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Desarrollo de dispositivo microfluídico para la visualización del fenómeno de la extravasación leucocitaria

Development of flow focusing device for the visualization of leukocyte rolling adhesion

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Resumen

La microfluídica es un área de la microtecnología basada en chips de PDMS que está siendo utilizada cada vez más en multitud de aplicaciones. Una de estas aplicaciones es la investigación biomédica. La microfluídica o "Lab on a Chip" se ha convertido en una manera de realizar experimentos biomédicos y diagnósticos de una manera barata, rápida y eficaz. Cuando se realizan estudios sobre la extravasación leucocitaria utilizando chips microfluídicos, podemos observar la inconsistencia en la trayectoria de rodadura de los leucocitos debido a un flujo laminar. En este trabajo de fin de grado presentamos un método para centrar la interfaz de células en el centro de canal microfluídico. Cuando las células circulan por los sistemas microfluídicos, las células tienden a circular de manera aleatoria por los canales. Por tanto, con el sistema propuesto en este trabajo, dichas células serán redirigidas a la porción central del canal con el fin de recrear el fenómeno de rodadura presente en nuestro sistema circulatorio y así obtener información más detallada. Los resultados de este trabajo muestran la utilidad y la versatilidad de este dispositivo para experimentos relacionados.

Palabras clave: Microfluídica, Lab on a Chip, Point of Care, Extravasación Leucocitaria, Litografía blanda, Inmunología, Modelado Multifísico, Método de elementos finitos.

Abstract

Microfluidics is an area of technology based on PDMS chips that is being increasingly used for many applications. One of said applications is biomedical research. Microfluidics or Lab on a chip poses as a great way of cutting costs, time, space and overall improving efficiency in medical diagnosis and biomedical experiments. When studying the rolling cell adhesion behaviour of leukocytes on microfluidic devices, we can observe the inconsistency in the manner cells roll due to laminar flow. In this final year dissertation we present a method to focus the flow of leukocytes on a rectangular microfluidic channel for rolling cell adhesion assays. When flowing in microfluidic channels, cells tend to circulate in a random manner, therefore with the system proposed, said cells will circulate in the central area of the channel, in order to optimally recreate the rolling cell adhesion phenomenon present in our circulatory system and achieve precise information regarding this cellular behaviour. The results of this work show the viability of the system and the versatility it may have with other related experiments.

Keywords: Microfluidics, Lab on a Chip, Point of Care, Rolling Cell Adhesion, Hydrodynamic flow focusing, Soft Lithography, Immunology, Multiphysics Modelling, Finite Element Method.

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Chapter 1: Introduction

1.1 Background and Motivation

Leukocyte rolling adhesion is the movement of leukocytes across the interior walls of capillaries and venules with the final goal of reaching the site where it will extravasate and ultimately combat the infection. Therefore the understanding of this phenomenon is paramount in comprehending how our immune system works.

Although this cellular behaviour has been thoroughly studied, it has never been approached from a biomechanical point of view. This was the idea behind the research conducted at Motlab in Tokyo University of Science. The goal was to try to shed a bit more light on this phenomenon that occurs in our body.

The initial research carried out by Sayaka Nomura, was aimed to observe the rolling adhesion of leukocytes along straight microfluidic channels. Once the observation and capture of images through fluorescence microscopy was achieved, she analysed the images with a self-written programme on python. This programme analyses the speed, angular velocity and centre of mass of the rolling leukocyte and outputs the numerical values of these parameters.

However, during the development of the research, one thing was experienced; there was a problem with the way leukocytes rolled in the channel. Due to the simplicity of the channel design and the physics that govern microfluidic flow, leukocytes rolled in random pathways and not in a constant area of the channel, making the analysis of the data non reliable. Due to the parabolic profiles of laminar flow, the conditions which govern the behaviour of the immune cells vary depending on the section at which the cell is located. Therefore, my work aims to solve this problem and create a device that could be used in other essays and experiments of the sorts.

This type of microfluidic device is called lab on a chip and belongs to a realm of microfluidic technology that is increasingly being used in biomedical engineering thanks to the advantages it poses over the classical glassware experiments.

The device created and explained in this work consists of a microfluidic system with a hydrodynamic flow focusing stage which ensures cells will flow through a controlled centred section of our microfluidic channel, for an optimal observation and analysis of the rolling of leukocytes along the device. Thanks to the design of the microfluidic channel, both top view and side view of the movement of the cells is possible.

