The ratio of the velocities of the target versus background cell types depends upon the duty cycle of the oscillation. We demonstrate the accumulation of mouse lymphoma cells in the microfluidic channel while maintaining cell viability. The system is simple, low-cost and label free.
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Chapter 1
Introduction

The separation of cells by phenotype from heterogeneous mixtures, such as whole blood, is important in a wide range of fields in medicine and biology. A notable example is the separation of white blood cells from whole blood which is required for many cellular assays. Cell separation methods can be classified as either chemical or physical. Chemical separation methods are based on affinity capture and flow cytometry to label and select for specific target cell species. These techniques can be limited by the lack of specific chemistry that uniquely select for the target cell types, as well as the inability to extract viable cells for propagation in culture. When chemical separation methods cannot be applied, it is sometimes possible to discriminate cells based on their physical properties. As material systems, cells have an enormous range in size and rigidity. In this research, we aim to use microfluidic techniques to develop new devices for separating cells based on these mechanical differences.

Microfluidics refers to a collection of techniques that enable the precise control over the flow of minute volumes of liquid. One of the pillars of microfluidics is microfabrication technologies developed for patterning intricate micrometer geometries to create complex integrated circuits. The combination of microfabrication and microfluidics enable the ability to create structures at the length scale of individual cells, and to do so with sufficient complexity in order to process a significant population of cells. One of the scions of traditional microfluidics techniques is multi-layer soft lithography of elastomeric materials, such as polydimethylsiloxane (PDMS). This technique has further enabled the ability to create dynamic structures in a microfluidic device using thin flexible membranes. Microfabrication, microfluidics, and multi-layer soft lithography forms the technical basis for the research presented in this thesis.

Fluid flow within microfluidics devices occur almost exclusively with Reynolds numbers less than 1 where viscous forces dominate over inertial forces. This property has two significant consequences for the research presented in this thesis: (1) the fluid flow in our microfluidics devices can be assumed to be exclusively laminar, and (2) the displacement of flexible membranes formed using multi-layer soft lithography is not limited by the inertia of the displaced fluid. We leverage these advantages to create a new mechanism for chromatographic separation of cells based on cell size and rigidity.
Our separation concept involves transporting a mixture of cells through a microchannel formed between a microstructured surface and a flexible membrane. As shown in Figure 1, the microstructured surface (layer 1) consists of an array of pockets that act as traps to capture the target cells, while the flexible membrane (layer 2) is designed to dynamically alter the geometry of the microchannel based on an externally applied pressure. This dynamic structure temporarily entraps the larger and more rigid cells in order to selectively impede their flow relative to that of smaller and more deformable cells. Specifically, the size- and rigidity-dependent flow rate is achieved by oscillating the flexible membrane between an ‘open’ position and a ‘semi-closed’ position. In the open position, the channel is sufficiently wide to allow all the elements of the mixture to travel. In the semi-closed position, only the smaller and less rigid cells can travel through the microchannel, while the larger and more rigid cells are trapped in the microstructured surface. Modulating the relative time period between the open and closed states enables precise control of the velocity of the target cells relative to the rest of the mixture. In addition, channel modulation minimizes the continuous interaction between target cells and the microstructure and thus can avoid potential clogging.

Our concept is similar in principle to chromatographic separation where a mixture is flowed through a column, which typically consists of a long microchannel or a porous matrix. Chemical and/or physical interactions between the mixture and the column selectively reduce the flow rate of the target components in order to enrich their concentration relative to the concentration of the background. Separation of cells based on physical interactions has not been practical because of the limited tolerance to shear forces and the tendency to clog the column. Separation based on chemical interactions has typically relied on flowing the sample through an affinity column that selectively captures the target cells onto its surface. The target cells can then be released by changing the solution pH or salt concentration, by increasing fluid shear flow, or by elastic deformation of the column.
material. Our technique uses dynamic microstructures to capture and release larger and more rigid cells from a mixture without the need for specific chemistry.

Using this concept, we designed, fabricated and tested two device prototypes. We validated the chromatographic behavior of the device using L5178Y mouse lymphoma cells (MLCs) and fixed human red blood cells (RBCs). We found the specific average velocities of the cells relies upon the percentage of the total time the device is in the semi-closed position. This can be defined as the duty cycle of the device and we found that with increasing duty cycles, cells experience increased relative displacement. Further, we studied the temporary displacement of fluid from the microchannels that is caused by the deflection of the membrane. We characterize the transient fluid velocity of the device using RBCs and determine the phenomenon to have no critical effect on cell separation. Finally, we demonstrate target cell accumulation in the microchannels while maintaining cell viability.

We can conclude this technique to be particularly well-suited for the enrichment of rare cells that are larger and more rigid than the background cells. This technique is simple, cost effective and label free. Future work aims to incorporate back and forth flow in order to accumulate rare cells, and to evaluate specific applications, such as the isolation of circulating tumor cells from the peripheral blood of cancer patients.

The presentation of this work include background, concept, design, methods, prototypes, testing, and conclusions. Two prototypes were designed, fabricated and tested. The design considerations and experimental methods of both prototypes were similar and is thus presented only once in chapters 4 and 5, while the specific device design and experimental results are presented separately in chapters 6 and 7.

**1.1 Research Objectives**

**1.1.1 Short-term Objectives**

The short term objectives are to study the concept of chromatographic cell separation using dynamic microstructures, demonstrate the feasibility of this concept, and develop a set of design rules for the separation of rare cells based on size and rigidity from heterogeneous mixtures.

**1.1.2 Long-term Objectives**

The long range objectives are to apply the techniques developed in the short-term objectives to the diagnosis and treatment of disease.
1.2 Organization of Thesis

This thesis is organized in the following order.

Chapter 1: The research objectives of this project are covered as well as the scope of this thesis.

Chapter 2: The motivation behind this research is introduced followed by a basic overview of the field of microfluidics including flow characteristics, flow control and current microfluidic applications. Also presented is a brief history of cell separation and its clinical importance. The fundamentals of cell mechanics are discussed as well as current physical cell separation methods in microfluidics.

Chapter 3: The concept behind our design is presented and the theoretical operation of the device is explored.

Chapter 4: The fundamental design considerations for the devices are presented.

Chapter 5: The fabrication procedure for the devices used in this project are discussed. The experimental setup and sample and device preparation are also described.

Chapter 6: The detailed design, experimental results and observations are discussed for the first prototype. Design recommendations are made for following designs.

Chapter 7: The detailed design, experimental results and observations are discussed for the second prototype.

Chapter 8: The conclusions drawn from the investigations are presented and recommendations are made for future designs.
Chapter 2

Background and Motivation

2.1 Microfluidics

The desire to create integrated circuits in microelectronics has led to the development of photolithographic processes capable of reliably creating complex structures with micrometer accuracy. The application of these techniques to create Microsystems with both electrical and mechanical function is what became known as micro electromechanical systems (MEMS) today. Notable examples of these types of systems include the MEMS accelerometer, the digital micromirror device, and the MEMS pressure sensor.

The desire to create MEMS devices to measure and manipulate fluids for applications in biology, chemistry, and medicine has led to the development of technologies for controlling the flow of minute volumes of liquid, or what became known as microfluidics. By leveraging the ability of photolithographic fabrication to create microstructures with great complexity, these techniques led to the development of products, such as DNA-chips, which enabled large numbers of biochemical reactions to be performed in parallel with minimal reagent consumption and therefore minimal cost. While these types of systems were initially created using micromachined silicon wafers as the substrate material, properties of these materials, such as expensive fabrication processes, intrinsic stiffness, opacity, and non-permeability to gases make this technology ill-suited to applications in fluid flow.

Many of these limitations could be overcome using soft polymer materials as the primary fabrication substrate. Specifically, polydimethylsiloxane (PDMS) is an optically transparent elastomeric material that is gas permeable (2). This material could be used to replicate microstructures originally fabricated on silicon wafers at a fraction of the cost of micromachining by using a technology developed by Xia and Whitesides known as “soft lithography” (3). Soft lithography greatly reduced the turnaround time and the cost for device fabrication, quickly fueling research innovation in the development of microfluidics devices. Some of the first functional microfluidic devices made using PDMS were developed for DNA analysis and cell sorting (4-6).

Shortly after the introduction of soft lithography, an extension of this process was developed to create flow control features, such as valves and pumps. This technique combined soft lithography with the capability to bond together multiple patterned layers of PDMS. Originally developed by Unger et al.,
“multilayer soft lithography” describes the fabrication of structures using separate layers of elastomer, each of which is separately cast from a micromachined mold (7). Now, ten years later, this method has led to the development of systems for biochemical assays, chemical synthesis, genetic analysis, drug screening, and cell separation to name a few.

The technological evolution from integrated circuit microfabrication to MEMS to microfluidics has presented several key new capabilities for creating systems with microscale features. Several key aspects, as listed below, are leveraged by the research presented in this thesis and are described in more detail in subsequent sections.

- Micrometer length scale features
- Device complexity
- Dynamic microstructures
- Negligible inertial forces in microscale flow
- Electrical circuit analogy of fluid flow in microfluidic devices

2.1.1 Micrometer Length Scale Features and Device Complexity
Microfabrication techniques have presented the ability to create features and hence fluidic structures at the length scale of individual cells. These structures enable micro scale mechanical manipulation of cells in order to discriminate them by their size, shape and deformability. This capability coupled with the ability to create structures with large complexity, allows sufficiently large numbers of cells to be processed. For example, a standard 100 mm diameter silicon wafer could be used to create approximately $10^8$ features if the average feature size is approximately 10 $\mu$m.

2.1.2 Dynamic Microstructures
The invention of multilayer soft lithography quickly popularized PDMS as a material for the fabrication of microfluidics devices containing sealed microchannels. As mentioned previously, PDMS is gas permeable which presents many design advantages such as device priming and the ability to manipulate the environmental conditions (8, 9). Another significant advantage is that PDMS is biocompatible, which when coupled with its gas permeability, makes it a suitable substrate for experimenting with and culturing mammalian cells (10).

PDMS is a two-part polymer compound, consisting of a base and a curing agent that undergo a hydrosilylation reaction upon cross linking(11). The two brands of PDMS that are frequently used in
microfluidics include RTV 615 (GE Silicone) and Sylgard 184 (Dow Corning). The following table presents generic physical and chemical properties of PDMS.

**Table 2.1:** Physico-chemical properties of PDMS(1).

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>Around 0.97 kg/m$^3$</td>
</tr>
<tr>
<td>Optical</td>
<td>Transparent, between 300nm and 2200nm, Index of refraction of 1.4</td>
</tr>
<tr>
<td>Electrical</td>
<td>Insulating, breaking field 20 kV/cm, Conductivity $4 \times 10^{-13}$ Ω m</td>
</tr>
<tr>
<td>Mechanical</td>
<td>Elastomeric, Young’s modulus 360-870 kPa, Poisson ratio 0.5</td>
</tr>
<tr>
<td>Thermal</td>
<td>Thermal conductivity ~0.15 W/mK</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Highly hydrophobic, contact angle 90-120°</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Nontoxic, Nonirritating to skin, no adverse effects on rabbits and mice, only mild inflammatory reaction when implanted</td>
</tr>
</tbody>
</table>

In multilayer soft lithography, two or more layers of PDMS, each cast from a separate mold, are bonded together via diffusion bonding. This bonding is achieved by offsetting the specific PDMS ratios of base:hardener so that when the layers are placed in contact for some time, the curing agent diffuses across the layer interface, resulting in a permanent bond. Typically, the bottom layer of these multilayer devices are constructed using a thin layer of PDMS, mixed at a base:hardener ratio of 20:1, that is spun onto a silicon wafer substrate. The thin layer is partially cured and bonded to the thicker top layer that is mixed at a base:hardener ratio of 5:1. This structure can then be peeled off the substrate and bonded to another thin layer or bonded to a silicon or glass surface by plasma activation. Figure 2.1 below show the addition of two separate molds to create a two-layer structure. In these cases the bottom layer is spun onto the substrate to achieve a predictable layer thickness.
Figure 2.1: A) A two-layer PDMS push-up microfluidic valve. B) A two-layer PDMS push-down microfluidic valve.

The intersection of microchannel cavities on two layers forms a thin flexible membrane, which can be used to create a valve when one channel is pressurized to obstruct the flow. Typically, the pressurized channel is known as the control channel, while the unpressurized channel is known as the flow channel. The sample liquid is typically injected into the flow channel. In order to ensure a complete seal, the flow channel must have a rounded profile in order to fully close when pressure is applied. As shown in Figure 2.1, when the flow channel is placed in the bottom layer, a “push-down” valve is formed, while the alternate arrangement yields a “push-up” valve. Push-down valves allow one to combine liquid control over a rigid substrate while push-up valves are better suited for taller flow channels, which is better for many applications including eukaryotic cell manipulations as is required by this research (12, 13).
As shown in Figure 2.1, the valve is essentially a thin flexible PDMS membrane. When a pressure is applied, the membrane deflects. The mechanical behavior of this membrane has been explored in several studies where force/deflection models have been developed(14, 15). Namely, a “thick beam” model, a “thin spring” model, and a “thick spring” model. These results were combined to produce a final model which produced the best agreement with the independently measured values in Kartalov’s study and good quantitative prediction in typically used systems of thin, wide membranes and low strains (15). The functional form of Kartalov’s final model is given below.

\[ P = E \cdot ln[1 + \left( \frac{16H^2}{3} \right) \left( W^{-2} + L^{-2} \right) + 4H(h^3 + \frac{16H^3h}{3} - \frac{16H^3}{5})/(W^{-4} + L^{-4})] \]

Where,

- \( P \) = Applied Pressure (Pa)
- \( E \) = Young’s Modulus (Pa)
- \( H \) = Flow Channel Height (m)
- \( W \) = Valve Width (m)
- \( L \) = Valve Length (m)
- \( h \) = Membrane Thickness (m)

Interestingly, Kartalov et al found that the thin spring model was closest to reality, meaning that valves do act approximately like thin springs. However, the inclusion of the other models were needed to take into account extreme conditions such as very thick membranes and narrow valves. The design of our microfluidic device is dependent on the accurate deflection of a PDMS membrane and thus Kartalov’s model is extensively used.

### 2.1.3 Negligible Inertial Forces in Microscale Flow

In microfluidic systems, the characteristic flow geometries typically range from a few microns to hundreds of microns. For fluid flow at this length scale, viscous forces dominate over inertial forces. This phenomenon is described by the Reynolds number, which is a dimensionless numbers that represents the ratio of inertial forces to viscous forces. Specifically, for the flow of water in a structure with characteristic length \( L \), where the fluid density, \( \rho = 1000 \text{ kg/m}^3 \) and shear viscosity, \( \eta = 10^{-3} \text{ Pas} \). For fluid velocities of less than 1 mm/s, which is typical for microfluidic system, the Reynolds number, \( \text{Re} = \rho L U / \eta \approx 10^{-2} \). Since this value is far less than 1, we can conclude that the viscous forces dominate over inertial forces in the fluid flow of these systems (16). The low Reynolds
number flow applies to the research presented in this thesis in two ways: (1) the fluid flow in our microfluidics devices can be assumed to be exclusively laminar, and (2) the displacement of the micro-valves, described in the previous section, is not limited by the inertia of the fluid the valve must displace.

Another way to approach the difference in physical phenomena between the micro- and macroscale is to apply a scaling law. This law states that if two quantities are proportional at one order of magnitude, then they must be proportional for another order of magnitude. Take for example capillary forces and gravitational forces. Capillary force is a surface force that arises due to a difference between adhesive intermolecular forces and cohesive intermolecular forces. The amount of capillary force is dependent on the surface tensions of the boundaries involved and can be interpreted as force per unit length (17). Thus, the force is proportional to \( L^2 \). Gravitational force is proportional to the mass (volume) or a body and therefore is proportional to \( L^3 \). Now it can be seen that at the macroscale, gravitational forces dominate capillary forces however at the microscale, the opposite is true.

### 2.1.4 Electrical Circuit Analogy of Fluid Flow in Microfluidic Devices

One of the principle laws of circuit design is Ohm’s law, which relates current, \( I \), and resistance, \( R \), to the electrical potential of the system \( \Delta V \) in the following form; \( \Delta V = IR \). Another is Kirchhoff’s law, deals with the conservation of charge in a circuit. Specifically, it maintains that the sum of the currents flowing into any node is equal to the current flowing out of that node and can be represented by \( \sum_{k=1}^{n} I_k = 0 \). These elementary equations have fluidic parallels when analyzing the transport of fluids in microfluidic systems as described in literature (18-20). Considering single-phase flow, we can apply a linear relation between the force applied to a fluid, the channel geometry and fluid viscosity, and the velocity of the liquid. Considering pressure to be the force applied (\( \Delta P \)), channel resistance to be a function of geometry and fluid viscosity (\( R_H \)), and fluid velocity as the flowrate (\( Q \)) we obtain the following equation,

\[
\Delta P = QR_H
\]
Considering Kirchhoff’s law, if we substitute Q for I, we obtain a relationship for the flowrate entering and leaving any particular node as such \( \sum_{k=1}^{n} Q_k = 0 \). A comparative figure illustrating fluidic and electrical equivalence is presented below.

\[ Q \rightarrow \sum_{k=1}^{n} Q_k = 0 \]

**Figure 2.2:** A) Pressure-driven flow in a system of parallel channels. The pressure difference, \( \Delta p \) is the same across each channel and results in a flowrate of \( Q_k \). B) The corresponding electrical representation with resistances, \( R_k \), in parallel and potential difference, \( V \). The electrical current is represented by \( I_k \).

To examine the hydrodynamic resistance, \( R_{H} \), of \( N \) channels with individual resistances \( R_m \), \( m=1,\ldots,N \), we consider the two simplest situations where the channels are either in parallel or in series. The same equations apply here as do in electrical circuits. Thus,

\[
R_{H(Total)} = \sum_{m=1}^{n} R_{H(m)}
\]  

3

\[
R_{H(Total)} = \frac{1}{\sum_{m=1}^{n} R_{H(m)}}
\]  

4

Where equation 3 represents the total \( R_{H} \) for channels in series, while equation 4 represents the total \( R_{H} \) for channels in parallel.

Finally, to calculate \( R_{H} \), the equation is similar to that for calculating the electrical resistivity of a circular wire; \( R = \rho L/\pi a^2 \), where \( \rho \) is the resistivity, \( L \) is the wire length and \( a \) is the radius. The fluidic equivalent, \( R_{H} \), is similar in form and is given as,

\[
R_{H} = \frac{12 \mu L}{wh^3}
\]  

5
where \( \mu \) is the fluid viscosity, \( L \) is the channel length, \( w \) is the channel width, \( h \) is the channel height and \( w \gg h \).

The following table compares the basic relations between electrical circuit and microfluidic systems.

**Table 2.2:** A comparison of basic electrical design equations and their microfluidic equivalents.

<table>
<thead>
<tr>
<th>Electrical Relation</th>
<th>Microfluidic Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ohm’s Law</strong></td>
<td>( \Delta V = IR )</td>
</tr>
<tr>
<td></td>
<td>( \Delta P = QR_H )</td>
</tr>
<tr>
<td><strong>Kirchhoff’s Law</strong></td>
<td>( \sum_{k=1}^{n} I_k = 0 )</td>
</tr>
<tr>
<td></td>
<td>( \sum_{k=1}^{n} Q_k = 0 )</td>
</tr>
<tr>
<td><strong>Resistances in Series</strong></td>
<td>( R_{Total} = \sum_{m=1}^{n} R_m )</td>
</tr>
<tr>
<td><strong>Resistances in Parallel</strong></td>
<td>( R_{H(Total)} = \sum_{m=1}^{n} R_{H(m)} )</td>
</tr>
<tr>
<td>( R_{Total} = \frac{1}{\sum_{m=1}^{n} R_m^{-1}} )</td>
<td>( R_{H(Total)} = \frac{1}{\sum_{m=1}^{n} R_{H(m)}^{-1}} )</td>
</tr>
<tr>
<td><strong>Resistivity</strong></td>
<td>( R = \frac{\rho L}{\pi a^2} )</td>
</tr>
<tr>
<td></td>
<td>( R_H = \frac{12\mu L}{wh^3} )</td>
</tr>
</tbody>
</table>

### 2.2 Cell Separation

The benefits of cellular analysis are widely recognized in medicine and the first complete blood test was performed in the 1960s by Alexander Vasten (21). Blood circulates the body through blood vessels for the purpose of providing cells with nutrients and oxygen, while transporting waste away from those cells. Theoretically, there is no change in the body that is not reflected by some change in the blood.

Cells in humans include red blood cells (RBCs), white blood cells (WBCs), and platelets. RBCs are the most populous variety and they contain a protein known as hemoglobin, which give blood its characteristic color. Hemoglobin is responsible for transporting oxygen to cells and removing carbon dioxide. RBCs do not contain a nucleus and are discoid in shape. These cells are approximately 8 \( \mu m \)
in diameter and approximately 2.5 µm in thickness. WBCs, also known as leukocytes, destroy and remove old or aberrant cells and cellular debris, as well as attack infectious agents and foreign substances. They are spherical cells and are typically 8-12 µm in diameter (13). Platelets are responsible for blood clotting and gather at a source of bleeding, where they clump together to form a plug that helps stop the bleeding (22).

The separation of cells by phenotype from heterogeneous mixtures, such as whole blood, is important in a wide range of fields in medicine and biology. For example, leukapheresis is a process where leukocytes in the blood are isolated or fractionated. This is important in blood transfusions to prevent adverse effects in the recipient as well as in leukemia patients to decrease their WBC count (23). In addition, many clinical assays require the prior separation of WBCs as the presence of other elements of blood can interfere with the assays (24).

Another important need for separation is in the isolation and enumeration of rare cell types as these cells cannot be separated by standard techniques. For example, the presence of circulating tumor cells (CTCs) in the blood of some cancer patients has been extensively noted in the literature to have significant diagnostic and prognostic relevance (25-27). Further, the isolation of these cells could be used as surrogate markers to monitor disease status and drug efficacy which has shown to have clinical significance in combating cancer as shown by Reuben et al and Urtishak et al (28-30).

Cell separation techniques can be categorized as two groups: Those that differentiate species based on physical methods and those that differentiate based on chemical methods. Current techniques for the chemical separation of cells are typically based on chemical affinity (Figure 2.3), which rely on specific chemical interactions between a cell and a surface. In flow cytometry, the marker/cell bond allows the cell to be separated based on the characteristics of the marker i.e. fluorescence-activated cell sorting (FACS) and magnetic cell sorting (MACS).
Figure 2.3: Some common cell separation strategies. Affinity capture separations operate based on specific cell adhesion to a matching antibody (A) where unmatched cells are washed away. Numerous capture antibodies can be used in an array (B). In this case different CD antigens are used in blood cell separation. These techniques can also occur in a column (C) which aids in elution and collection (31).

These techniques often require complex chemical processing steps which can make it difficult to extract viable cells after separation for further analysis and propagation in culture. Further, in many instances cells this approach is inadequate because of the lack of markers that specifically identify the target cells (31).

When affinity based separation methods cannot be used, it is sometimes possible to distinguish certain cell types by their mechanical properties. For physical separation, cells can be separated if there is a significant difference in the mechanical properties of the phenotypes i.e. size, shape, deformability, dielectric potential, buoyancy or other parameter. Particular methods include dielectrophoresis, field-flow fractionation, sedimentation and cellular sieving. For the majority of these methods, the separation processes lack specificity. For example, dielectrophoresis relies on the difference in dielectric potential of cells which is related to a difference in cell size. This difference in dielectric potential is often extremely small which limits the accuracy of the technique (31). Field-flow fractionation and sedimentation rely upon the density of the cells which again is often very similar. Physical separation is based on size and rigidity (13, 32) has the potential to be a selective process due to differences in these mechanical properties as well as the capability to fabricate accurate systems at the cell length scale using microfluidics. Before reviewing current work regarding cell separation based on mechanical properties, a brief review of cell mechanics is presented.

2.3 Cell Mechanics

Studies into the mechanics of single cells, sub-cellular components and biological molecules have rapidly evolved during the past decade with significant implications for biotechnology and human health. A large contributing factor to this progression has been the discovery of new methods for measuring force and displacement at the micro and even nano scale as well as bio-imaging. As material systems, cells are far more complex than engineered materials such as metals, ceramics, polymers and composites. They are dynamic and perform functions including metabolism, control, sensing, communication, growth, remodelling, reproduction and apoptosis (33). To serve all these functions, cells have a wide variety of characteristics and to measure these properties, several experimental techniques have been developed. These techniques can be broadly classified into three types: first, local probes in which a portion of the cell is deformed (type I); second, mechanical
loading of an entire cell (type II); and third, simultaneous mechanical stressing of a population of cells (type III). In Figure 2.4, a and b comprise type I; c and d comprise type II; e, f comprise type III.

Figure 2.4: Schematic representation of experimental techniques used to characterize living cells. Atomic force microscopy (a) and magnetic twisting cytometry (b) probe cell components (force resolution: $10^{-10}$ and $10^{-12}$ N respectively; displacement resolution: 1 nm). Micropipette aspiration (c) and optical trapping (d) deform an entire cell (force resolution: $10^{-10}$ and $10^{-11}$ N respectively). Shear flow (e) and substrate stretching (f) methods are capable of evaluating the mechanical response of a population of cells (34).

Alternative approaches using MEMS devices have been attempted such as that based on a cluster of micro-needles or a cantilever beam (35, 36). The reported data have shown the elastic moduli of living cells to have an enormous range as shown in Figure 2.5 below.
Specific mechanical properties of cells occurring in blood were recently characterized using atomic force microscopy (AFM) by two separate research groups. Rosenbluth et al in 2006 used a MEMS approach to trap white blood cells in micro-wells and then quantify the mechanical properties. Cross et al in 2008 examined metastatic cancer cells and found them to be far stiffer than benign cells as well as having a stiffness distribution over six times narrower. Interestingly, they also found that non-specific cell adhesion inherent to tumor and normal cells collected from patients showed the surface adhesion of tumor cells is ~33% less adhesive compared to that of normal cells (37, 38). A table with some results from these studies is shown below. Note, the elastic modulus of RBCs is effectively zero since these cells can be deformed without resistance. This is due to the absence of rigid structures within these cells such as a nucleus.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Approximate Size (µm)</th>
<th>Elastic Modulus ± SD (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Blood Cell</td>
<td>8 x 2.5 (discoid)</td>
<td>~0</td>
</tr>
<tr>
<td>White Blood Cell</td>
<td>8 - 12</td>
<td>156±87 (37)</td>
</tr>
<tr>
<td>Cultured Cancer Cells</td>
<td>8 - 15</td>
<td>530±200 (38)</td>
</tr>
</tbody>
</table>

These large differences in size and rigidity have sparked several different cellular separation techniques exploiting these differences. Before reviewing some of the methods used to separate cells based on size and rigidity, the following two phenomena should be noted as it affects cells flowing through narrow channels.

### 2.3.1 Cytoskeletal Remodelling

Living cells are dissimilar to typical materials in that they are dynamic and they can alter their structure in response to mechanical load. This is known as cytoskeletal remodelling and it occurs in cells in the bloodstream. When a RBC or a WBC is placed under a mechanical load, the cell structure undergoes changes. Specifically, the actin filaments within the cytoskeleton remodel which results in a decrease in shear modulus by > 60% (39). This phenomenon allows these cells to travel through extremely narrow capillaries.

### 2.3.2 Protein Adsorption

An extremely important factor relating to cell mechanics is due to protein adsorption occurring between proteins embedded in the cell membrane and the microstructure. This phenomenon results in a irreversible bond between a cell and the interacting surface and is a limiting factor in almost all cell assays (40). Two directly controllable factors determining the frequency and strength of protein adsorption are the specific surface properties of the interacting structure as well as the time the cell is in contact with the interacting structure. It has been established that the interaction between a protein and a surface increases with increasing hydrophobicity of the surface and increases with increasing hydrophobicity of the protein (41). Although we cannot alter the hydrophobicity of the protein without altering the chemical form of the cell, we can decrease the hydrophobicity of the interacting
surface via surface modification (42). In work presented by Andrade and Hlady, they conclude that as contact or residence time increases, the protein tends to orientationally and conformationally adjust to the interface, leading to stronger bonding (43). This factor is of specific importance in our research as our concept seeks to minimize the continuous interaction time between the cells and the microstructure.

2.4 Target Applications

Cancer is the leading cause of death worldwide: it accounted for 7.9 million deaths (approx. 13% of all deaths) in 2007 (44). It is a generic term for a large group of diseases that result in the rapid creation of abnormal cells that grow beyond their usual boundaries. These cells can then disseminate through the body, mainly by intravascular routes, via a process known as metastasis (45). Specifically, parts of the primary tumor (“original cancerous tissue”) break away and enter the lymphatic or blood vessels where they circulate the blood system. These cells are known as CTCs and some are eventually deposited in normal tissue where they can begin a new tumor. Metastases are the primary cause of cancer related deaths.

One of the primary motivations for this research is to establish a technique for isolating and enumerating CTCs. The idea arose due to the physical differences observed between CTCs and other hematological cells, namely size and rigidity. These physical differences may be able to be exploited in other cell types such as white blood cells (WBCs), cardiac myocytes and mesenchymal stem cells (MSCs). This can be realized using precise microfluidic systems combined with the particular physical properties of the cells to be separated.

2.5 Size- and Rigidity-Based Cell Separation

Here we present a brief review of the work done in the field of microfluidics applied to cell separation based on size and rigidity. Previous methods have focused on the use of sieve filters to separate cells by their ability to transit through a series of small pores at a particular pressure. The separation of three specific phenotypes from their respective background cells will be discussed, namely WBCs from RBCs, cardiac myocytes from non-myocytes and CTCs from hematological cells.

The separation of WBCs by physical means has been attempted by several research groups over the past 20 years. A key component of most genetic tests is a polymerase chain reaction (PCR) and considerable effort has been expended miniaturizing this reaction. Haemoglobin in RBCs is an inhibitor of the PCR reaction and thus the presence of RBCs is detrimental in PCR reactions (46).
This phenomenon motivated research in WBC enrichment with Carlson et al in 1997 and Wilding et al in 1998 (47, 48).

Wilding and colleagues managed to separate WBCs from RBCs using microfluidic filters of decreasing feature size (Figure 2.6). They achieved adequate separation for genomic amplification processes (e.g. PCR) which isolates WBCs with 10% efficiency and removes RBCs with 99.9% efficiency (48). Even at this early stage, they made reference to the potential application of isolation of rare cell types such as foetal cells in maternal circulation and CTCs. This research eventually culminated in 2003 with a portable PCR analyzer incorporating this WBC enrichment device (49).

![Figure 2.6: One of the decreasing weir type structures used to trap WBCs in the narrow gap between the top of the weir and the under surface of the cover (49).](image)

This device demonstrated effective isolation of WBCs from RBCs based on their larger size and greater rigidity. However, they experienced target cells becoming permanently stuck in the traps. This results from protein adsorption which negates the possibility of extracting viable cells as the adsorption process is considered irreversible (40). Further, permanently trapped cells reduces the throughput of the filter and compromises its selectivity and increases the hydrodynamic resistance of the filter over time.

In an attempt to prevent clogging and facilitate continuous separation, a similar device was developed by Sethu et al (32, 50). In this device, the sieves were arranged on the sides of the channels, connecting the channel to a diffuser to attempt continuous flow operation. To ensure continuous volumetric flow through every filter element, it was designed with a flared geometry (Figure 2.7). This device was initially designed for use in leukapheresis however it was modified for use in the separation of myocytes from non-myocytes from neonatal rat myocardium.
This approach was relatively successful and managed to demonstrate enriched subpopulations of neonatal rat heart cells, myocytes and non-myocytes, on the basis of size and deformability while maintaining viability. In the case of WBC separation, the device managed to achieve close to 99% depletion of WBCs and isolation of approximately 50% of RBCs. In the literature however, they make reference to filter elements becoming clogged at the interface of the main channel and the filter elements due to improperly oriented RBCs and large WBCs (50). This they could overcome using low flow rates however non-specific cell adhesion remained troublesome.

In 2007 VanDelinder et al describes a device operating with a continuous cross-flow to separate WBCs from whole blood (13). They reported a ~4000-fold enrichment of WBCs in a single passage with continuous flow and without cell lysis. This device retained 98% of WBCs and was made up of a single deep channel with a large number of orthogonal, shallow side channels. As a suspension of particles advances through the main channel, a cross flow washes away particles that are small/deformable enough to enter the side channels. This is illustrated in the sequence of images below.

**Figure 2.7:** Schematic of the diffusive filter for size based continuous flow fractionation of myocytes and non-myocytes from neonatal rat myocardium. The insert shows the 40 µm x 5 µm sieve structure and the arrangement connecting the main channel to the diffuser. In WBC separation, the sieve structures were fabricated as 40 µm x 2.5 µm (32).
Figure 2.8: Images of RBCs entering side channels from the main channel. The horizontal (main) channel is 25 µm deep and the vertical (side) channels are 3 µm deep. No WBCs are seen in (h) due to their low concentration and short exposure time (13).

This approach successfully separated WBCs from whole blood and reported an enrichment 2 orders of magnitude higher than previous microfluidic devices utilizing continuous flow without RBC lysis. Once again however, the separation mechanism relies on continuous cell/microstructure interaction.

To minimize continuous cell/microstructure interaction, numerous suggestions are made in the literature towards potential applications of the sieve based approach to rare cell enrichment and isolation. One of the ideas behind this is that if the number of large and rigid cells is extremely small in comparison to the background, cell/microstructure interaction and hence non-specific cell adhesion will not prove a significant problem. Another attractive reason for rare cell isolation based on mechanical properties is because of the lack of specific markers for rare cells and separation based on physical parameters often yields viable cells which can then be propagated in culture. Recently, much research has been carried out in the hope of isolating CTCs from whole blood using physical means.

In 1964, Seal recognised the larger and more rigid nature of cancer cells as compared with haematological cells (51). To take advantage of this fact, he designed a sieve-based filter utilizing pore sizes in the range of 3 - 5 µm. He found this to be successful in separating the cancer cells however fabrication of consistent pore sizes was a problem as well as clogging. Even in this early
stage of research into rare cell isolation, Seal makes reference to the potential benefits of preserving the cells viability.

Advances in microfabrication and microfluidics technologies have sparked more research into CTC isolation. Vona et al in 2000 demonstrated a new method to isolate epithelial cells based on their size (52). A membrane consisting of 8 µm cylindrical pores was used to filter the cells via gentle aspiration. The cells were then stained and the membrane could be examined. This method was useful for identifying cancer cells however it is at the expense of viability.

More recently have been studies successfully isolating viable CTCs. In 2004, Mohamed et al developed a rare cell fractionation device that separated individual cells based on their mechanical characteristics (53). In this study, they isolated cultured neuroblastoma cells from whole blood, based on their size and rigidity, using a sieve of progressively narrowing channels (Figure 2.8).

![Figure 2.9: Schematic for the Mohamed et al device showing size- and rigidity-based cell separation (53).](image)

This device successfully isolated cells, however obtaining the cells from the device after separation was difficult. This method had successfully been applied to the isolation of rare cells however it was still plagued by cell adhesion. In 2009, Tan et al presented a microfluidic device isolating CTCs based on their size and mechanical deformability (54). This device again sought to filter CTCs based on their size and rigidity but tried to minimize microstructure/cell interaction. This was designed to prevent cell adhesion and allow for viable CTC extraction after separation.