The system created in this work, poses of great relevance due to all of the fields it may be used in and all the applications it may have. Rolling cell adhesion is not only studied in immunology, but also in cancer and thrombus research. It has been discovered that E-selectin mediated rolling facilitates pancreatic cancer cell adhesion (Shea, et al. 2017). There are many recent studies that explain the importance of rolling adhesion in these areas of medicine, therefore the development of a system that has the ability to mimic this movement *in vitro* is of great relevance when testing new therapies, drugs and experiments.

This work was developed at the Tokyo University of Science during a 3 month stay as a visiting student in Motlab, a micro/nanoscale thermofluidics research group directed by Professor Masahiro Motosuke in Tokyo, Japan.

1.2 Objectives

The main objective of this work is to develop a system that solves the problem raised previously: non uniform, non-centred flow of leukocytes along the microfluidic channel. The system will be reusable and biologically active in order to be used systematically in leukocytic experimentation.

The specific objectives are as follow:

- Systematically review an extensive bibliography on microfluidics, rolling cell adhesion and lab on a chip.
- Understand the state of the art in microfluidic and rolling cell essays.
- Develop a system capable of centring a stream of leukocytes with the goal of observing rolling cell adhesion.

1.3 Content Outline

The following document is divided into seven chapters and a bibliographical section. This is the **introductory** first chapter of the seven chapter dissertation.

In **Chapter 2: The Immune System**, we review and analyse an extensive bibliography regarding the **immune system**, **Leukocytes** and **rolling adhesion**. Here we aim to explain all the relevant information needed to understand the underlying biological principles and phenomena related to the works presented in this dissertation.

In **Chapter 3: Microfluidics**, we aim to explain what **microfluidics** is and relate the equations that govern microfluidic flow to our work. In this chapter many aspects of **microfluidic dynamics** are covered with the goal of providing the reader of this work, the relevant information for understanding how the system behaves.

In **Chapter 4: Simulations**, a series of simulations performed on **COMSOL,** the multiphysics simulation software, have been included. This chapter is of extreme relevance because it helps us visualize and comprehend the principles that dictate the behaviour of the fluid and the system we aim to design. Here we can observe **hydrodynamic fluid focusing** by two methods: **particle tracing** and **transport of diluted species**.

In **Chapter 5: Materials and Method**, I have included all the necessary information needed to build the system. The materials, machinery and process was depicted in this chapter and supported with pictures. Both the microfluidic and the biological aspects of this work have been included.

In **Chapter 6: Experimental Analysis**, the information regarding experimental setup and results has been included, alongside a discussion for each of the four experiments performed.

In the final chapter, **Chapter 7: Conclusion**, we conclude this dissertation and propose future lines of work.

Chapter 2: The Immune System

2.1 The Immune System

Like all mammals, humans possess a specialized system destined for the protection and the defence of our body; we refer to this mechanism as the Immune System. This complex is composed by very specialized cells that function inside very well organized anatomical structures. Therefore to understand the importance of these specialized cells we must study them in conjunction with the tissues and organs in which these cells interact with.

If we go back in history, evolutionary speaking, our immune system was developed in order to combat the infections caused by viruses, bacteria, protozoa, fungus and helminths. These pathogens can be responsible for many infections both intracellular and extracellular, for which the response must be different. The immune system has developed a variety of appropriate defence mechanisms in order to fight off each of the different pathogens that infect us.

In order to eradicate a pathogen that has infected its host, the immune system must first detect its presence and then take the necessary steps to destroy it. For the destruction of pathogens, the human body has developed diverse immune mechanisms which are both innate and adaptive; the difference rests in the fact that they use different methods to detect threats and infections.

In order for the innate response to work, a series of molecular patterns that are present in same group pathogens must be detected. All members of a same animal species are born with an innate and immediate capacity of detecting and destroying numerous pathogens which we have not been in contact before. The innate immune response is very effective; however, it cannot protect us from all threats. The majority of pathogenic agents are single celled organisms that divide very quickly; therefore, they evolve at higher rates and evade the body's innate response. In order to avoid this, vertebrates have developed a pathogen reconnaissance strategy which enables the detection of foreign agents that have never been encountered before. This defence mechanism is referred to as

the adaptive immune system. Lymphocytes are the cells which will be responsible for this adaptive immunity.