To accomplish this, filters were designed as crescent-shaped isolation wells with gaps of 5 µm within each microstructure. These wells were spaced 50 µm apart and consecutive lines of wells were offset to enhance hydrodynamic efficiency. The trapping system operates by allowing smaller/more deformable cells to travel around the isolation wells or pass through them. Once target cells have been captured, their presence causes the flow pattern to divert to help avoid clogging. In this way the
design maintains hydrodynamic efficiency by diverting incoming cells to the next level of wells. When removing cells, the flow direction is simply reversed and the cells can leave the traps (Figure 2.10).

![Image of MCF-7 breast cancer cells](image)

**Figure 2.10:** Above: CTCs trapped in crescent-shaped structures. Flow downward. Below: Time sequence images showing flow reversal for cell retrieval. Flow upward (54).

In this test, human breast cancer cells (MCF-7) are added to whole blood at a concentration of 100 cells/ml and filtered at 0.7 ml/hr. The device achieved a 80% isolation efficiency and isolated cells were retrieved using flow reversal. The retrieved cells were then successfully cultured to verify viability.

This filter design attempts to decrease the surface area of cell/microstructure interaction. Although the authors found this technique to yield viable cells. The time the cells are in contact with the traps may prove a problem, as well as cytoskeletal remodelling as the cells are under a continuous mechanical load. After a certain time (~ 30 s), the cells may experience a significant decrease in shear modulus and thus escape from the trap.

### 2.5.1 Key Trends

Physical interaction with the cells is a prerequisite to separating cells based on size and rigidity, . A reoccurring limitation of the approaches described here is the non-specific adhesion of cells, causing a loss of specificity in numerous sieve devices. These results illustrate the need to reduce the
undesirable adhesive tendencies of the cell/device interface which can be done in three ways: (1) by reducing the surface area of the cell/microstructure interaction, (2) reducing the time of continuous cell/microstructure contact or (3) modifying the surface of the device to decrease hydrophobicity.

In this thesis, we propose a method to modify the size of the microchannel in order to minimize the continuous contact between cells and the microstructure. This is hoped to be utilized in tandem with surface modification to effectively manage the clogging problem while allowing for continuous cell separation based on size and rigidity. Furthermore, by separating based on physical properties, we endeavor to maintain cell viability.
Chapter 3
Concept

3.1 Introduction

The separation of cells based on physical parameters has recently become feasible due to precise microfabrication and microfluidics technologies. These technologies have enabled the ability to create structures at the length scale of individual cells and the ability to precisely control the flow of minute volumes of liquid. Multi-layer soft lithography of elastomeric materials, such as polydimethylsiloxane, has further enabled the ability to create dynamic structures using thin flexible membranes (7, 55). In this thesis, we apply these capabilities to create a microfluidic channel with dynamic geometry that acts in a chromatographic fashion to separate cells based on differences in their size and rigidity. One of the key novelties of this work is the selective attenuation of the velocity of target cells through the use of dynamic microstructures, which is made possible by a dynamic membrane and the negligible inertia of the liquid it displaces at the length scale of microfluidics.

3.2 Separation Concept

Our separation concept involves transporting a mixture of cells through a microchannel formed between a microstructured surface and a flexible membrane. As shown in Figure 3.1, the microstructured surface (layer 1) consists of an array of pockets that act as traps to capture the larger and more rigid (target) cells, while the flexible membrane (layer 2) is designed to dynamically alter the geometry of the microchannel based on an externally applied pressure. This dynamic structure creates temporary entrapment of the target cells in order to selectively impede their flow relative to that of smaller and less rigid (non-target) cells. Specifically, the size- and rigidity-dependent flow rate is achieved by oscillating the flexible membrane between an ‘open’ position and a ‘semi-closed’ position. In the open position, the channel is sufficiently wide to permit the travel of all elements in the mixture. In the semi-closed position, only the non-target cells are permitted to travel through the microchannel, while the target cells are arrested in the traps. Modulating the relative time period between the open and semi-closed states enables precise control of the velocity of the target cells relative to the rest of the mixture. In addition, this modulation minimizes the continuous interaction between target cells and the microstructure and thus can manage potential clogging.
Our separation concept is similar in principle to liquid chromatography where a mixture is flowed through a column, which typically consists of a long microchannel or a porous matrix. Chemical and physical interactions between the mixture and the column selectively reduce the flow rate of the target components in order to enrich the concentration of these species relative to the concentration of the background. Chromatographic separation of cells based on differences in their mechanical properties is not currently possible because of the lack of a consistent method to mechanically interact with the cells. Consequently, these techniques typically use flow through an affinity column that selectively captures the target cells onto its surface. The target cells can then be released by changing the solution pH or salt concentration, by increasing fluid shear flow, or by elastic deformation of the column material (56-58). Our physical separation technique employs a similar capture-and-release approach, but is applied repeatedly to enable consistent attenuation of the velocity of the larger and more rigid cells. Modulation of the channel geometry has the additional benefit of continuously disturbing the contact between the cells and the microstructures, which reduces the potential for cell adsorption and clogging.

The ability to discriminate various cell types using the dynamic microchannel can be characterized by the retention factor ($R_f$), which is defined as the ratio of the time required for non-target versus target cells to traverse a unit length of channel (59). For $R_f$ values near 1, the ability of the channel to discriminate target cells is weak and a long channel length is required for separation. For $R_f$ values near 0, the ability of the channel to discriminate target cells is strong, but there is increased potential for clogging when the target cell density is high. Unlike traditional liquid/gas chromatography columns where $R_f$ is fixed, the $R_f$ for the dynamic microchannel is controlled by the duty cycle of the membrane oscillation, which is defined as the ratio between the amount of time spend in the semi-closed state versus the period of the oscillation cycle. The adjustable retention factor of the dynamic microchannel opens the possibility to adjust the properties of the column during the separation.
process depending on the concentration of the target cells. For example, the Rf could be lowered while the target cell concentration is low and then increased for a higher target cell concentration.

3.3 Theoretical Operation

Consider fluid flowing through the channel with a constant forward flow rate. In the open state, the channel is sufficiently high such that both target and non-target cells can pass through the filter at a certain velocity. In the semi-closed state, target cells are arrested in the traps however non-target cells can still travel. We first analyze the flow of cells in both of these situations without considering the transient flow caused by fluid displaced by the channel height modulation. To demonstrate device operation we will assume that in the open state all cells travel at $x \, \mu m/s$ and in the semi-closed state target cells are stationary while non-target cells travel at twice the open state velocity ($2x \, \mu m/s$).

The ability of this mechanism to separate target and non-target cells is dependent on the duty cycle (DC) of the membrane deflection. The DC is defined as the fraction of time that the system is in the semi-closed state as follows,

$$DC = \frac{T_{SC}}{T_{SC} + T_{OPEN}} \times 100\%$$

Where, $T_{OPEN}$ and $T_{SC}$ are the periods when the system is in the open and semi-closed state respectively.

Assuming that the membrane actuates uniformly, non-target cells travel with a constant velocity in both states, target cells are completely trapped in every semi-closed state, and the system responds with either a semi-closed or open state flow velocity without any backflow or pressure build-up, the following graph illustrates the predicted motion of the target and non-target cells.
Figure 3.2: Theoretical cell displacement versus time graph. Cells are introduced into the filter with a constant fluid velocity of x μm in the “open-state”. The duty cycle of the oscillating membrane is overlaid.

Figure 3.2 shows the displacement versus time graph of target and non-target cells with a DC of 0.36. The instantaneous cell velocity is represented by the slope of the graph. In the semi-closed state (Region A), target cells are stationary while non-target cells travel at an increased velocity. In the open state, both types of cells travel at the same velocity as shown by the identical slopes. The dynamic capture and release of the target cells causes them to travel at a lower average velocity than non-target cells and thus enabling separation.

To investigate the effect of the percentage of time the membrane is semi-closed, we can extend this theory over a range of DCs. Setting the $T_{SC}$ at a constant 4 s, we choose four values of $T_{OPEN}$, including 10 s, 7 s, 4 s, and 2 s. We then plotted the theoretical cell displacements for DCs associated with each of these parameters.
Table 3.1: A range of duty cycles for the microfluidic system

<table>
<thead>
<tr>
<th>$T_{SC}$ (s)</th>
<th>$T_{OPEN}$ (s)</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>0.29</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Figure 3.3: A theoretical plot of target and non-target cell displacements when the system is operated at various DCs.

Figure 3.3 shows the displacement between target and background cells at various duty cycles. The graphs show greater relative cell displacement at higher DCs as target cells are trapped for a greater proportion of the total time. The relative displacements over time between each target and non-target cell pair in Figure 3.3 are plotted in Figure 3.4. These graphs indicate the relative displacement for each duty cycle and tend towards a steady-state value. As shown in Figure 3.4, for every DC the relative cell displacement tends to a certain value over time. With decreasing DC we can observe a
decrease in relative cell separation. When this relative displacement is plotted against DC, the following dimensionless graph can be obtained.

**Figure 3.4:** Theoretical relative displacement between target and non-target cells ($S_{\text{Target}}/S_{\text{Non-target}}$) versus time.

**Figure 3.5:** This graph shows the impact of the system’s duty cycle on the relative displacement of target to non-target cells.

Figure 3.5 shows that with decreasing duty cycle, less cell separation is observed. This is expected as a duty cycle of zero (i.e. constant open state) would result in no separation and therefore a relative cell displacement of 1. Conversely, a duty cycle of 1 (i.e. constant semi-closed state) would allow zero target cell displacement and thus a relative cell separation of zero. Interestingly, this plot is independent of flowrate in which case the device characteristics (i.e. geometry, hydrodynamic resistance) govern the separation efficiency.

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Chapter 4
Design Considerations

4.1 Channel Design

In order to achieve membrane deflection necessary to achieve dynamic channel geometry, this device is fabricated as a two-layer polydimethylsiloxane (PDMS) microstructure using multilayer soft lithography. Traps are placed along the length of the channel in the flow layer. The control channel will encompass the entire channel and the following cross section will result.

![Figure 4.1: Left: A cross-section across the fluid flow in the channel (front view). Right: A cross-section along the flow of the device (side view). Horizontal arrows indicate direction of flow.](image)

The control channel is filled with water and serves to actuate the membrane by pressure as shown in Figure 4.2.

![Figure 4.2: A front view cross section of the two layer device. When pressure is applied in the control channel the membrane deflects accordingly.](image)

As shown in Figure 4.2, deflection of the membrane in this structure would nearly seal the channel, which would prevent the passage of all cells and possibly cause cell damage. Thus, a mechanical stop is required to control the membrane deflection between open and semi-closed states. A locating fin is
incorporated into the design to act as a mechanical stop thereby controlling membrane deflection in the flow channel and preventing excess pressure from being applied to the target cells.

![Diagram](image)

**Figure 4.3:** A front view cross section of the two layer device. A locating fin in the centre of the channel serves to prevent the membrane from sealing the flow channel. **A)** Open state, **B)** Semi-closed state.

The target cells used to validate this cell separation mechanism are cultured cancer cells. As discussed in Chapter 2, these cells range between 8 and 15 µm. The non-target cells used will be human RBCs which are approximately 8 µm discoids and extremely deformable due to their lack of a nucleus. Thus, to be sure to trap the cancer cells, a semi-closed channel height of 5 µm is used. An open channel height of greater than 15 µm should be used to allow all cells to flow. Thus total membrane deflection will most likely range from 10 µm - 20 µm.

The width of the channel is governed by a 10:1 aspect ratio inherent to PDMS microstructure design. Due to the elastic nature of PDMS, a channel should be no more than 10 times wider than it is tall in order to prevent the channel collapsing onto the glass slide during fabrication. A channel height of 20 µm means our maximum channel width should be no greater than 200 µm. To gain insight into the pressures required to deflect a membrane of this width, empirical data from a study by Studer et al. (60) are presented.
Figure 4.4: The mapped actuation pressures required to deflect a 5 µm thick membrane of varying dimensions a distance of 54 µm (60).

As shown in Figure 4.4, the pressures required to deflect a 200 µm wide membrane a distance of 54 µm lie in the range of 0 - 20 psi providing the other dimension does not lie in the extreme. With oxygen plasma bonding the pressures PDMS devices can withstand is approximately 40 psi (61). In addition, the total membrane deflection required ranges from 10 µm - 20 µm, so it can be reasoned that the membrane deflection required by this design is possible.
A 3D computational model of the device was developed using Solidworks 2009 (Dassault Systemes, Solidworks Corp.) to better understand the channel geometry and the deflection characteristics of the membrane.

![Diagram of the device model](image)

**Figure 4.5:** Simplified device models. Not to scale. **A)** Isometric view of channel construction with an offset cross-section along the direction of flow. **B)** Device model with zero control channel pressure. Open state. **C)** Device model with pressure applied in the control channel. Semi-closed state.

In the FEA simulation shown in Figure 4.5(C), the membrane has come into contact with the fin however there is still some deflection around it. This continual deflection may result in the channel becoming blocked or damage being done to the cells. However, a crucial design aspect is the central location of the fin. When the membrane makes contact with the fin, the width of the membrane is essentially halved. By looking at Figure 4.4, it can be seen that in the region of 200 µm, halving the channel width significantly increases the rigidity of the membrane and thus raises the pressure required for further deflection. This two-state membrane structure is important because not only is the applied pressure variable, the fabrication process is subject to many inconsistencies which may affect the properties of the membrane. Thus a greater than necessary pressure can be applied to the membrane in order to ensure complete actuation without fear of channel blockage or cell damage.

More detailed calculations regarding membrane dimensions and actuation pressures will be presented in section 4.3 Membrane and Control Channel Design.

Another potential problem is the chance that when the membrane has deflected and the pressure is released, it may not return to its original position. This potential “membrane sticking” should not
prove troublesome due to three factors. First, there is a positive pressure in the flow channel in order to have constant fluid flow which would aid in forcing the membrane to its original position. Second, the membrane is made of PDMS which is an elastic material and will tend to resume its original form. Thirdly, the membrane thickness can be easily increased should this prove a problem. This would increase membrane rigidity causing it to exhibit stiffer spring characteristics and may be beneficial as a thicker membrane may prove more robust in the fabrication process.

A final foreseeable issue is the deflection profile of the membrane. Figure 4.5(C) shows a lack of membrane deflection at the outer edges of the flow channel. This is to be expected however may prove crucial as the height of the channel (in this area) will remain large in both states. To prevent target cells from travelling in this area, boundary fins will be introduced. They will have the same height as the centre fin due to restrictions (placed by the Stanford Microfluidics Foundry) on the number of layers of a design.

![Diagram](image)

**Figure 4.6:** A front cross-section of our two-layer device in the semi-closed state. A) Membrane deflection at the edges of the channel is minimal allowing target cells (1) and non-target cells (2) to flow. B) Boundary fins (3) prevent target cell motion in areas of minimal membrane deflection.

As shown in Figure 4.6, the deflected membrane forces target cells upward into the traps. To prevent cell damage, the traps will be made a uniform depth of no greater than 20 µm and will be placed in various configurations across the width of the channel. The different configurations will be explored in greater depth in the detailed device designs in chapters 6 and 7.

The control channel will be made 25 µm high as a standard height specified by the Stanford Microfluidics Foundry. The final channel profile is shown in the schematic below.
The thickness of the membrane, $t$, separating the two channels is unknown. This is a crucial dimension and will be addressed in section 4.3 Membrane and Control Channel Design.

To increase the number of devices on a single wafer, the flow channel length was restricted to 6000 $\mu$m. This length was chosen as a tradeoff between space limitations on the actual silicon wafer and the desire to make the channels as long as possible to allow the maximum time for particle separation. This increased number of devices can be used to vary certain parameters however the main motivation for designing several devices on a single wafer is to increase the number of devices manufactured per successful fabrication. This is important as the fabrication process is still being perfected and is not always reliable.

4.2 Hydrodynamic Resistance

Given a required flow rate, the pressures needed to generate it can be calculated using the product of the volume flow rate, $Q$, and the hydrodynamic resistance, $R_{H}$. The specific equations are given below.

$$\Delta P = QR_{H}$$

$$Q = WHU$$
where $\Delta P$ is the required pressure, $W$ is channel width, $H$ is channel height and $U$ is flow velocity.

Note that the following formula for $R_{H}$ is for a rectangular channel where $W >> H$, and

$$R_{H} = \frac{12\mu L}{WH^3}$$

Where $\mu$ is fluid viscosity and $L$ is the length of the channel.

**Figure 4.8:** Front view cross section of the device split up into regions of differing hydrodynamic resistance. In the open state, the flow area is represented by 1 & 2 while in the semi-closed state, the flow area is represented by 3. Note that region 2 encompasses region 3.

The flow channel profile has an non-rectangular cross-section. Thus, to calculate $R_{H}$, we will treat the open state as five rectangular channels running in parallel (regions 1 & 2) while in the semi-closed state we will assume only two channels running in parallel (regions 3). Now $R_{H}$ can be calculated using the following formula.

$$\frac{1}{R_{H}} = \sum \frac{1}{R_{I}}$$

When the channel is in the semi-closed state, we will treat the channel as two rectangular channels 77.5 µm across and 5 µm tall and thus calculate $R_{H}$ using equation 3. Including approximates for the hydrodynamic resistance of the system outside the trapping channel, the $R_{H}$ for the separate flow regions and specific states can be calculated:
Table 4.1: The specific $R_H$ for the flow channel regions and in open and semi-closed states.

<table>
<thead>
<tr>
<th>Region</th>
<th>$\text{Pas/m}^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$5.3E+14$</td>
</tr>
<tr>
<td>2</td>
<td>$5.3E+13$</td>
</tr>
<tr>
<td>3</td>
<td>$6.6E+15$</td>
</tr>
<tr>
<td>Open</td>
<td>$1E+14$</td>
</tr>
<tr>
<td>Semi-closed</td>
<td>$3.4E+15$</td>
</tr>
</tbody>
</table>

A limiting factor in previous attempts at chromatographic cell separation is the tolerable shear stress. In previous studies measuring RBC velocity in vivo, it was found that in capillaries of approximately 20 µm diameter experienced cell velocities at rest of $0.84 \pm 0.53$ mm/s with a range of 0 - 3.47 mm/s (62, 63). In order to keep cell shear stresses within the physiological range, similar fluid velocities will be used. Using these velocities, the required flow rate can be calculated using equation 8 and the subsequent required channel pressure using equation 2. The results are presented in the table below.
Table 4.2: This table compares the pressures required (in a single micro-channel) to achieve physiological velocities in both the open and semi-closed states.

<table>
<thead>
<tr>
<th>Velocity (mm/s)</th>
<th>Required Flowrate (m³/s)</th>
<th>Required Channel Pressure (mbar)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Open</td>
<td>Semi-closed</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>4.78E-13</td>
<td>7.75E-14</td>
</tr>
<tr>
<td>0.5</td>
<td>2.39E-12</td>
<td>3.88E-13</td>
</tr>
<tr>
<td>1</td>
<td>4.78E-12</td>
<td>7.75E-14</td>
</tr>
</tbody>
</table>

The channel pressures needed to generate these flow velocities are very small. These pressures will also decrease if any channels are placed in parallel in proportion to the number of parallel channels (i.e. if 2 channels were in parallel the pressures required would be halved). In general, it is very difficult to achieve such low pressures with a high resolution. The external pressure controller currently available for this research is a Fluigent MFCS-4C which has a pressure range of 1000 mbar with a resolution of approximately 1 mbar. This is insufficient to generate and regulate these pressures at flow velocities below 0.5 mm/s.

We increase the hydrodynamic resistance in order to increase the required pressure to levels obtainable by our external pressure controller. This goal was accomplished by placing additional hydrodynamic resistance in series with the device as shown below.
Figure 4.9: A simple top view schematic of device design. The flow channel is in black while the control channel is in gray. Additional hydrodynamic resistance is placed in series to increase the total resistivity of the system.

As we have a finite area in the microfluidic devices (<2cm), we can add $R_{hi}$ by connecting external tubing in series with the channel. For example, connecting an additional $R_{hi}$ of $5 \times 10^{14}$ Pas/m$^3$ in series results in the new input pressures shown in Table 4.3.
Table 4.3: This table compares the pressures required (in a single micro-channel) to achieve physiological velocities in both the open and closed states.

<table>
<thead>
<tr>
<th>Velocity (mm/s)</th>
<th>Open</th>
<th>Semi-closed</th>
<th>Open</th>
<th>Semi-closed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>4.78E-13</td>
<td>7.75E-14</td>
<td>20.5</td>
<td>5.9</td>
</tr>
<tr>
<td>0.5</td>
<td>2.39E-12</td>
<td>3.88E-13</td>
<td>102.7</td>
<td>29.4</td>
</tr>
<tr>
<td>1</td>
<td>4.78E-12</td>
<td>7.75E-14</td>
<td>205.3</td>
<td>58.8</td>
</tr>
</tbody>
</table>

This result shows a marked increase in required pressure to values more easily attained by the pressure generating device. Since the addition $R_n$ is far greater than that of the open channel, the difference between the pressures required by different states is now smaller. An important reason to reduce the overall change in $R_n$ is the tendency of the system to behave similarly to a displacement-driven system with certain characteristics of a pressure-driven system.

4.3 Displacement-driven Versus Pressure-driven Flow

In the case of a displacement-driven flow (i.e. generated using a syringe pump), the flow rate ($Q$) is constant and thus the fluid velocity in the channel varies proportionally with the change in cross-sectional area. In the case of pressure-driven flow, the velocity change is dependent on both the change in $H_R$ and cross-sectional area. Consider a rectangular channel of length $L$, width $W$ and height $H$ where $W >> H$.

For the case of constant $Q$:

Using equation 8,
\[ Q = \text{const.} \]
\[ WHU = \text{const.} \]
\[ U = \frac{\text{const}}{H} \]

Thus we can conclude that the fluid velocity in the system will increase with decreasing channel height. This is beneficial to the operation of our device as cell separation occurs when the target cells are held stationary so an increase in fluid velocity while the membrane is deflected would increase relative cell separation.

For the case of constant \( \Delta P \):

Using equation 7,

\[ QR_{H} = \text{const.} \]

Substituting in equation 9,

\[ WHU \frac{12\mu L}{WH^3} = \text{const.} \]

Therefore,

\[ U = \text{const.}\times H^2 \]

Thus we can conclude that the fluid velocity in the system will decrease with decreasing channel height. This will decrease the relative cell separation seen by our device as the non-target cells will slow down when the target cells are trapped.

With the inclusion of additional \( R_H \) the system becomes one where the velocity of the fluid in the closed state is related to the change in cross-sectional area of the channel as well as the change in \( R_H \).

Thus, under a constant pressure and equation 7,

\[ Q_{\text{OPEN}}R_{H(\text{OPEN})} = Q_{SC}R_{H(SC)} \]

Using equation 7,

\[ A_{\text{OPEN}}U_{\text{OPEN}}R_{H(\text{OPEN})} = A_{SC}U_{SC}R_{H(SC)} \]
\[ U_{\text{OPEN}} = \frac{A_{\text{SC}}}{A_{\text{OPEN}}} \times \frac{R_{\text{H(SC)}}}{R_{\text{H(OPEN)}}} \times U_{\text{SC}} \]

To further decrease the change in $R_H$ without affecting the change in cross-sectional area, we can place several channels in parallel. In a similar manner to an electric circuit, placing resistances in parallel decreases the total resistivity of the system. Thus, by increasing the number of trapping channels in parallel and keeping the additional $R_H$ in series, we can decrease its proportion of the total resistance which reduces the change in $R_H$ between states. We will design the device with 4 channels in parallel to reduce the change in $R_H$, increase the throughput of the device and to design for some potential blockages.

Specifically, when the device actuates from the open state to the closed state,

\[
\frac{R_{\text{H(SC)}}}{R_{\text{H(OPEN)}}} = 1.19 \quad \text{while} \quad \frac{A_{\text{SC}}}{A_{\text{OPEN}}} = 0.16 . \quad \text{Thus equation 17 simplifies to,} \]

\[ U_{\text{OPEN}} = 0.19U_{\text{SC}} \]

Thus we can see that under a constant pressure the average cell velocity will be approximately 5 times larger in the closed state than in the open state.

### 4.4 Membrane and Control Channel Design

Modulation of the microfluidic channel height is accomplished using pneumatic pressure in order to deform the PDMS structure. The mechanical stop created by the locating fin is used to precisely control channel height modulation and to allow for a two-state membrane structure. The thickness of the oscillating membrane is determined by:

1. The distance the membrane needs to deflect.
2. The length and width of the channel.
3. The pressure the device can withstand.
4. The achievable thickness using PDMS spinning.

1&2. The distance the membrane needs to deflect is determined by the height difference between the base of the flow channel and the locating fin (Figure 4.7). The difference of 20 µm results in a channel width of 200 µm. The channel length was set as 6000 µm.

3. Preliminary tests confirmed that devices bonded using oxygen plasma can withstand pressures as high as 40 psi (approx. 2750 mbar) and sometimes greater. This is far higher than the pressure the
Fluigent can generate thus the limiting factor is the maximum deliverable pressure that which is 1000 mbar.

4. Achievable thicknesses using PDMS spinning have been shown to range between 20 and 150 µm (64). Using Kartalov’s membrane deflection model shown in Chapter 2, we obtained the following figure relating the pressure required to deflect a membrane of varying thicknesses a distance of 20 µm.

![Required Pressure vs Membrane Thickness](image)

**Figure 4.10:** This plot shows the pressures required to actuate a 200 x 6000 µm membrane of variable thickness a height of 20 µm.

We can see from this figure that there is a notable increase in the required pressure above membrane thicknesses of 60 µm. When fabricating the control layer, there is a certain amount of uncertainty in the nominal thickness of the layer as well as local maximum and minimum thicknesses. With this in mind, it is beneficial to restrict the thickness of the membrane to below 60 µm where a small difference in thickness has little effect on membrane deflection. Conversely, a thicker membrane is more desirable as it is more robust under construction and less prone to sticking to the top of the channel or sagging. Thus we conclude that the membrane thickness should be in the range of 40 to 60 µm.

Finally, the concept of a two-state membrane design is presented in the diagram below.
**Figure 4.11:** A front view cross-section of the flow channel. The hash lines represent different membrane positions. $P_{\text{OPEN}}$ corresponds to the pressure required to deflect the membrane enough to contact the locating fin while $P_{\text{SC}}$ corresponds to the pressure required to further deflect the membrane.

As shown in Figure 4.11, once the membrane has deflected to $P_{\text{SC}}$, the width of the membrane is essentially halved. This is significant because the pressure required to deflect a membrane half the original size is much greater than the original pressure. We validated this concept with data obtained using Kartalov’s model for a 50 µm thick membrane.

**Figure 4.12:** This plot shows the pressures required to actuate a 6000 µm long, 50 µm thick membrane varying heights. The open state curve represents the stiffness of the membrane before making contact with the locating fin while the semi-closed state represents membrane stiffness after.

This plot shows the effect of membrane width on deflection on a 50 µm thick membrane. As shown in figure, the pressures required to further deflect the membrane after it makes contact with the locating fin are much larger than those required for initial deflection. A further 1 µm deflection after the membrane width is halved requires approximately 25 mbar of additional pressure while a further 5 µm (enough to block the channel) requires approximately 175 mbar. As our pressure control apparatus has a resolution of ±1 mbar, we can effectively control the membrane deflection without fear of channel blockage or cell damage.
Chapter 5
Methods

5.1 Silicon Wafer Fabrication
The microstructures used for cell separation are fabricated on a silicon wafer by the Stanford Microfluidics Foundry using a photolithographic process. The 3D microstructures are initially designed using Solidworks. Subsequently, the 3D structure is decomposed into layers of 2D structures, which are exported to and edited using the Solidworks DWGeditor to create optical photomasks. The designs of optical photomasks are then submitted to the Stanford Microfluidics Foundry, where the photomasks and the layered microstructures are fabricated.

The microfluidics devices presented in this thesis are two-layer structures composed of a flow layer and a control layer. The flow layer microstructure is fabricated on a silicon wafer substrate using a four-layer photolithographic process. First, an alignment layer of positive photoresist (SPR 220, Rohm & Haas) is spun onto a 4 inch silicon wafer and exposed to UV light through a specially designed photomask. The subsequent three layers of negative photoresist (SU 8 2010;SU 8 2015;SU 8 2025, Microchem) are separately spun on top of the alignment layer and each exposed to a specific photomask. Finally, the wafer is developed and the completed mold baked to improve bonding between the silicon and photoresist.

**Figure 5.1:** Cross-sectional front view of the completed master mold for the flow layer fabricated using photolithography. The SU 8 2025 layer is not continuous along the length of the device and forms the cell traps. H1, H2, H3, represent the heights of the specific layers in each design.
The control layer microstructure is fabricated on a silicon wafer substrate using a negative photoresist (SU 8 2025) and exposed to UV light through a specially designed photomask. The wafer is developed and the completed mold baked to improve bonding between the silicon and photoresist.

![Figure 5.2: Cross-sectional front view of the completed master mold for the control layer fabricated using photolithography. All dimensions in microns. Not to scale.](image)

The specific spin speeds used to obtain the required layer thicknesses are determined by the Stanford Microfluidics Foundry.

### 5.2 Device Fabrication

The microfluidic device fabricated in this research is designed as a two layer PDMS structure. We used the procedure outlined in Figure 5.3 to achieve this structure. The initial silicon wafers are replicated using polyurethane (Smooth-Cast® 300, Smooth-On Inc.) to preserve the delicate master molds. The polyurethane molding process developed by Desai et al dramatically reduces the cost and effort required for microfabrication (65). The polyurethane molds are made in two steps. First, a PDMS (Sylgard 184, Dow Corning Corp.) mold is made from the silicon wafer and this is then used to cast the polyurethane replica. The PDMS mold can be reused many times which means the master silicon is preserved. The polyurethane molding process is outlined in detail in appendix A.
Figure 5.3: Process diagram for the fabrication of a two layer PDMS device using replica molding and multilayer soft-lithography. 1) PDMS replicas made from master polyurethane molds. 2) Diffusion bonding of PDMS layers. 3) Plasma bonding of PDMS device to glass slide.

The separate layers of the multilayer structure are fabricated by allowing liquid PDMS to cure while in contact with the replica polyurethane structures (Step 1). For the upper layer, this is achieved by simply pouring PDMS in a ratio of 5:1 onto the polyurethane structures and allowing it to cure. This yields a thick layer of PDMS that can be manipulated by hand. The lower layer (control layer) is fabricated by spinning a thin layer of PDMS onto the specific polyurethane structure in a ratio of 20:1 and allowing it to cure. This yields a thin PDMS layer of predictable thickness.
The upper layer is then removed from the polyurethane, aligned on top of the control layer, and bonded by allowing the curing agent to diffuse between them (Step 2). Once this has occurred, the entire structure can be peeled off the polyurethane wafer. This is then trimmed and punched which will allow the introduction of fluidic samples. Finally, the device is plasma bonded to a glass slide (Step 3) and is ready for use. The upper layer consists of a system of channels which the cell sample is forced to travel through. The lower layer consists of a system of dead end channels which are used to control the actuating membrane as well as fluidic valves. What follows is a more detailed explanation of Steps 1 - 3.

**Step 1**

The devices were fabricated using a specific brand of PDMS (RTV 615, GE Silicone) as it exhibits greater permeability to air and superior bonding characteristics. The devices were constructed using two layers bonded together using diffusion bonding via the multilayer soft lithography technique developed by Unger et al, which enabled the fabrication of valves and pumps in PDMS (7).

To fabricate the upper layer (flow layer), the polyurethane dish mentioned in appendix A is used. The procedure is outlined below:

- Weigh 50 g of PDMS base (Part A) and 10 g of PDMS hardener (Part B) in a paper coffee cup.
- The mixture is briefly mixed by hand before placed in a mixer (ARE-250, THINKY Corp) and set to mix for 3 min and defoam for 1 min.
- The mixture is then poured into the polyurethane dish and degassed using a vacuum for 5 min.
- The dish and mixture is then placed in a convection oven at 65 °C for 1.5 hours.
- The dish is then removed from the oven and allowed to cool to room temperature before removing the PDMS layer.
- The PDMS layer should be kept covered until further bonding.
- If care is taken when removing the PDMS layer, the dish can be covered and reused many times.

To fabricate the second layer (control layer), the polyurethane wafer mentioned in appendix A is used. The procedure is outlined below:
• Weight 20 g of PDMS base (Part A) and 1 g of PDMS hardener (Part B) in a paper coffee cup.

• The mixture is briefly mixed by hand before placed in a mixer (ARE-250, THINKY Corp) and set to mix for 3 min and defoam for 1 min.

• The mixture is then degassed in a vacuum dessicator for 5 min.

• The polyurethane wafer (Figure B.6, Right) is centered in a spinner (model and company) and the mixture is carefully poured on the centre of the wafer until about 50% is covered.

• The wafer is spun using the following process:
  - Step 1: 30s, 500rpm, 110rpm/s
  - Step 2: 50s, 1600rpm, 110rpm/s
  - Step 3: 20s, 0rpm, 110rpm/s

• The wafer is removed and placed in a convection oven at 65 °C for 1.5 hours.

• Once removed from the oven, the wafer should be kept covered until further bonding.

If the base/hardener PDMS ratio, the mixing/defoaming process and the spinning time/acceleration are kept constant, the spin speed (Step 2 above) of the device can be altered in order to achieve a predictable PDMS thickness. In this design Step 2 was set at 1600 rpm to achieve a PDMS thickness of approximately 50 µm.

**Step 2**

The next step is to align and bond the two layers together using diffusion bonding. Alignment is performed manually using a stereo microscope with a magnification of 10x - 50x. Manual alignment has been made possible by building sufficient alignment tolerances (500 µm) into the design (Section 3.1). The polyurethane wafer is placed under the microscope and the thick PDMS layer is carefully brought into contact with it. The two layers can be gently squeezed together to removed any visible air bubbles and then baked for 1.5 hours at 65 °C. The concentration difference between the two layers enables the hardener to diffuse across the interface causing them to bond together irreversibly. After the baking is complete, the bonded PDMS structure is allowed to cool to room temperature.
Step 3

Once cooled, the PDMS chip can be carefully peeled off the polyurethane wafer. The polyurethane wafer may be reused or discarded if too dirty. The PDMS chip cut into individual test pieces and fluidic ports punched. These final steps should be carried out in a laminar flow hood to prevent particles from polluting the devices. The completed PDMS devices are stored upside down in a clean environment (i.e. closed Petri dish) until needed for testing. The final step for device fabrication is to bond the individual test pieces to a glass slide. Bonding is accomplished by activating the PDMS and glass surfaces to oxygen plasma in accordance with (66). The multilayer PDMS test piece and a clean glass slide are oxygen plasma treated using a plasma cleaner (Model PDC-001, Harrick Plasma) for 30 s before the PDMS is brought into contact with the glass slide and the PDMS-glass bond is allowed to propagate. Moderate downward pressure is often needed to expedite this process. Plasma bonding is done right before testing so that the PDMS surface remains activated for channel coating purposes. This will be described in section 5.5 Device Preparation.

![Fabricated microfluidic device after plasma bonding. Fluidic lines have been introduced into the device.](image)

5.3 Experimental Setup

The device is visualized using an inverted stage microscope (Nikon Ti-U) which is connected to a CCD camera (Nikon Digital Sight DS-2MBW). Particle images and videos were recorded using commercial image gathering software (NIS-Elements, Nikon) and these were analyzed using open source software, ImageJ (National Institute of Health, USA). The external pressure control system (Fluigent MFCS-4C) is connected to the fluid and control samples via 1.6 mm ID tubing as pictured below. These are then introduced into the device via 0.51 mm ID tubing (Masterflex, Cole-Parmer,
Illinois) fitted with a short steel needle and the pressures set independently through a PC. The basic setup is shown below.