Immune cells are the key players in our immune system, enabling detection and destruction of foreign pathogens. There are many types of cells, each with different functions and characteristics. However, their origin is similar. Immune cells, commonly referred to as white blood cells or leukocytes, originate from the bone marrow, a semi-solid tissue found in the trabecular bone tissue. In this myeloid tissue, leukocyte precursors are created. These "parent" cells will then differentiate through a process called haematopoiesis to create white blood cells.

There are many types of cells in our immune system as seen in Figure 2.1, all with different characteristics but same objective: to destroy foreign pathogens.

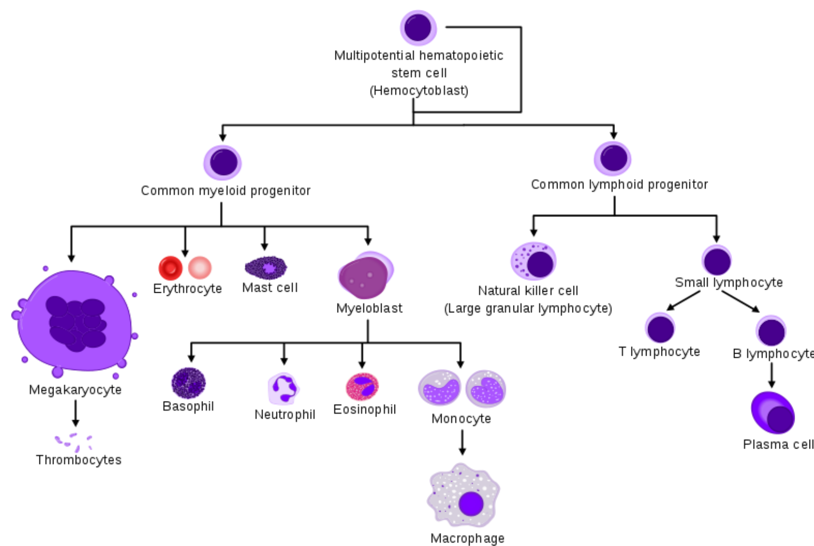

Figure 2.1: Leukocyte development, from progenitor to mature differentiated cells **(Mikael Häggström 2009)**.

2.1.1 Haematopoiesis

All blood cells arise from a type of cell called the hematopoietic stem cell (HSC). Stem cells are undifferentiated cells which have the unique capacity of differentiating into many other types of cells (see Figure 2.1). Also, they possess many different characteristics which enable them to self-renew themselves indefinitely and regulate their population by cell divisions (Kuby, et al. 1992). In humans, haematopoiesis begins in the embryonic yolk sac during

the first two weeks of development, by the third month, hematopoietic stem cells have migrated to the foetal liver; after that, the differentiation of HSCs in the bone marrow becomes the mayor factor in haematopoiesis. By birth there is little or no haematopoiesis in the liver and spleen (Kuby, et al. 1992, 23).

The first step to haematopoiesis consists in the differentiation of a multipotent stem cell, creating either a myeloid progenitor cell or a lymphoid progenitor cell. These cells have now lost the capacity of self-renewal and are therefore destined to give rise to cells of its particular cell lineage. Lymphoid progenitors will then differentiate into Natural Killers (NK) cells, T lymphocytes and B lymphocytes. Analogously, myeloid progenitors will give rise to erythrocytes (red blood cells, RBCs), platelets, basophils, neutrophils and other immune cells commonly referred to as white blood cells. Each of these cells are created and induced under different circumstances, different signals and microenvironments will promote growth and differentiation of these cells. These microenvironments are occasioned by scaffold like structures created by stromal cells and by growth factors that arrive to their target cells by diffusion. To summarise, it can be said that the different cells that descend from the HSCs will come to be due to the different interactions of the different factors related with its growth and differentiation.

Haematopoiesis is regulated at genetic level, the development of the stem cells into the different cell types requires the expression of different sets of lineage-determining and lineage-specific genes at appropriate times and in correct order (Kuby, et al. 1992, 24). Therefore, we can say with certainty that the proteins expressed by these genes are of utmost importance and will determine the outcome of cellular differentiation. Much remains to be learnt in this field; however, there are some discoveries that have provided a very useful insight into understanding how this process works. One of these advances in the immune-genetic research led to the discovery of Leukocyte Adhesion Deficiency (LAD), a heritable genetic disease that we will discuss in the following pages due to the close relationship with our research.