![Experimental setup](image)

**Figure 5.5**: Experimental setup.

As shown above, the Fluigent places the samples under pressure through a system of sample loading valves. The reason these are used is three-fold. Firstly, the pressure source generates an air pressure which needs to be introduced into a fluid pressure. Secondly, fluid back flow into the pressure source would damage the sensitive Fluigent and thirdly, the easily removable test tubes allow for easy, inexpensive refilling and replacement of samples and working fluid.

The 100 µm ID polyaryletheretherketone (PEEK) resistance tubing (Fisher Scientific, Inc., ON, Canada) used in preliminary tests to add \( R_{t1} \) is connected in series and fitted with the same steel needle as the 0.51 mm tubing.

### 5.4 Sample Preparation

#### 5.4.1 Particle Preparation

Some of the initial separation experiments were performed using microparticles since the flow channel of the first prototype was mistakenly fabricated as 40 µm high. Thus, the oscillating membrane caused a 20 µm change in channel height which meant target cells would need to be greater than 20 µm in diameter. Since our research facilities do not have cells this size, we decided to
run preliminary experiments solely with polymer beads and use these results to design an improved set of devices. Synthetic particles are easier to obtain than cell cultures and typically have a narrow size range distribution. However, a significant drawback is that in comparison to mammalian cells, polymer beads are infinitely rigid. Thus, to simulate the difference between rigid and deformable cells, large and small beads are used. The small beads represent deformable cells, while the large particles simulate rigid cells.

30 µm and 20 µm polymer microspheres were obtained from Thermo Scientific (Microgenics Corp.) They are made from polystyrene divinylbenzene (PSDVB) which is a non-toxic, non-hazardous synthetic material and are packaged in a fluid suspension. The size distribution of the particles was measured using (Cellometer, Nexcelom) and the results are presented in the Table 4.1. Although there may be a large range in size, this shouldn’t prove a problem as particle diameter can be verified inside the microchannels.

Table 5.1: Polymer bead size distributions and concentration.

<table>
<thead>
<tr>
<th>Nominal Size (µm)</th>
<th>Mean Diameter (µm)</th>
<th>Std. Dev. &amp; CV</th>
<th>Concentration (Particles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>19.5 µm</td>
<td>1.4 µm (7.1%)</td>
<td>4.36x10⁶</td>
</tr>
<tr>
<td>30</td>
<td>29.4 µm</td>
<td>2.5 µm (8.5%)</td>
<td>7.42x10⁵</td>
</tr>
</tbody>
</table>

For experiments, the 20 µm beads were used to simulate smaller, more deformable cells while 30 µm beads were used to simulate larger, more rigid cells. Experimental samples were diluted with phosphate buffer solution (PBS) to a concentration of 4.36x10⁶ particles/ml and 7.42x10⁵ particles/ml respectively. PBS was used to dilute the mixture as it is isotonic and non-toxic to cells, which will be important in later experiments.

5.4.2 Cell Separation
The flow channel of the second prototype was fabricated as 17 µm. Thus, the oscillating membrane caused a 7 µm change in channel height which meant target cells would need to be greater than 7 µm in diameter. To test the operation of the device, human RBCs and mouse lymphoma cells (MLCs) are used.
The RBCs to be used are preserved with 0.03% neomycin and 0.05% chloramphenicol and suspended in a buffered isotonic medium (Reverse-Cyte, Medion Diagnostics AG, Switzerland. They are at a concentration of $6.74 \times 10^8$ cells/ml. The cells were sized using an automated cell counter (Cellometer, Nexcelom) and the results presented below.

![Histogram showing the size distribution of human RBCs.](image)

**Figure 5.6:** Histogram showing the size distribution of human RBCs.

The RBCs were found to have a mean cell size of 5 µm. Although there is a large range in cell size, 2.46 - 9.53 µm, this is not expected to be a problem due to the deformable nature of RBCs. The coefficient of variance was found to be 24.9%. This large range in size is most likely due to the discoid shape of the RBCs and their varying positions while being measured. To investigate this large size range, a drop of RBCs mixed with PBS is placed on a glass slide and the sample is flattened using a cover slip. The cells can then be observed and counted using the NIS software. Using this software 121 cells were measured and the mean cell diameter was found to be 6.6 µm with a standard deviation of 0.4 µm.

The MLCs used are cultured in suspension in media consisting of a solution of RPMI Medium 1640 (Gibco) with 10% Fetal Bovine Serum and 1% of a Penicillin/Streptomycin solution PEG coating. All cell culture is performed in a Class II Type A2 biological safety cabinet (Thermo Scientific) to ensure sterility of the sample and to protect the user. The cabinet is wiped with 70% ethanol prior to use and everything entering the cabinet was wiped with 70% ethanol to ensure sterility. Cells are cultured in 25cm² canted neck polystyrene tissue culture flasks (Corning) and incubated in a Steri-Cycle CO₂ Incubator (Thermo Scientific) at 37°C and 5% CO₂. Cells and media are transferred using 5ml and
10ml serological pipettes (FisherBrand) and a Pipette Aid (Drummond Scientific). Cell counting is performed using a hemocytometer (Hausser Scientific) under an inverted stage microscope. Cells need to be split every 3-4 days and they can be harvested for testing every 2-4 days. The concentration has to be established for every sample preparation. The MLCs were sized using an automated cell counter (Cellometer, Nexcelom) and the results presented below.

![Figure 5.7: Histogram showing the size distribution of cultured MLCs.](image)

The MLCs were found to have a mean cell diameter of 10.5 µm after 4 days culture. Approximately 10% of the cells are smaller than 7 µm which is the critical size for the device. These cells will not be obstructed by the microstructure in the device. This however should not prove detrimental to testing as the cell diameters can be examined once in the device and MLCs < 7 µm can be ignored. Only 3.5% of the MLCs exceed 17 µm in diameter so clogging due to large cell blockage is not expected to prove a problem. The coefficient of variance was found to be 33.4%.

For experiments a mixture of RBCs and MLCs is prepared in phosphate buffer solution (PBS) (Gibco, Invitrogen) with 5% of 7.5% bovine serum albumin (BSA) to help prevent non specific cell adhesion. When a mixed sample is required, it is prepared to concentrations of 7x10^6 RBCs/ml and 0.1x10^6 MLCs/ml unless otherwise stated.

### 5.5 Device Preparation

Prior to each experiment, the microfluidic channels are coated with poly ethylene glycol (PEG) in minimize protein adsorption in order to reduce friction between cells and the surface of the microfluidic structure. The PEG coating is applied using a 1mg/ml aqueous solution of poly(L-
lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) prepared according to the procedure described by Lee and Vörös (42). The constituents of this solution are 150 µl of 1mg/ml PEG diluted with 840 µl DI water and 10 µl HEPES buffer solution (Gibco, Invitrogen).

The final step for device fabrication is to bond the individual test pieces to a glass slide as described. While the surfaces are activated, the PEG solution is introduced into the fluid layer to modify the surface of the PDMS by the adsorption of poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) from an aqueous solution in accordance with (42) to minimize protein adsorption and surface friction. Simultaneously, the control channel is filled with de-ionized (DI) water. Both the flow and the control channel are filled at 200 mbar until all air bubbles are cleared from the device. Once this has occurred, the pressure can be decreased and the PEG sample replaced with particle/cell sample. The devices are now ready for testing and appear as in Figure 5.4.
Chapter 6
Prototype I

6.1 Detailed Device Design

6.1.1 Flow Channel

The first prototype is designed with an open-state channel height of 25 µm. The resulting total membrane deflection is 20 µm. A channel width of 200 µm is used. To maximize the trapping area, the width of the boundary fins are limited to 15 µm. The final design of the flow channel is shown in Figure 6.1. The cross-section of the channel is shown in dark gray, while the cross-section of the periodic traps is shown in light grey. Several variations of the trap and side fin design are included on the wafer in order to determine the optimal geometry in testing. These variations are discussed below.

![Figure 6.1: A front view cross-section of the flow channel. The dark grey sections represent periodic traps while the light grey section represents the continuous part of the flow channel. Dimensions are shown in microns.](image)

The first parameter to be varied is the trap placement within the channel. Ideally, the traps should be as wide as possible in order to force every target particle to pass over every trap. As shown in Figure 6.1, the trap extends fully from fin to fin. This situation may not be ideal as traps occupied with target particles may cause a blockage. Consider an occupied trap as shown in Figure 6.2.
When the large traps are occupied by target cells they may act as obstacles to fluid flow, this situation may lead to blockages in the channel in the absence of alternate routes. Thus we staggered the traps across the width of the channel. This is shown in configuration 1.

The average size of the target cells in our experiments is 8-15 µm. A 40 µm trap length was selected to provide sufficient trap length for the trapping of target cells. Several devices were designed with a 20 µm trap length in order to test if a smaller trap could be feasible. Such a configuration increases the number of traps in a given channel and can improve particle separation at lower flow velocities however it was believed that the traps should be larger in order to accommodate larger cells. The depth of the traps was kept uniform at 20 µm.

The following configuration maximizes the cross sectional area of the traps while forcing the target cells to pass over the traps. The staggered configuration is designed to prevent channel blockages due to occupied traps.
Figure 6.4: A top view of trap configuration II. All dimensions shown in microns. Not to scale.

A final trap configuration was suggested to extend the traps over the entire width of the channel. This was thought to be helpful when establishing proof-of-concept with low concentration cell analysis. This could also be used to verify whether occupied traps did, in fact, cause an obstruction to non-target cells.

Figure 6.5: A top view of trap configuration III. All dimensions shown in microns. Not to scale.
6.1.2 Control Channel

The control channel was initially designed as a long, thin channel running parallel underneath one flow channel. This design was abandoned in favor of a wide control channel that would span over several flow channels at once. This configuration significantly improves alignment tolerance to ±500 µm. The width of the control channel is 2500 µm. The height of the channel is 25 µm to match with one of the standard channel heights designated by the Stanford Microfluidics Foundry. Since control channel geometry greatly exceeds the maximum 10:1 width:height ratio required to prevent channel collapse, the control channel is supported by a 30 µm square pillars placed every 200 µm. Additional push-up valves formed between crossed control and flow channels have dimensions of 200 µm x 200 µm. The designed thickness of the membrane between the control channel and the flow channel is 50 µm.

6.1.3 Final Device Design

A representative side view cross-section of the prototype I device is shown in Figure 6.6.

![Cross-sectional schematic diagram of device after bonding. The 100 µm diameter PEEK tubing placed in series to increase system hydrodynamic resistance is represented by (i). Cells are introduced on the left and flow through the modulated trap section under a constant pressure. The membrane is oscillated to impart differential velocities to specific cells. All dimensions in microns. Not to scale.](image)

As shown in Figure 6.6, cell samples are introduced through the inlet on the left of the figure under a constant pressure-driven flow and forced to flow through additional lengths of microchannels (indicated i), placed in series with the device before entering the trapping channels. As the cells pass through the device, the pressure in the control layer is varied with some duty cycle to actuate the
membrane to alternatively trap and release target cells. The non-target cells are eluted through the outlet on the right of the device.

There are several versions of this device on the complete prototype I wafer. The following features are included in every device unless otherwise stated in table 6.1:

- The flow layer for each device contains four parallel channels where the flows are evenly distributed. A plurality of parallel channels increases throughput and makes the device robust against blockages. An eventual device will be designed with 64 channels in order to handle the large volumes that are likely to be required for the isolation of CTCs. Each device contains two inlet channels and two outlet channels. The inlet channels are connected to the sample fluid, while the outlet channels are connected to the waste container. Using a back and forth flow, the target cells can be accumulated in the microchannel, while the non-target cells can be eluted into the waste container.

- A simple hemocytometer is incorporated into every inlet and outlet channel. It is simply an enlarged section of the channel with posts forming 100 µm square areas to facilitate manual particle counts.

- Membrane control channels serve to actuate the membrane with a certain frequency. They are dead-end channels that require only one connection and will be controlled using an external pressure controller.

- Valve control channels are 200 µm wide and serve to open and close valves controlling the flow input channels. They are dead-end channels requiring only one connection and are controlled using an external pressure controller. Three valves are placed in parallel which could double as a peristaltic pump. These however were not used in favor of pressure driven flow.

- The alignment features for this design include cutting lines surrounding each die with pronounced corners to assist with alignment.

- Die identification is simple and is found at the base of each die in the centre. They are only present on the control layer so that misalignment would not cause label distortion.

The generic device design is shown in the figure below for configuration 4-111.
Figure 6.7: Top view of the flow layer and control layer for configuration 4-111. The control layer includes the actuating membrane and the control valve channels. The flow layer includes 4 parallel channels making up the trapping section and the connected fluidic lines. Note alignment and cutting marks are present on both layers however device identification is only on the control layer.

In brief, the final device operation is envisioned as follows: The sample will be introduced into a fluidic inlet while oscillating the membrane. After a given time (i.e. enrichment of target cells), the control membrane will be placed under a constant pressure, the sample fluidic inlet will be shut using the control valve, and a buffer solution will be run through another inlet in order to flush out non-target cells. Then, the control membrane will be allowed to relax and the target cells can be removed through a separate outlet. In this way, filtering/enrichment can be accomplished.

The following figure is an overlaid schematic of the device designs. The flow layer is in black and the control layer is in grey. Following this figure is a table outlining the specific features of each die.
Figure 6.8: Top view of the flow layer and control layer for the final design. The control layer is shown in gray while the flow layer is shown in black.
Table 6.1: Die identification with corresponding features

<table>
<thead>
<tr>
<th></th>
<th>Device Identification</th>
<th>Features &amp; comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11 - 1</td>
<td>20 ( \mu )m trap length, Trap configuration 1</td>
</tr>
<tr>
<td>2</td>
<td>11 - 111</td>
<td>20 ( \mu )m trap length, Trap configuration 11</td>
</tr>
<tr>
<td>3</td>
<td>11 - 111</td>
<td>20 ( \mu )m trap length, Trap configuration 111</td>
</tr>
<tr>
<td>4</td>
<td>4 - 1 (Long)</td>
<td>40 ( \mu )m trap length, Trap configuration 1, Increased length to better investigate particle separation</td>
</tr>
<tr>
<td>5</td>
<td>4 - 11</td>
<td>40 ( \mu )m trap length, Trap configuration 11</td>
</tr>
<tr>
<td>6</td>
<td>4 - 111</td>
<td>40 ( \mu )m trap length, Trap configuration 111</td>
</tr>
<tr>
<td>7</td>
<td>4 - 1 (64)</td>
<td>40 ( \mu )m trap length, Trap configuration 1, large multiplexor for increased channel number observations</td>
</tr>
<tr>
<td>8</td>
<td>4 - 11</td>
<td>40 ( \mu )m trap length, Trap configuration 11</td>
</tr>
<tr>
<td>9</td>
<td>4 - 1 NF</td>
<td>40 ( \mu )m trap length, Trap configuration 1, No side fins in order to investigate the impact of these on flow patterns and particle capture</td>
</tr>
</tbody>
</table>
6.2 Results and Discussion

6.2.1 Wafer Fabrication

Once the silicon wafers were fabricated by the Stanford Microfluidics Foundry, they are examined using a optical interferometer (Wyko NT1100, Veeco) to verify their geometry. An image obtained using the Wyko is shown in Figure 6.9.

Figure 6.9: Optical interference image gained using the Wyko for trap configuration 4-11. Note the 16 µm offset in the scale bar. The light blue layer represents the channel height, the green layer the boundary and locating fin height and the red layer the trap height. To scale in the vertical direction.

Upon inspection, we found the height of the channel to be approximately 20 µm while the height of the fins is approximately 40 µm. This is not in agreement with the original design (Figure 5.13) which specified the fin height to be 5 µm and the channel height to be 25 µm. The trap height is approximately 20 µm. The actual fabricated channel dimensions are shown below.
As we can see, the height of the “5µm Layer” is actually fabricated as 20 µm leading to a new critical channel height. Thus, synthetic particles were used to obtain preliminary results regarding the operation of the device.

The control wafer was examined using the Wyko and found to comply with the design.

Using these master molds, PDMS devices could be fabricated and prepared for testing using the procedures outlined in Chapter 2.

**6.2.2 Flowrate Validation**

A 4-111 device was used for initial flow rate validation. A mixture of 20 µm diameter beads was introduced into the flow port of the microfluidic device so that the fluid velocity could be easily visualized. The fluid was driven through 150 cm of PEEK tubing at a constant pressure using the Fluigent. The pressure was varied between 0 and 50mbar in increments of 5mbar. The time taken for particles to travel through the entire trapping channel was measured for each pressure increment. For each pressure variation, the flow was allowed to stabilize. Figure 6.11 show the results from this experiment.
As shown in Figure 6.11, particle velocities measured in the semi-closed state are higher than those in the open state. On average, particles were found to travel 3.3 times faster in the semi-closed state than the open state as compared to theory which predicted an approximate increase of 5 times.

Measurements were difficult to obtain at higher pressures due to the high velocity of the particles. The negative fluid velocity seen when a zero pressure is applied can be attributed to the back-pressure in the device, as well as the small height difference between the device and the fluid source. These results are lower than expected which may be attributed to additional friction in tube connections and fittings, as well as small air leaks. No fluid leaks were observed in the device.