2.1.2 Immune System Organs

Like many other systems, the Immune system has distinct organs and tissues with specialized functions, destined to contribute to the development of immune responses. Immunologists classify these organs by function, dividing them into primary and secondary lymphoid organs. The thymus and bone marrow belong to the central o primary lymphoid organs, whereas the secondary lymphoid organs are comprised of lymph nodes, the spleen and other tissues. Maturation of lymphocytes takes place in the primary lymphoid organs, whereas in the previously mentioned secondary lymphoid organs, the matured lymphocytes interact with antigens. These lymphocytes will then circulate around the body through the circulatory and lymphatic system (Figure 2.2), detecting and fighting off possible pathogens.

Primary lymphoid Organs

Lymphocytes generated in haematopoiesis are yet to be matured and become committed to antigenic specificity. Only after the cell has matured within a primary organ, is the cell immunocompetent. T-lymphocytes are called as such due to the fact that they mature in the thymus, a primary lymphoid organ.

The thymus is a specialized organ formed by specialized cells called thymocytes separated by connective tissue. This organ is the site for t-lymphocyte development and maturation. The thymus is flat and bi-lobed, being located above the heart (see Figure 2.2), it is very complex and of utmost importance. Its role in the immune system can be studied in mice by examining the effects of neonatal thymectomy. Thymectomized mice show a dramatic decrease in circulating lymphocytes of the T-cell lineage and an absence of cell-mediated immunity (Kuby, et al. 1992, 41).

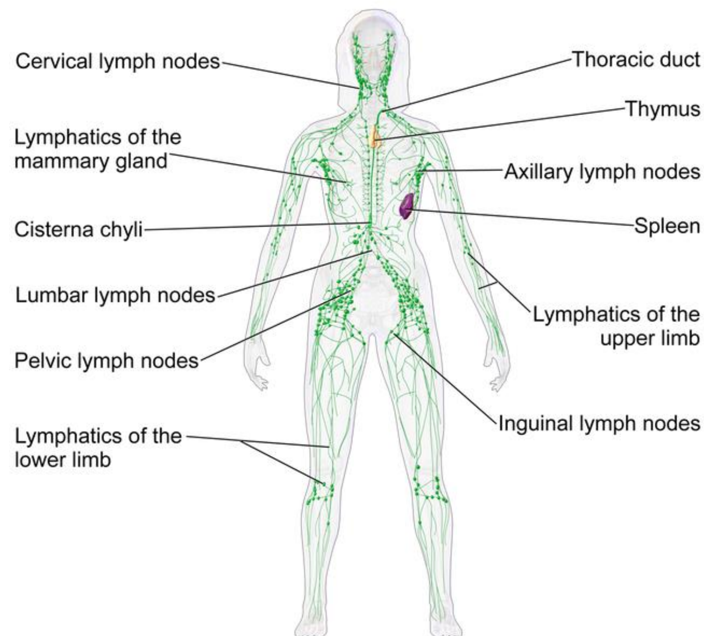

Figure 2.2: Schematic of the Lymphatic System **(Blaus 2013)**.

The other primary lymphoid organ is the bone marrow. The bone marrow is a sponge-like tissue situated inside of bones; most of the cells of our immune system are produced and developed here and, once they develop and mature, they migrate to the bloodstream to circulate the body. At birth, many bones contain red bone marrow, which actively builds defence cells. During the course of life, more and more of this red bone marrow turns into fat tissue. Eventually, all the red bone marrow transforms, except in a few bones, such as the ribs, the breast bone and the pelvic bone (Schmidt, Lang and Heckmann 2010). Lymphocytes that originate from this organ are referred to as B-lymphocytes.

Lymphatic System

The circulatory system is a pressurised system, because of this as blood circulates the body, the plasma seeps through the capillary's endothelium, this fluid is called interstitial fluid and bathes all the surrounding cells. Due to the high volumes of this fluid being permeated through the capillary walls, a lymphatic system needs to collect it to prevent swelling of the nearby areas (edema) which would slowly become life threatening. This lymphatic system forms a network of lymphatic vessels which collect all the lymph (plasma) and then drains it back into the blood. When a foreign antigen gains entrance to the

tissues, it is picked up by the lymphatic system and is carried to the lymph nodes where it is trapped and treated accordingly.

2.2 Leukocyte Activation and Migration

When we experience an infection from an external agent, our bodies react in many different ways to signal our immune system that the integrity of a tissue has been compromised. An inflammatory response is generated as a response to a local injury or trauma, which the immune system detects as a distress signal. This inflammation is a complex response that involves several immune system cells and numerous mediators. Once the innate immune system reaches the area of infection, it mounts the initial attack against the foreign pathogen. However this initial defence may not suffice and lymphocytes are recruited and become activated in response to antigens with the goal of helping in the battle against foreign pathogens. This process is very complex therefore we shall study it in more detail in the following pages.

2.2.1 Cell Adhesion Molecules

The human body is made out of many cells and tissues joint together forming bigger and more functional structures. All these cells are bonded together by what we call cell adhesion molecules (CAMs), and it is these same molecules that help the leukocytes and the immune system cells interact with other tissues.

Leukocytes are circulating around the body waiting for an infection, ready to attack foreign pathogens. Therefore, vascular endothelium serves as an important entry point for these leukocytes to arrive at the targeted tissue. Due to the high pressure in the circulatory system, cells are under high mechanical stresses and find it impossible to attach themselves to the vascular endothelium and trespass the vascular barrier. Therefore, they need some sort of aid to migrate from the blood to the lymphoid organs or surrounding tissues. This process is called extravasation and it happens partly due to the presence of leukocyte specific CAMs that will bind white blood cells to the vascular walls.

Endothelial cells express leukocyte adhesion molecules, some are cell specific and others are response specific, e.g. cytokine produced during an inflammatory process. All in all, we can say that regardless the origin of the response or specificity; these CAMs are paramount in the migration and movement of white blood cells across the body.

In addition to their role in leukocyte adhesion to vascular endothelial cells, CAMs on leukocytes serve to increase the strength of the functional interactions between cells of the immune system. Various adhesion molecules have been shown to contribute to the interactions between T_H cells and APCs, T_H and B cells, and CTLs and target cells (Stein and Nombela-Arrieta 2005). Most of these CAMs belong to four families of adhesion proteins: selectin[1], mucin-like, integrin and the immunoglobulin family.

Selectins

The selectin family of cell adhesion protein are a group of glycoproteins that has a specific domain that enables biding with other molecules due to the affinity of this lectin-like domain to specific carbohydrates. The selectin protein family groups three molecules: L-, E- and P-selectin. E- and P-selectin are mostly found on vascular endothelium whereas L-selectin is expressed on the surface of most leukocytes. E-selectin is a very interesting protein that needs synthesis of new proteins for it to express itself on the vascular walls. These new proteins commonly appear after the stimulation of inflammatory cytokines. Selectins are the initial bonders of leukocytes to vascular endothelium; therefore they play a prominent role in leukocyte extravasation.

Chemokines are another family of proteins relevant in leukocyte extravasation, these molecules are responsible for certain CAMs activation, e.g. E-selectin. Consequently they are a mayor regulator in lymphocyte and leukocyte traffic (Stein and Nombela-Arrieta 2005). Chemokines are typically induced due to an inflammation process, commonly as a response for tissue infection. These molecules are of high importance because they essentially activate E-selectin

[1] Due to the importance of selectin family adhesion protein in our research we shall only focus on this protein for the time being.

molecules. In the event of chemokines not being expressed, leukocytes would not be correctly recruited due to E-selectin not being activated (Collins, et al. 1991).

2.2.2 Leukocyte Extravasation

Lymphocyte and Leukocyte extravasation as a whole is a multistep process that occurs in blood vessels and regulates the traffic of white blood cells in the body. This process is normally initiated by an inflammatory response due to the infection of a tissue by a foreign agent. Once this inflammatory response develops, cytokines and other inflammatory mediators act on the local blood vessel walls, inducing the expression on endothelial CAMs. Once the site is activated, leukocytes can then proceed to extravasate to the site of infection. For this to happen, leukocytes will have to strongly attach themselves to the vessel wall to avoid being swept away by the highly pressurised flow of blood.