### 6.2.3 Membrane Deflection Validation

A mixture of 30 µm beads was introduced to a 4-111 configuration device as described in Chapter 4 with a constant 15 mbar pressure. In this case, pressure was applied to the control fluidic port and gradually increased until the motion of these 30 µm beads was impeded. The flow impedance to the beads became noticeable at approximately 180 mbar control pressure (i.e. to the membrane), however only some beads were completely stopped. When the pressure was further increased to 210 mbar, it was observed that all the beads were arrested. Over this pressure range, a visible change in the shade of the centre locating fin in the flow layer and the support posts in the control layer could also be seen. When the pressure was released to beneath 180 mbar, the 30 µm beads could once again travel through the device.
The pressures required to deflect the membrane were in keeping with theory. Particle adhesion was rare but observed in three separate cases throughout the experiments. Firstly, excess membrane deflection forces particles to stick to the roof of the channel regardless of the particle’s location in the channel. Secondly, particles became stuck at either side of the channel because they were propelled into the sides of the channel by membrane deflection. Thirdly, particles became stuck after extended periods of immobility, which was largely caused long periods in the semi-closed state coupled with extremely low flow velocities (0 - 5 µm/s). Clogging, or the tendency for particles to stick to each other, also caused particle immobility. However this phenomenon was rarely observed.

6.2.4 Fluid Velocity Fluctuations

Initial tests showed that the deflections of the control membrane can create significant fluctuations in the flow velocity. When the membrane deflects as a result of the increase of applied control pressure, fluid is temporarily repelled from the channel and causes a transient flow of the liquid. The magnitude of the transient flow is dependent on the location of the cell with respect to the center of membrane deflection. Since the membrane is inflated starting from its center and propagating outwards along the channel, the transient flow rate is greater at the edge of the membrane than at the center of the membrane because of the accumulated displaced flow. Thus the transient velocity of the cell can be described as a sinusoidal curve increasing in amplitude from the centre of the membrane (zero) to outwards to the edge of the membrane (maximum).

Although the flow fluctuations do not affect the net motion of the particles, it can make it difficult to monitor the motion of the particles as the optical setup has a limited field of view (FOV) and excessive velocities may damage cells in later experiments. The particle motion is depicted in the figure below.
Figure 6.12: A) A top view diagram representing a section of trapping channel overlaid with an actuating membrane. B) A graph representing fluid velocity at different regions within the microchannel under a constant pressure driven flow with a duty cycle of 1. At (i), the transient velocity is zero as this corresponds to the centre of membrane deflection. The positive velocity is due to the constant pressure-driven flow. At (ii) the velocity fluctuations increase due to accumulated displaced flow until (iii) where the transient stabilizes. Consider downstream fluid velocity to be positive.

As shown by Figure 6.12, there is a constant fluctuating fluid velocity caused by the oscillating membrane. The large flow fluctuations are due to large membrane deflection. To remedy this, a non-zero base pressure was incorporated into the duty cycle. Specifically, 30µm particles were allowed to flow through the device while constantly increasing the pressure in the control layer. In this way the membrane was deflected continuously until the particles became trapped. Once this occurred the semi-closed pressure ($P_{SC}$) was noted. The control pressure was then continuously decreased until the particles could once again flow. This was determined as the open pressure ($P_{OPEN}$). Using this we could minimize the deflection of the membrane and the subsequent flow fluctuations.

We then used 20 µm particles to characterize the fluid velocity profile as they are unaffected by the microstructure. A duty cycle of 0.29 ($P_{SC} = 200 mbar; P_{OPEN} = 100 mbar$) was used for membrane control with a semi-closed time ($T_{SC}$) of 4 s. A constant pressure of 15 mbar was used in the flow channel. The following figure represents the fluid velocity over time as related to control pressure.
As can be seen, at no point is the 20 µm particle stationary and the flow is cyclical. At $P_{\text{OPEN}}$ the particle velocity is constant travelling at an average of 60 µm/s. At $P_{\text{SC}}$ the particle velocity is approximately 150 µm/s although a $P_{\text{SC}}$ time of 4 s does not allow the flow velocity to stabilize. The semi-closed fluid velocity is 2.5 times that of the open state. This can be attributed to the non-zero base pressure which decreases the change in cross-sectional area. The peak velocity caused by the fluctuating membrane is approximately 850 µm/s which is in the physiological range as mentioned in section 4.1.2.

As can be seen, membrane inflation occurs at a faster rate than membrane deflation. In particular, stable semi-closed pressure is reached after ~1.5 s while stable open pressure after ~3 s. This phenomenon may be due to the limited control channel area that the pressure can escape from as well as the small elastic forces causing the membrane to relax. This results in particles experiencing a greater initial transient velocity upon a pressure increase than a pressure decrease. The velocity profile is also indicative of the particle’s position relative to the membrane. When the membrane is pressurized, the particles initially experience an increased negative (upstream) velocity and upon
membrane deflation, an increased positive (downstream) velocity. This profile is shown by Figure 6.13. Assuming downstream particle velocity to be positive, we can conclude that the particle is upstream of initial membrane deflection (i.e. the pressure inlet). On the opposite side of the pressure inlet, the opposite profile exists. This velocity profile mirror will be shown in later results. Finally, the local maxima and minima in the control pressure are due to stabilizing inaccuracies from the Fluigent.

In the large devices, (40 - 1 (64) & 40 - 1 (Long)) it was observed that the membrane took a far longer time to deflect than in the small devices. Due to their size, this propagation happened very quickly in the small devices however it was seen to begin at the pressure source and originate outwards. Because of this, far longer semi-closed pressure times are needed to ensure complete membrane deflection which results in continual particle/microstructure interaction and particle adhesion. If normal semi-closed pressure times are used, incomplete membrane deflection occurs which results in a loss of function. Pressure is introduced to the control channel via a single 200 µm channel located in the center of the membrane (Figure 6.7). This channel has a large hydrodynamic resistance and thus prevents the membrane from inflating/deflating quickly.

6.2.5 Chromatographic Particle Behavior

In preliminary tests to verify the chromatographic behavior of the separation mechanism, we observe the motion of 20 µm and 30 µm particles in a range device configurations. It is noted that with correct control pressure calibration, both sizes of particles can travel in the open state while only 20 µm particles can travel in the semi-closed state.

For demonstrative purposes, we measured the velocity of 20 µm and 30 µm particles in a 4-111 configuration device. First, a mixture of 20 µm and 30 µm beads is introduced into the flow port at a constant pressure of 30 mbar until particles are seen flowing through the microchannels. DI water is introduced into the control port and the membrane deflection is calibrated by varying the control pressure incrementally as mentioned in 6.2.3. In this way the control pressure is set to vary between 100 mbar and 200 mbar. The data shown in the following figure was obtained using a DC of 0.36 where the time the channel is in the semi-closed state, T_{SC}, is set to remain constant at 4 s while the time the channel is to remain in the open state, T_{OPEN}, is set to 7 s.
Figure 6.14: The displacement of 20 µm and 30 µm particles when the membrane deflection is oscillated with a duty cycle of 0.36 (4 s at 200 mbar and 7 s at 200 mbar) and an inlet pressure of 20 mbar.

In the open state (region B), both particles travel at the same average velocity (approximately 100 µm/s) shown by the similar slope of the graph. This velocity is higher than the open state velocity measured in section 6.2.2 however this can be explained by smaller cross-sectional area of the open state caused by a non-zero $P_{\text{OPEN}}$. In the semi-closed state (region A), the 30 µm particle is trapped by the microstructure and the 20 µm particle travels at an increased average velocity (approximately 260 µm/s) as shown by the slope of the graph.

It was observed that target particles experienced non-specific adhesion to the microstructure under certain conditions. Most commonly, adhesion occurred when particles were in continuous contact with the microstructure (i.e. stationary) which is most often caused when the target particles are trapped in the semi-closed state. Particle adhesion was also seen to occur when the membrane is over-deflected.
Although minimized by using a non-zero $P_{\text{OPEN}}$, large velocity fluctuations caused by the oscillating membrane still proved troublesome when measuring specific particle velocities. Further, the 100 $\mu$m PEEK tubing is easily clogged and difficult to integrate into the experimental procedure.

### 6.2.6 Lateral Particle Motion

Preliminary tests showed a tendency of particles to travel towards the outsides of the channel, regardless of their starting point. This was found to be due to the profile change of the membrane as it is deflected. We found that because membrane deflection is initiated at the center, a resultant force was being generated perpendicular to the flow direction, originating at the centre.

![Diagram showing lateral particle motion](image)

**Figure 6.15**: *Top View*: Device schematic with a digital image of a traced trajectory of a non-target particle and a trapped target particle (enlarged section). In this sequence, the non-target particle begins near the centre of the channel and over about 9 traps (approx 600 $\mu$m) it is forced to the side of the channel. *Front View*: Membrane deflection schematic. The solid lines represent the start and end positions of the membrane while the hash lines represent the intermediate stages of deflection.
The diagram above shows the deflection of the membrane in stages and the resultant force move cells towards the boundary of the device where there is a much higher occurrence of non-specific adsorption to the surface of the microstructures. This phenomenon is shown by the traced particle trajectory shown in Figure 6.15 (enlarged section). Further, the compression in the corners due to membrane deflection may result in cell damage or even cell lysis.

In some cases, target particles moving along the outer edges of the channel are able to travel in the closed state. Upon inspection, it is noted that the majority of the un-trapped target particles are smaller than average and that in all cases observed, the particle was travelling adjacent to the side fins. This can be attributed to the larger channel area along the outer edges of the channel in the semi-closed state as previously mentioned.

6.2.7 General Observations

In the flow channel, the centre locating fin served to prevent over-deflection of the membrane under normal operation. In the control channel, the posts served to prevent the actuating membrane from sagging during fabrication, and did not impede the membrane from trapping target particles.

The two layers are easy to misalign despite the 500 μm tolerance. This is due to the alignment marks being near the edges of the mold and thus minor differences in alignment are magnified on the other side of the mold.

If the membrane is misaligned and covers the part of region A in the figure below, an accidental valve is formed which prevents particles from entering the trapping channel which renders the device useless.
It is observed that the integrated cell counters caused particles to slow down which resulted in increased particle/microstructure interaction. This was often a site for non-specific particle adhesion. Further, channel collapse was observed to occur frequently in this region despite the design including support posts. The outline of the posts is visible however the posts may have been damaged or broken in the master fabrication process which would explain the frequent channel collapse.

**Figure 6.16:** Top view schematic of flow layer design (Configuration 4-11).
6.3 Design Recommendations

Here we have shown the design, fabrication and testing of a microfluidic device that employs a new technique for size based separation of microparticles. In this section we will discuss the functionality of the device, elaborate on the specific shortcomings and suggest improvements for the next round of design.

6.3.1 Transient Flow

To reduce the transient flow resulting from membrane deflection, the change in area between open and semi-closed states should be reduced. Although this problem can be addressed by incorporating a non-zero base pressure, the percentage of the channel unaffected by membrane deflection (i.e. fin height) should be greater.

6.3.2 Membrane Deflection

The deflection of the membrane is not instantaneous owning to the limited flow rate available to fill the control channel. The slow deflection of the membrane is most prominently observed in the long device (4-1 Long). The narrow width of the inlet channel causes an increased hydrodynamic resistance which increases the time for the liquid to fill the control channel. This situation can be remedied by increasing the width and number of channels used to inflate the membrane. Long devices should specifically be designed with several inlets in parallel to observe membrane deflection over a greater area as well as cell separation over a longer channel distance.

6.3.3 Lateral Particle Motion

In the semi-closed state, smaller than average target particles often have a tendency to move toward the edge of the channel, which causes them to travel adjacent to boundary fins. To minimize this, the optimal side fin width needs to be addressed. Further, cells traveling near the outer edges of the channel may be crushed by the membrane deflection. Therefore additional mechanisms should be developed to minimize lateral motion.

6.3.4 Non-specific Particle Adhesion

As mentioned previously, particles appear to experience more non-specific adhesion when exposed to greater continuous particle/microstructure interaction. Thus to minimize particle adhesion, the time that the membrane is deflected to the semi-closed position should be minimized.
6.3.5 General Design Recommendations

The following recommendations are made for future design iterations:

- To avoid the difficulties associated with additional hydrodynamic resistance in the form of external tubing, devices should incorporate the additional resistance within the device. This would allow the sample to be visualized throughout the sample loading process.

- To aid in membrane placement, alignment marks should be incorporated in close proximity to the trapping region of the devices.

- Due to the limited field of view (FOV), a single device should be designed to observe cells over a longer channel length.

- Cell counters should be removed.

- The channel width should remain narrow enough to prevent accidental valve creation due to membrane misalignment.
Chapter 7
Prototype II

7.1 Detailed Device Design

This prototype will address the issues outlined in section 6.3, namely correct feature height during initial silicon wafer fabrication; minimizing channel height change; optimal fin width; reduced lateral particle motion; increasing channel FOV; incorporation of additional hydrodynamic resistance; accidental valve creation due to layer misalignment; and regulation of membrane inflation.

7.1.1 Flow Channel Design

In order to evaluate this concept using mammalian cells, it is imperative that the initial feature heights fabricated on the silicon wafer are correct. The critical height required will remain 5 µm as mentioned previously due to the cell size and rigidity differences between mouse lymphoma cells (MLCs) and red blood cells (RBCs). However, to help minimize transient flow due to membrane deflection, the height of the first layer will be 10 µm. In this case, membrane deflection will cause the channel height to change from 15 µm to 5 µm rather than 25 µm to 5 µm as in prototype I. This configuration results in a 66.6% volume change as opposed to the previous 80% volume change. The height of the traps is reduced from 20 µm to 10 µm to avoid potential problems with feature fabrication. The width of the channel will be reduced to 150 µm however it will remain 6000 µm in length.

Although the boundary fins were effective in preventing target particles from travelling at the sides of the channel, they may not function as well considering the smaller size difference between MLCs and RBCs. Another factor which may affect their function is the deformability of the cells. Thus, to optimize boundary fin width, FEA of membrane deflection was carried out. When the membrane makes contact with the centre locating fin, there is a certain portion at the channel boundary that experiences minimal deflection. In order to characterize this channel profile, a range of membrane thicknesses were simulated using COSMOS (Dassault Systemes, Solidworks Corp.). In this way, the minimum boundary fin width can be established to prevent target particle boundary motion, while still maximizing the total channel width.

Consider the figure below. When the membrane deflects to the height of the locating fin (10 µm), there is a certain distance, x, that represents the width of boundary fin necessary to prevent particles ≥10 µm from travelling. This width varies for different membrane thicknesses and theoretical data for this variance was obtained using COSMOS.
**Figure 7.1:** A) Simple front view cross section of the flow channel. The distance, $x$, represents the width of channel with a height greater than 10 $\mu$m in the semi-closed state. B) A plot of the results for $x$ obtained using FEA of the deflection of the membrane for various thicknesses.

Using these results, we can see that a boundary fin width of 25 $\mu$m would be suitable over a range of membrane thicknesses. Finally, the centre fin width will be increased from 15 $\mu$m to 20 $\mu$m. The final flow channel dimensions are shown in the figure below.
As discussed in the previous design, additional hydrodynamic resistance was incorporated into each device using a length of PEEK tubing placed in series. This approach was expensive, the PEEK tubing was prone to clogging, and the experiment was time consuming to prepare and clean after use. Thus, the new design incorporates a 17.5 cm length of 80 x 15 µm serpentine channel in every device which corresponds to an additional $R_H$ of $6.92 \times 10^{15}$ Pas/m$^3$. It will switch back on itself to minimize its footprint and have two separate inlets, allowing one to easily regulate the amount of additional hydrodynamic resistance. Using the new channel dimensions we can calculate the new hydrodynamic resistance of the device in a similar manner as previously and these can be used with equation 8 to yield the theoretical fluid velocities in the open and semi-closed states.

Using equation 17,

$$U_{OPEN} = \frac{A_{SC}}{A_{OPEN}} \times \frac{R_{H(SC)}}{R_{H(OPEN)}} \times U_{SC}$$

$$U_{OPEN} = 0.211 \times 2.063 \times U_{SC}$$

$$\therefore U_{SC} = 2.3U_{OPEN}$$

As can be seen, the velocity in the semi-closed state ($U_{SC}$) is predicted to be 2.3 times greater than in the open state ($U_{OPEN}$).

To prevent lateral particle motion, as mentioned in section 6.3.3, target particles require a resultant force towards the centre of the channel greater than the lateral force generated by the membrane. To
accomplish this, angled traps are incorporated into the design as shown in the figure below. This design relies on the relatively small lateral force due to membrane deflection as compared to the normal force.

**Figure 7.3:** Top View. A diagram representing the previous design’s trap orientation (I) and the proposed trap orientation (II). $F_V$, $F_N$, $F_M$ and $F_{RES}$ represent the forces due to viscosity, normal reaction, membrane deflection and the resultant respectively. $\beta$ represents the angle of the trap.

The angle of the traps in configuration II ($\beta$) will affect the movement of target particles. With this in mind, $\beta$ will be varied between 45° and 60°. As this trap configuration restricts the flow to one direction, some devices will be designed with $\beta = 90^\circ$ (see Table 7.1).
Figure 7.4: A top view of trap configuration II & III. All dimensions shown in microns. The angle of the traps, β, is varied between 45, 60 and 90°. Not to scale.

To address the general design recommendations made in section 6.3, the following changes to the flow layer will be made:

- The camera used to visualize the device has a FOV restricted to 2900x2200 µm at the smallest magnification (4x objective). In order to observe particle motion through a larger portion of the channel, we will incorporate a flow channel design that switches back on itself.

- To prevent accidental valve creation the channels near the trapping region, and thus the membrane, will remain narrow for 800 µm providing a greater alignment tolerance.

7.1.2 Control Channel Design
Inflation of the membrane originates in the center of the membrane and propagates outwards. This was easily observed in the large device. The single pressure inlet caused slower than ideal membrane inflation/deflation due to the hydrodynamic resistance of the inlet channel. To minimize this problem, we incorporated several inlet channels over the length of the membrane. This is expected to help regulate the velocity fluctuations.
The width of the flow channel results in a membrane width of 150 µm in the open state. To investigate the two-state membrane behavior, Kartalov’s model was again used to obtain the following theoretical predictions.