Leukocyte extravasation can be divided into four steps: leukocyte rolling, activation, arrest and migration. We shall focus on the rolling phase of the cell's movement. **Rolling** is mediated by selectins; this movement is comprised of a loosely attached bond that will bind the cell to the endothelium by a low-affinity selectin-surface carbohydrate interaction. As we've mentioned previously, E-selectin is activated by chemokines, therefore once they are properly expressed in the endothelium they can adhere to carbohydrates expressed in leukocyte membranes. Being such a weak adhesion, leukocytes will only bond briefly and soon detach itself due to the shear force of the circulating blood. Once this happens it will rapidly attach itself to another endothelial cell. This tumbling of the cell is repeated over and over, creating a rolling motion (see Figure 2.3).

This process is fairly slow; therefore it slows down the speed of the cell just enough to allow interactions between chemokines present in vessel wall and the receptors on the leukocyte surface to interact and generate a tighter bond that will completely adhere the cell to the wall. Once the movement of the leukocyte has been stopped by this tight adhesion, the cell will squeeze through two neighbouring endothelial cells and traverse the endothelial barrier and enter the infected tissue (Anderson and Anderson 1976).

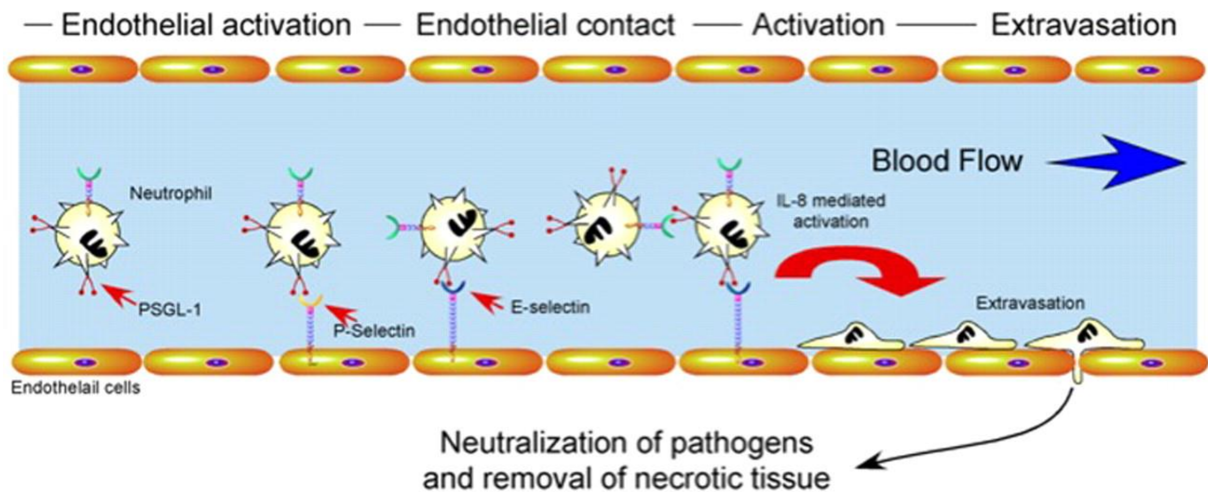

Figure 2.3: Steps in Leukocyte (Neutrophil) extravasation **(H. Shaz, R. Stowell y D. Hillyer 2011)**.

2.2.3 Leukocyte Adhesion Deficiency

Leukocyte Adhesion Deficiency or LAD is a genetic disorder that is characterized by a poor leukocyte adhesion to the capillary walls, making it impossible for the immune system cells to adhere properly and therefore not extravasate to the infected tissue. This disease results in the immunodeficiency of the patient and concludes in the patient having recurrent infections.

LAD affected patients will suffer from bacterial infections from a very early age and can become a life threatening condition due to the infant's inability to combat foreign pathogens. This inability to combat bacteria and other agents is due to the non-existing leukocyte migration. Besides the notable absence of a defence system, LAD patients will also have high counts of leukocytes in the blood and will not be able to form pus (Abbas, Lichtmann and Pillai 2012).

As we speak there is no cure for this disease, the only current curative therapy is intensive antibiotherapy or haemopoietic stem cell transplant. I wanted to include this section in this chapter because although there is no cure, the prototype and experiment that we propose in this work could be used as a tool to conduct experiments and assays to design a novel drug and, eventually, test its suitability for curing this disease.

Chapter 3: Microfluidics

3.1 What is Microfluidics?

Microfluidics is a technology that is given on submillimetre scales: microfluidics studies the interactions of fluids in channels with microscopic dimensions. This area of fluidics is providing a new environment in which we can carry out numerous experiments and will continue to do so in the following years, making it a very promising technology that will be used in multiple fields of science and research.

Historically, microfluidics became widely used after the end of the cold war. Due to the military and terrorist threats chemical and biological weapons posed, scientists realized microfluidics could be used on this field; therefore, the Defence Advanced Research Projects Agency (DARPA) of the US Department of Defence supported a series of programmes in the 1990s aimed at the development of microfluidic systems for field-deployable chemical and biological threat detection. These programmes were the main stimulus for the rapid growth of academic microfluidic technology (Whitesides 2006).

In vitro microfluidics take place in a series of structures called microfluidic channels that manipulate low volumes of fluids (10^{-9} to 10^{-18} litres). These channels are designed and characterised using technologies borrowed from the field of electronics and microelectronics and are normally in the range of hundredths of a micron. Within these channels, diverse fluids flow and interact with each giving microfluidic chips their incomparable advantage. These chips are designed in order to analyse, synthesize chemicals and re-enact diverse phenomena, they are becoming a tool that is increasingly being used due to their ability of performing all these processes with the following advantages: low cost, short reaction times, low reagent volume and increased performance. Besides the aforementioned advantages, the two main advantages microfluidics exhibit are their small size and their laminar flow, this last one being of extreme importance.

3.2 Properties of Fluidics

There are certain important intrinsic properties we should highlight and discuss in this introductory section; they are presented in the following lines.

- Mass density (ρ; Kg/m^3): It is the mass distribution over a unit volume.
- Specific volume (V; m^3 /Kg): It is the volume occupied by a unit mass.
- Weight density or Specific weight ($\gamma; \mathrm{N}/m^3$): It is the force due to gravity on the mass in a unit volume and is expressed as:

$$\gamma = g \cdot \rho \, . \quad (3.1)$$

- Specific gravity or Relative density (δ): it is the ratio of density of the fluid to the density of water.
- Viscosity: It is the resistance offered by the fluid to gradual deformation by neighbouring fluid layers under an external force, namely shear stress or tensile stress.
- Dynamic Viscosity (η; $Poiseuille\ (PI); Pa \cdot s$): It is the resistance offered by a fluid layer to adjacent layers where all the layers are moving parallel to each other but at different speeds. Thus, is also called shear viscosity. Therefore, when top layer is moving slow, then ideally all the layer will be parallel to each other and speed of layers will be 'zero' in the bottom layer and maximum in the top layer. Here, each layer will oppose the forward motion of the layer above it and make the layer beneath it to move forward. In such conditions, an external force will be required to overcome the fluid viscosity and keep it in motion. This force is Newton's shear force (Dixit and Kaushik 2016).

$$F = \frac{\eta\, A\, U}{y} \, . \quad (3.2)$$

These properties are given in all fluids and the alteration and difference between them will alter significantly the behaviour the fluid will exhibit. Therefore by understanding the intrinsic properties of a fluid, we will be able to design better microfluidic systems that behave as we expect them to behave, giving optimal results.

3.3 Shear Stress

Due to the viscosity of real fluids, the friction caused by the boundary conditions, in essence, the channel walls will cause a shear stress on the border. The no-slip condition dictates that the speed of the fluid at the boundary is zero, but at some height from the boundary the flow speed must be equal to that of the fluid (Day 1990). In Newtonian unidirectional fluids flowing in a laminar manner, the shear stress is given by the following formula:

$$\tau(y) = \mu \frac{\partial u}{\partial y}, \qquad (3.3)$$

where μ is the dynamic viscosity, u the flow velocity and y the position along the width of the channel.

To be precise, the wall shear stress is defined as:

$$\tau_w \equiv \tau(y=0) = \mu \frac{\partial u}{\partial y}\bigg|_{y=0}. \qquad (3.4)$$

Let us note that the channel wall is where the highest shear stress is manifested.