**Figure 7.5:** This plot shows the pressures required to actuate a 6000 µm long, 50 µm thick membrane varying heights. The open state curve represents the stiffness of the membrane before making contact with the locating fin while the semi-closed state represents membrane stiffness after contact with the locating fin.

As shown in Figure 7.5, the pressures required to further deflect the membrane after it makes contact with the locating fin are much larger than those required for initial deflection. The difference between the deflection pressures for each state is greater than in the first design due to the narrower membrane width. Finally, the control channels is a 25 µm high membrane that is supported every 220 µm by 30 µm square support pillars.
7.1.3 Final Device Design

The generic device design is shown in the figure below for configuration 11-11.

Figure 7.6: Top view of the flow layer and control layer for configuration 4-111. The control layer includes the actuating membrane and the control valve channels. The flow layer includes 4 parallel channels making up the trapping section and the connected fluidic lines. Note alignment and cutting marks are present on both layers however device identification is only on the control layer.

The following figure is an overlaid schematic of the device designs. The flow layer is in black and the control layer is in grey. Following this figure is a table outlining the specific features of each die.
Figure 7.7: Top view of the flow layer and control layer for the final design. The control layer is shown in gray while the flow layer is shown in black.
Table 7.1: Die identification with corresponding features

<table>
<thead>
<tr>
<th>Device Identification</th>
<th>Features &amp; comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>111 - 11 (Switchback) Trap configuration 111, 60° trap angle, increased channel length in FOV</td>
</tr>
<tr>
<td>2</td>
<td>111 - 1&lt;&gt; Trap configuration 111, reversed 45° trap angle</td>
</tr>
<tr>
<td>3</td>
<td>111 - 1&lt;&gt; Trap configuration 111, reversed 60° trap angle</td>
</tr>
<tr>
<td>4</td>
<td>111 - 11 (Long) Trap configuration 111, 60° trap angle, increased length to better investigate particle separation</td>
</tr>
<tr>
<td>5</td>
<td>11 - 1 Trap configuration 11, 45° trap angle</td>
</tr>
<tr>
<td>6</td>
<td>11 - 11 Trap configuration 11, 60° trap angle</td>
</tr>
<tr>
<td>7</td>
<td>11 - 111 Trap configuration 11, 90° trap angle</td>
</tr>
<tr>
<td>8</td>
<td>111 - 1 Trap configuration 111, 45° trap angle</td>
</tr>
<tr>
<td>9</td>
<td>111 - 11 Trap configuration 111, 60° trap angle</td>
</tr>
<tr>
<td>10</td>
<td>111 - 111 Trap configuration 111, 90° trap angle</td>
</tr>
</tbody>
</table>
7.2 Results and Discussion

In this section, the specific conditions used in each experiment will be described. For each experiment, the microfluidic channel system is fabricated and prepared for experiments as described in Chapter 5. This section will begin with general experimental observations before describing velocity fluctuations characterized using RBCs as well as lateral MLC motion. Size- and rigidity-dependent cell motion is then presented, including the effects of duty cycle on relative cell displacement. Finally, cell viability results are presented and preliminary work regarding target cell enrichment.

7.2.1 Wafer Fabrication

Once the silicon wafers were manufactured by the Stanford Microfluidics Foundry, they were examined using an optical interferometer (Wyko NT1100, Veeco) to verify correct fabrication. Images obtained using the Wyko are shown below.

![Figure 7.8: Optical interference image gained using the Wyko for trap configuration 111-111. Note the 2.9 µm offset in the scale bar. The light blue layer represents the channel height, the green layer the boundary and locating fin height and the red layer the trap height. To scale in the vertical direction.](image)

As shown in Figure 7.8, the height of the light blue layer (locating fin and side fin height) is approximately 10 µm while the height of the orange layer (channel height) is approximately 17 µm. The height of the red layer (trap height) is approximately 24 µm. This is in close agreement with the
original design (Figure 7.2) which specified the fin height to be 10 µm, the channel height to be 15 µm and the trap height to be 25 µm. The actual fabricated channel dimensions are shown below.

![Enlarged Section diagram](image)

**Figure 7.9:** A front cross-section of the actual fabricated flow channel geometries. The desired layer heights are shown in parenthesis. All dimensions in microns.

The critical height is the 7 µm difference between the locating fin and the top of the flow channel. Thus, MLCs and human RBCs were selected to test the operation of the device. The control wafer was examined using the Wyko and found to comply with the design. Using these master molds, PDMS devices could be fabricated and prepared for testing using the procedures outlined in Chapter 5.

### 7.2.2 General Observations

The boundary fins were observed to be effective in forcing target cells to travel in the centre region of the flow channel. Despite decreasing the width of the flow channel in the immediate vicinity of the membrane, we still observed some accidental valve creation associated with membrane misalignment. This was rare however, due to improved alignment marks.

In the long device (111 - 11 (Long)), we observed the membrane deflected with greater uniformity across the length however a lag in membrane deflection was seen across the width of the membrane. This suggests that further work is needed to achieve constant membrane deflection across the channels at an acceptable rate.
7.2.3 Fluid Velocity Fluctuations

To establish the control pressures required to trap MLCs, a sample of MLCs is prepared to a concentration of approximately $0.1 \times 10^6$ cells/ml as outlined in 5.4 and introduced at a constant pressure of 20 mbar. The exact concentration is not important as this experiment is performed solely to establish the pressures required to capture and release MLCs. In a similar fashion to section 6.2.4, the control pressure is steadily increased until the MLCs are trapped in the microstructure and the pressure is noted ($P_{SC}$). This occurs at 180 mbar which is larger than the 118 mbar pressure predicted by theory (Figure 7.5). The control pressure is then steadily decreased until the MLCs can once again flow and the pressure is noted ($P_{OPEN}$). This occurs at 100 mbar which again is higher than expected. These differences in control pressures are most likely due to inaccuracies in membrane fabrication (i.e. thicker than expected). The control pressures used are consistent with a 70 µm thick membrane. The deformability and size variation of MLCs also contributes to variations in control pressures as compared to theory.

RBC motion was tracked over a period of 60 s in order to characterize the fluid velocity profile. The position of the RBC was downstream of the centre of membrane deflection. A duty cycle of 0.167 ($P_{SC} = 180$ mbar; $P_{OPEN} = 100$ mbar) was used for membrane control with a peak pressure time of 2 s. A constant pressure of 20 mbar was applied at the inlet of the flow channel. The following figure represents the fluid velocity over time as related to control pressure.
As shown in Figure 7.10, the flow is cyclical and the RBCs are not stationary. At $P_{\text{OPEN}}$ the cell velocity is a constant $\sim 70 \, \mu m/s$ while the peak velocity due to membrane fluctuation is approximately $1000 \, \mu m/s$. The flow does not have an opportunity to stabilize at $P_{\text{SC}}$ due to two factors: (1) the limited time $P_{\text{SC}}$ is applied, and (2) the finite time the membrane takes to inflate. Figure 7.10 shows a $P_{\text{SC}}$ time of 2 s and a $P_{\text{OPEN}}$ time of 10 s, however the pressure takes approximately 1.5 s to reach $P_{\text{SC}}$ and approximately 3 s to decrease to $P_{\text{OPEN}}$.

As discussed in section 6.2.4, membrane deflation occurs more slowly than inflation. This phenomenon is apparent in the RBC velocity profile which shows a faster velocity transient associated with membrane inflation than compared with membrane deflation. Furthermore, the RBC’s downstream position is caused by the initial positive (downstream) velocity increase followed by a negative (upstream) velocity increase. The RBC velocity profile from the prototype II device shows a similar shape to that of the 20 $\mu m$ particle velocity profile in section 6.2.4. However, the velocity profile here is an inverted version as the position of the RBC is downstream of the center of membrane deflection. Figure 7.11 illustrates this.
Figure 7.11: A simplified flow channel including traps with an overlaid actuating membrane. The velocity time graphs show data representing fluid velocity profiles inside the microstructure under a constant pressure driven flow with a given DC. A) Trapping region upstream of the centre of membrane inflation. B) The centre of membrane inflation. C) Trapping region downstream of the centre of membrane inflation.

For a RBC in region A, we can see an initial negative fluid velocity (1) due to membrane inflation temporarily forcing fluid away from the centre of membrane deflection. This negative velocity is followed by a positive velocity (2) due to membrane deflation. The acceleration and peak velocity is greater in (1) as compared to (2) as well as occurring over a shorter period of time. This occurs because the membrane inflates faster than it deflates as mentioned previously. This fluid velocity profile (Region A, Figure 7.11) is consistent with a region upstream of initial membrane deflection.

For a RBC in region B, there is a negligible transient velocity. For a RBC in region C, the fluid velocity profile is the reverse of that in A. Specifically, there is an initial positive velocity (3) caused by membrane inflation (indicated by the steep gradient and short transient time) followed by a negative velocity (4) (indicated by the more gentle gradient and longer transient time). This fluid velocity profile is consistent with a region downstream of initial membrane deflection.

7.2.4 Lateral Cell Motion

To oppose the lateral target cell motion due to membrane deflection, angled traps were incorporated into the flow channel design. It was observed that the angled traps caused target cells to behave as shown in Figure 7.12.
In region A, the initial membrane deflection traps the target cell and the transient fluid flow forces the cell to travel along the path through 1 until 2. When the membrane is relaxed, transient fluid flow forces the target cell to follow the path through 3 until its final release position. This property is consistent with experimental observations. The reason the target cell is not immediately released from the trap at 3 is due to the finite time taken for the membrane to relax. Thus, transient fluid flow is reversed and the cell travels laterally before the membrane has relaxed sufficiently to release target cells.

In region B the transient flow effect is the mirror of that in region A. Consequently, the traps will focus target cells to the outer edges of the channel. As such, this trap configuration is effective in focusing target cells to the centre of the channel for half the channel length.

### 7.2.5 Chromatographic Behavior

In this series of experiments, the short devices were exclusively used. The following configurations were used; 111 - 11<> ; 111 - 1<> ; 11 - 1 ; 111 - 1 ; 111 - 11. Although the angled traps did not function to focus target cells over the length of the device, the ability of the dynamic microchannel to act as a chromatography column for cells is observed.

To characterize the chromatographic behavior of the dynamic microchannel, we measured the relative flow velocities of a single MLC and RBC. A sample of MLCs and RBCs is prepared with a concentration of $7 \times 10^6$ RBCs/ml and $0.1 \times 10^6$ MLCs/ml. The sample is introduced into the inlet channel at a constant pressure of 20 mbar. The data shown in the figure below is obtained with a duty cycle of 0.222, where the control membrane oscillates between 180 mbar for 2s and 100 mbar for 7s.
**Figure 7.13:** The membrane oscillation corresponds to a DC of 0.222 [2s at 180 mbar and 7s at 100 mbar]. Displacement time graphs showing the specific displacements of both cell types under a constant pressure of 20 mbar.

During the experiment, we noticed the MLCs would increase in velocity as the membrane deflected but would still be trapped before the RBCs achieved their highest forward velocity. This is consistent with the idea that the MLCs would slightly deform and slide between the membrane and the microstructure before settling into a trap.

In the open state (region A) the velocity of the MLC and RBC is similar as shown by the slope of the graph. This corresponds to approximately 70 µm/s. In the semi-closed state (region B), the MLC is trapped by the microstructures and is nearly stationary, while the RBC continues to travel at a similar average velocity as the open state. The negligible change in average RBC velocity can be attributed to the reduced change in cross-sectional area resulting from the non-zero base pressure of the membrane. Because the RBC is progressing while the MLC is stationary, it leads to a difference between the average velocity of RBCs and MLCs that is determined by the duty cycle of the membrane oscillation.

To investigate this relationship, a mixture of cells are introduced into the inlet channel at a constant pressure of 20 mbar. The DCs used to oscillate the control membrane are 0.167; 0.222 and 0.333 and operate between a pressure of 100 mbar and 180 mbar. To minimize the continuous interaction between cells and the microstructure, the time at 180 mbar is kept constant at 2 s. The relative
displacement of a single pair of MLC and RBC is then measured for each DC. Due to the limited FOV, cell positions are monitored over a channel length of 3000 µm. The metric for the ability to impart different velocities is the relative displacement, which is defined as the ratio between the displacement of the MLCs and the RBCs over the same unit of time. These values are obtained on average every 0.3 s and plotted in Figure 7.14. In this system, deformable cells travel at a greater average velocity than rigid cells. Thus a smaller value of relative displacement refers to greater separation efficiency.

![Relative Particle Displacement vs Time](image)

**Figure 7.14:** Relative cell displacement ($S_{MLC}/S_{RBC}$) over time for various DCs under a constant 20 mbar input pressure. Membrane oscillation pressures vary between 100 mbar and 180 mbar.

With decreasing DC we observe a decrease in relative separation. 0.222 and 0.167 tend towards a specific relative displacement while 0.333 appears to slowly decrease with time. This phenomenon is believed to be caused by the actuation of the membrane which inflates at a higher rate than it deflates. At high DCs, the membrane does not have sufficient time to deflate and rigid cells cannot make forward progress. Since deformable cells can still travel, an increasing separation efficiency is observed.

To validate the results from Figure 7.14, the relative time taken for MLCs and RBCs to travel over the same unit of distance is investigated. To measure the relative time over the entire channel length,
individual cells were tracked at a constant pressure of 30 mbar. The DCs and membrane operating pressures are identical to the previous experiment. The average time taken for the cell to negotiate the channel was calculated, recorded and the inverse of the relative time plotted in Figure 7.15. Duty cycles of 0.167, 0.222 and 0.333 were used and the number of individual RBCs tracked was 9, 6 and 3 respectively. These measurements were repeated with MLCs.

These experimental relative displacement results are summarized with theoretical predictions in Figure 7.15. The theoretical results are obtained by assuming that when the membrane is deflected, (1) the target cells are trapped instantaneously by the microstructure, (2) the background cells can travel unobstructed, and (3) the velocity of the background cell is proportionally altered by the change hydrodynamic resistivity. In this case, we use Kartalov’s model to estimate a membrane height change of approximately 4 µm between when inflated at 100 mbar versus 180 mbar. Using this, we can approximate $\frac{R_{H(\text{SC})}}{R_{H(\text{OPEN})}}$ as 1.3 and the $\frac{A_{\text{SC}}}{A_{\text{OPEN}}}$ as 0.4. Using equation 17,

$$U_{\text{OPEN}} = \frac{A_{\text{SC}}}{A_{\text{OPEN}}} \times \frac{R_{H(\text{SC})}}{R_{H(\text{OPEN})}} \times U_{SC}$$  \hspace{1cm} (22)

$$U_{\text{OPEN}} = 0.40 \times 1.18 \times U_{SC}$$  \hspace{1cm} (23)

$$\therefore U_{SC} = 2.12U_{\text{OPEN}}$$  \hspace{1cm} (24)
Figure 7.15: Theoretical and experimental plots relating relative cell displacement ($S_{MLC}/S_{RBC}$) to DC for a constant pressure-driven flow. The experimental results are gained by oscillating the membrane between a pressure of 100 mbar and 180 mbar with a constant $T_{SC}$ of 2 s and 30 mbar input pressure. Theoretical results are plotted using the same DC and assuming $U_{SC} = 1.5U_{OPEN}$.

As expected, both theoretical and experimental results show the relative cell displacement increase with decreasing duty cycle. Interestingly, the experimental relative displacement is more efficient than the theoretical results which can be explained by the membrane actuation asymmetry previously mentioned. This phenomenon causes target cells to be caught at a faster rate than they are released resulting in an increased target cell retention time than what is predicted by theory. Finally, it can be seen that this separation mechanism does indeed operate independently of flow rate, verified by comparable relative cell displacements to Figure 7.14.

7.2.6 Cell Enrichment and Viability

The ability to accumulate target cells in the dynamic microchannel has been quantified using the following experiment: A mixed sample containing $7 \times 10^6$ RBCs/ml and $0.1 \times 10^6$ MLCs/ml is prepared and introduced into the device at a constant pressure of 30 mbar while oscillating the membrane between 100 mbar and 180 mbar with a DC of 0.222. Prior to mixing, the MLCs are stained using a two-color live/dead fluorescence assay (ethidium homodimer-I and calcein acetoxymethly, Invitrogen) and the RBCs are added to this sample. As the RBCs are of an extremely high concentration to begin with ($168 \times 10^6$ RBCs/ml), only a very small volume is required. Thus the bulk of the sample contains the live/dead stain which can be used to both visually identify MLCs and to monitor their viability. The microscope FOV is fixed on a particular section of the microchannel and
images are acquired every minute over a 20 minute period. The number of MLCs and RBCs at each time point is counted and plotted in Figure 10.

![Cell concentration over time](image)

**Figure 7.16:** A plot of the concentration of MLCs and RBCs over 20 min under a constant 30 mbar input pressure. A DC of 0.22 was set to oscillate between 100 mbar and 180 mbar and the active state was kept constant at 2 s. The cell counts were done within a fixed FOV over a channel length of 3000 µm.

This figure clearly indicates that the number of RBCs remain constant while the number of MLCs are accumulated until reaching a steady-state value after approximately 600s. This plateau effect occurs when cells initially reaching the FOV begin to exit at the same rate as new cells are reaching the FOV.

Finally, to ascertain cell viability, the sample was imaged under a red filter and a green filter both before and after sorting. The results of this test are shown in Figure 7.17. We observed no notable change in the viability of MLCs before and after separation in the dynamic microchannel. This observation confirms that the target cells are not damaged by the membrane deflection, which is consistent with the observations of (7).
Figure 7.17: A) Images of MLCs before enrichment under a green filter (1) and a red filter (2). B) Images of MLCs after enrichment under a green filter (3) and a red filter (4). Green Filter Ex/Em(nm):494/517 ; Red Filter Ex/Em(nm):517/617.
Chapter 8

Conclusion

8.1 Conclusion

We designed, constructed and tested a new technique for chromatographic separation of cells based on size and rigidity using a dynamic microstructure. We demonstrated that rigid cells travelling through this microstructure experienced a reduced velocity relative to that of deformable cells. The flow velocity of the target cells can be precisely controlled by the duty cycle of the membrane oscillation. We further demonstrated that this device could be used to accumulate rigid cells without chemical labels while maintaining viability. The channel geometry can easily be modified to target specific cell populations. This technique is simple, cost-effective, and label-free.

8.2 Summary

The field of microfluidics was discussed with particular application to mechanical cell separation. Subsequently, specific mechanical properties of cells were investigated before a review of the literature regarding size- and rigidity-based cell separation in the field of microfluidics was presented including some key trends.

The concept of separating cells based on size and rigidity using dynamic microstructures was presented and designed as a multilayer PDMS device. The master silicon molds were fabricated using four-layer photolithography, while the microfluidic devices were fabricated using replica molding and multilayer soft lithography.

We demonstrated chromatographic cell separation using a mixture of L5178Y mouse lymphoma cells (MLCs) and fixed human red blood cells (RBCs). We found the relative separation to rely upon the duty cycle of the oscillating membrane. We observed and measured a transient flow arising from membrane deflection using RBCs. We found this transient profile to rely upon the position of the cell within the channel as well as the rate of deflection of the membrane. Finally, we demonstrated target cell accumulation in the microchannels while maintaining cell viability.
8.3 Design Recommendations and Future Work

The following recommendations are made for future design iterations:

8.3.1 Short-term Goals

To completely remove the effects of membrane misalignment on the flow channels in the vicinity of the trapping area, either the membrane length should be shortened or the trapping channel length extended to result in a design as shown in Figure 8.1.

![Figure 8.1](image_url)

*Figure 8.1:* Top view of a simple device design where the trapping channel extends past the end of the actuating membrane.

The height of the continuous flow section (Figure 8.2 Region 2) should be maximized bearing in mind that the height of the variable flow section (Figure 8.2 Region 1) determines the width of the channel (10:1 ratio). If the height of the constant flow section is maximized, it will make the fabrication of the master mold more robust as larger variations in the heights of the photoresist layers would be tolerable. In addition, a greater proportion of the channel area would be a region of continuous flow therefore decreasing the change in cross-sectional area between states and thus minimizing flow fluctuations.

![Figure 8.2](image_url)

*Figure 8.2:* Front cross-section of the flow channel. Region 1 represents non-continuous flow while regions 2 represent continuous flow.

Lateral particle motion can be addressed by reversing the angle of the traps based on their location within the channel. As described in 6.2.4, transient fluid motion due to membrane deflection operates in a consistent manner. With membrane inflation, fluid is temporarily driven away from the centre of
the membrane only to return with membrane deflation. Using the behavior of target cells in angled traps, we can exploit this phenomenon as shown in the figure below.

**Figure 8.3:** Top view of a single flow channel with overlaid actuating membrane. The enlarged sections illustrate target cell motion on either side of the centre of membrane deflection.

In region A, the initial membrane inflation traps the target cell and the transient fluid flow forces the cell away from the center of membrane deflection. The cells thus follows the path until 1 where the normal force from the microstructure forces the cell until 2. When the membrane is relaxed, transient fluid flow forces the target cell to travel back towards the centre of membrane deflection. Thus the cell follows the path through 3 until its final release position where the membrane has deflated sufficiently to allow the cell to leave the trap. This is consistent with observations described in 7.2.4.

In region B the effect of the transient is negligible and thus angled traps have little effect. In region C the transient effect is the mirror of that in region A. By angling traps according to their relative position to the actuating membrane, we can prevent lateral target particle displacement using the transient flow velocity. Thus, this trap configuration allows for reversible flow as the transient motion is independent of the direction of average bulk flow.

Transient fluid flow should be accurately characterized within the channel using PIV. This could be done using large fluorescent particles to represent target cells and small fluorescent particles to represent non-target cells. An accurate transient flow profile would allow for a more optimal membrane design as well as optimal trap orientation.

A system to periodically reverse the flow of the device. We demonstrated the accumulation of target cells within the system, however this number is limited due to the finite length of the channel. If the sample was oscillated back and forth through the channel, a greater target cell enrichment could be obtained as described below.
The above figure demonstrates a potential device design for reversible flow. The sample is split and introduced in both inlets at an identical pressure. A certain DC is applied to the actuating membrane and the device is allowed to run by opening valves 1 & 2 and closing valves 3 & 4. Non-target cells will be eluted while the target cells progress along the channel. Before target cells leave the trapping system, the direction of flow can be reversed by opening valves 3 & 4 and closing valves 1 & 2. In this way target cells could be further enriched without affecting the background population. A system would need to be effected in order to prevent the loss of target cells when changing direction however there are several ways this could be done.

A final idea to potentially reduce transient flow, would be to cross-linking between flow channels. The height of the cross linking should be large enough to allow target cells to traverse to prevent clogging. Cross-linked flow channels would allow some of the fluid displaced by the membrane to move laterally between channels and reduce the velocity of the transient along the channel.

### 8.3.2 Long-term Goals

Device used in the separation of rare cells from whole blood. Particular applications could include CTCs. Relying on the success of this application, the technique could be integrated into a biomedical device to isolate and enumerate CTC’s from the peripheral blood of cancer patients.
References


Appendix A
Polyurethane Molding

The chemicals used to create the replica molds of the silicon wafers are:

- Polyurethane (Smooth-Cast® 300, Smooth-Sil® 910.)
- Polydimethylsiloxane (Sylgard 184, Dow Corning Corp.)
- Silicone Rubber (Smooth-Sil® 910, Smooth-On Inc.)

The following procedure was used to fabricate the polyurethane dishes for the flow molds and the polyurethane wafers for the control molds. I would like to acknowledge Jenny Reimer for her work done in developing and recording these procedures as well as providing the images used. It must also be noted that, where possible, any steps that expose the microfeatures are carried out in a laminar flow hood. At all other times the features should be kept covered. This helps prevent particles from settling on the features and possibly affecting the final device.

Polyurethane Dish Fabrication

The polyurethane dish is fabricated in three steps. Firstly a silicone rubber dish is made. This is followed by a PDMS mold of the silicon wafer. Lastly, the PDMS mold and the silicone rubber dish are used to make the final polyurethane dish. The result is a polyurethane dish with the micro-features imprinted on the bottom. Provided sufficient care is taken, the three components can be reused many times.

Silicone Rubber Dish

- Place a 6-inch plastic dish face down in 9 inch diameter baking dish.
- Measure 150ml of each of the two parts of silicone rubber in separate containers and mix well.
- Add two parts together and mix them until a homogeneous mixture is formed.
- Pour the mixed silicone rubber into the baking pan (Note: There should be enough silicon rubber to fill the baking dish and to completely cover the plastic dish).
- Allow 7 hours to cure at room temperature.
• Remove the silicone rubber mold from the dish (Pictured below).

Figure A.1: Finished silicone rubber dish.

**PDMS Mold**

• An aluminum foil (tinfoil) cup is made to tightly fit the silicon wafer.

• 80g of a 10:1 mixture of PDMS prepolymer and curing agent is prepared using a centrifuge mixer set to mix for 5 min and to defoam for 2 min.

• The mixture is then carefully poured into the tinfoil cup to avoid introducing air bubbles and degassed in a vacuum dessicator until all the air bubbles are gone.

• It is then baked in a convection oven at 65 °C for 3 hours. The tinfoil cup is then removed from the oven and allowed enough time to cool to room temperature.
The PDMS mold is then removed from the tinfoil cup and carefully separated from the silicon wafer.

Figure A.2: Silicon wafer in tinfoil cup.

**Polyurethane Dish**

- Degas the PDMS mold to remove the air trapped in the porous PDMS (approx 15min).
- Shake the two polyurethane parts well to prevent sedimentation.
- Measure 100ml by volume of each of the two parts of polyurethane in separate containers (Note: There should be enough polyurethane to fill the dish and to completely cover the PDMS mold).
- Degas the polyurethane in a vacuum dessicator until all of the air bubbles are gone.
- Mix the two polyurethane parts carefully by hand until a homogeneous mixture is formed.
- Pour the polyurethane carefully over the PDMS to fill the dish. Remove any visible air bubbles from the features using a thin piece of wire or similar device.
- Allow the polyurethane 2-4 hours to cure.
• Remove the polyurethane mold from the dish and remove the PDMS layer from the polyurethane mold (Final dish pictured below).

![Final polyurethane dish](image)

**Figure A.3:** Final polyurethane dish.

**Polyurethane Wafer Fabrication**

The polyurethane wafer is once again fabricated in three steps. Firstly a silicone rubber ring is made. This is followed by a PDMS mold of the silicon wafer. Lastly, the PDMS mold and the silicone rubber ring are used to make the final polyurethane dish. The result is a polyurethane dish with the micro-features imprinted on the bottom. Again, provided sufficient care is taken, the three components can be reused many times.

**Silicone Rubber Dish**

• Place a 6-inch plastic dish face down in 9 inch diameter baking dish.

• Place a beaker with a diameter of approximately four inches inside a dish with a six inch diameter.

• Measure 150ml of each of the two parts of silicone rubber in separate containers and mix well.

• Add two parts together and mix them until a homogeneous mixture is formed.

• Pour the mixed silicone rubber into the dish around the beaker.

• Allow 7 hours to cure at room temperature before removing the final silicone ring.
**PDMS Mold**

- Place the silicon wafer on a sheet of tinfoil slightly larger than itself on top of a 6-inch plastic dish (pictured below).

![Silicon wafer on tinfoil on plastic plate](image)

**Figure A.4:** Silicon wafer on tinfoil on plastic plate.

- A few drops of HMDS (hexamethyldisilazane) are placed on the silicon wafer. A monolayer of HMDS helps prevent PDMS sticking to the wafer (Note: This step should be done in the fume hood as opposed to the laminar flow hood as HMDS is a dangerously reactive chemical).

- Measure 200g of PDMS with a 10:1 base to hardener ratio and mix in a centrifuge mixer set to mix for 5 min and defoam for 2 min.

- Pour the PDMS directly on top of the silicon wafer, taking care not to introduce air bubbles.

- Vacuum the PDMS in a vacuum dessicator until all air bubbles are gone.

- Bake the PDMS at 65°C for 3 hours.

- Remove the PDMS from the oven and allow sufficient time to cool to room temperature.
• Carefully remove the PDMS mold (pictured below) from the dish and extract the silicon wafer.

![PDMS mold](image)

**Figure A.5: PDMS mold.**

*Polyurethane Wafer*

• Degas the PDMS mold to remove the air trapped in the porous PDMS (approx 15min).

• Shake the two polyurethane parts well to prevent sedimentation.

• Measure 20ml by volume of each of the two parts of polyurethane in separate containers.

• Degas the polyurethane in a vacuum dessicator until all of the air bubbles are gone.

• Mix the two polyurethane parts carefully by hand until a homogeneous mixture is formed.

• Pour the polyurethane carefully over the PDMS to fill the ring to a height of approximately 5mm.

• Remove any visible air bubbles from the features using a thin piece of wire or similar device.

• Allow the polyurethane 2-4 hours to cure.
• Remove the polyurethane mold from the dish and remove the PDMS layer from the polyurethane mold. The mold may need to be trimmed to a round shape.

• Due to the opacity of the polyurethane, the micro-features are extremely difficult to see under a stereo-microscope. Hence, non-crucial features (such as cutting/alignment features) are marked with a permanent marker.

![Image of polyurethane mold and marked wafer]

**Figure A.6:** Curing polyurethane (left) and the final marked wafer (right).
Appendix B
Supplementary Data

This appendix will present some noteworthy data obtained. It is split into three sections. In the first section a sequence of images is shown depicting cell motion within the device. These images are taken in a single section of microchannel over a period of \(~ 20\) s with a fixed field of view. In the second section velocity time plots are shown for a single pair of cells, namely a RBC and MLC followed by an overlaid displacement time plot. These data are given for three separate duty cycles (DCs). These are followed by a brief discussion of the figures. In the third section a table is presented showing the times taken for both RBCs and MLCs to travel through a 6000 \(\mu\)m section of microchannel under varying DCs.

Section 1

(Figure on next page)
Figure B.1: A sequence of digital images using a fixed field of view taken every second using a 111-11 device. Constant $P_{IN} = 20$ mbar from left to right flow. DC of 0.333 with membrane oscillated between 100 mbar and 200 mbar. $T_{SC} = 2$ s.
Figure B.1 shows the chromatographic behavior of the device. The MLC has little displacement in comparison to the RBCs. Upon close inspection, movement of the MLC within the trap can be seen. The transient velocity of the device can also be observed as shown by the path of the RBCs however the net displacement of the RBCs is indicative of the constant forward fluid flow.

Section 2

The following results are obtained using experimental procedures similar to those outlined in section 7.2.3. Images were obtained on average every 0.15 s and analyzed using ImageJ.

**Figure B.2:** Velocity profile of a RBC over time. Particle downstream of pressure inlet. Duty cycle 0.167 (2 s at 180 mbar; 10 s at 100 mbar). Flow input pressure 20 mbar.
**Figure B.3:** Velocity profile of a MLC over time. Particle downstream of pressure inlet. Duty cycle 0.167 (2 s at 180 mbar; 10 s at 100 mbar). Flow input pressure 20 mbar.

**Figure B.4:** The membrane oscillation corresponds to a DC of 0.167 [2s at 180 mbar and 7s at 100 mbar]. Displacement time graphs showing the specific displacements of both cell types under a constant pressure of 20 mbar.
Figure B.5: Velocity profile of a RBC over time. Particle downstream of pressure inlet. Duty cycle 0.222 (2 s at 180 mbar; 10 s at 100 mbar). Flow input pressure 20 mbar.

Figure B.6: Velocity profile of a RBC over time. Particle downstream of pressure inlet. Duty cycle 0.222 (2 s at 180 mbar; 10 s at 100 mbar). Flow input pressure 20 mbar.
Figure B.7: The membrane oscillation corresponds to a DC of 0.222 [2s at 180 mbar and 7s at 100 mbar]. Displacement time graphs showing the specific displacements of both cell types under a constant pressure of 20 mbar.
**Figure B.8:** Velocity profile of a RBC over time. Particle downstream of pressure inlet. Duty cycle 0.333 (2 s at 180 mbar; 10 s at 100 mbar). Flow input pressure 20 mbar.

**Figure B.9:** Velocity profile of a RBC over time. Particle downstream of pressure inlet. Duty cycle 0.333 (2 s at 180 mbar; 10 s at 100 mbar). Flow input pressure 20 mbar.
Figure B.10: The membrane oscillation corresponds to a DC of 0.333 [2s at 180 mbar and 7s at 100 mbar]. Displacement time graphs showing the specific displacements of both cell types under a constant pressure of 20mbar.

In the above figures, we show the velocity profile of the RBC, the velocity profile of the MLC and finally an overlaid plot of the displacement of both the cells over time.

**RBC velocity profile:**

A cyclical velocity profile can be observed in these plots. Initially, the RBC experiences a large positive increase in velocity. The steep gradient and short period of this peak in comparison to the following peak is indicative of membrane inflation. The positive value is consistent with a cell location downstream of the center of membrane deflection. Following this, the RBC experiences a negative velocity however with a gentler gradient and longer period than the positive peak. This is consistent with membrane deflation and the negative value verifies the downstream cell position. With smaller DCs we can observe the cell velocity to stabilize between membrane deflation and inflation. This is expected and corresponds to the open state of the channel.

**MLC velocity profile:**

A cyclical velocity can also be observed in these plots. Once again, the MLC experiences a positive increase in velocity however this is usually over a short period of time. The steep gradient and short period of this peak in comparison to the following peak is indicative of membrane inflation. In this
case however, the MLC is typically arrested by the cell trap and the velocity profile reflects this state with a zero value. Following this stationary period, the MLC frequently experiences a negative velocity that corresponds with the RBC velocity profile for those experimental conditions. With smaller DCs we can observe MLCs travelling with a constant velocity that corresponds to cell motion in the open state.

**Combined cell displacement profile:**

In these plots, the higher average velocity of the RBC compared to the MLC can be seen. With increasing DC, we observe an increase in cell separation. The fluctuations in RBC displacement are indicative of the transient velocity. The periods of zero displacement of the MLC indicate the semi-closed position of the channel. Once again, at smaller DCs we can observe regions where the cells travel with similar velocities as shown by the slope of the graph. These regions correspond to the open state of the channel.
Combined MLC displacement profile:

In Figure B.11, the displacement of a single MLC is plotted for various duty cycles using the data from B.4; B.7 and B.10.

Figure B.11: The membrane oscillates between 180 mbar and 100 mbar. Displacement time graphs showing the specific displacements of a single MLC under a constant pressure of 20mbar for varying duty cycles.

At higher duty cycles, the displacement of the MLC is smaller. It can also be seen that the number of times that the cell is entrapped increases with increasing duty cycle, as shown by the number of horizontal sections of the graph which indicates a velocity of zero. This is critical to the operation of the device as the velocity of the RBCs is largely unaffected by the duty cycle and thus the average velocity of the MLC indicates the relative cell velocity (or retention factor) of the system for a given duty cycle.
Section 3

Table B.1: Times for cells to traverse a 6000 \(\mu\)m section of oscillating microchannel using various duty cycles.

<table>
<thead>
<tr>
<th>Test</th>
<th>DC 1 [0.1667]</th>
<th>Test</th>
<th>DC 2 [0.222]</th>
<th>Test</th>
<th>DC 3 [0.333]</th>
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<tbody>
<tr>
<td></td>
<td>Time (s)</td>
<td></td>
<td>Time (s)</td>
<td></td>
<td>Time (s)</td>
</tr>
<tr>
<td>MLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>311.9 333.73</td>
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<td>580.57</td>
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<td>360 342</td>
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