3.4 Types of Fluids

When it comes down to fluids there are two main classifications: Newtonian and Non- Newtonian fluids. These fluids have different characteristics due to their different properties. It is important to study the types of fluids and their implications, because they can hugely impact the way we work with them on a microfluidic scale.

Viscosity is proportional to shear stress and shear rate. Therefore the two types fluids are categorized into are given by the relation between viscosities. If the viscosity is constant the fluid will be a Newtonian fluid. On the contrary, if the viscosity changes, the fluid will be a non-Newtonian fluid. Some examples of Newtonian fluids include: water, alcohol, glycerol, honey… These fluids are only temperature dependant meaning, they will only change their viscosity with the action of temperature.

However the majority of fluids are Non- Newtonian, their viscosity changes due to shear rate or shear stress, making viscosity a very important factor in fluidics.

In our case, this study will only work PBS. PBS is an aqueous mixture which contains sodium chloride (common salt) and disodium hydrogen phosphate in proportion to the concentrations present in the human body making it Newtonian fluid.

3.5 Types of Flows

In fluidics there are two types of flow: Laminar and Turbulent. These two types of flow are given, among other, by the trajectory of particles.

Turbulent flows have the characteristic that the particles do not follow a straight path, their movement is chaotic. However when we analyse the flow in, say, a pipe, the average speed of the particles is the same making the flow profile a straight line.

However, in laminar flow this does not happen. Laminar flows behave in such a way that causes the flow to exhibit a parabolic profile.

Note that these two flows can be more or less laminar or turbulent depending on the values of the parameters that characterize the Reynolds number, as we will see in the following section.

3.5.1 Laminar flow

The flow of the fluid through a microfluidic channel can be characterised by the Reynolds number, defined as:

$$Re = \frac{L\, V_{avg}\, \rho}{\mu}, \tag{3.5}$$

where L is the length, V is the average velocity of the flow, ρ is the density of the fluid and μ is the viscosity. Here the most relevant number is L due to the small dimensions of the channel, therefore the Reynolds number will always be much less than 100 making it a laminar flow.

Flow in microfluidics exhibits laminar flow as opposed to macroscopic fluidic systems that usually behave in a turbulent manner. This turbulent flow is chaotic and violent, making particles move along the channels in an erratic and random manner (see Figure 3.1). Turbulent flow in microfluidics is most of the time a disadvantage rather than a beneficial factor, this is because these types of flows are very hard to control and even harder to model, and therefore are practically never used as opposed to laminar flows.

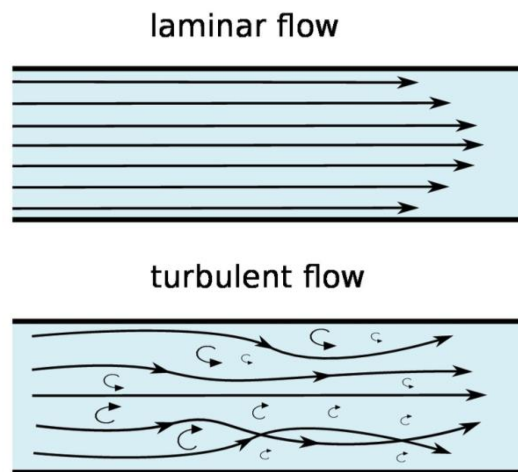

Figure 3.1: Illustration depicting the difference between laminar flow and turbulent flow.

Microfluidic devices mostly behave in a laminar manner. In laminar flow each particle along the width of the channel travels in a straight path and do not interfere in the other particle's movement, this is shown in the absence of turbulence, as previously mentioned. However due to the near perfectly linear motion particles have and their viscosity component, a new effect arises: parabolic flow profile.

Fluids have different grades of viscosity, this viscosity is nothing more than the resistance a particle has to the flow of the fluid. This viscosity affects the fluid particles and makes them more prone to interacting with its neighbouring particles. This is why there is a gradient of velocity in laminar flow. The walls of the channel are fixed therefore the frictional force between the channel wall and the fluid is highest cause the particles in this domain of the channel to barely move. This effect alters the behaviour of all the particles creating a parabolic profile which has its peak velocity in the central section. If we observe Figure

3.2, we can clearly see this behaviour in a straight channel. In this figure, the flow in the central section has a maximum velocity as opposed to the lateral sections where a substantial decrease in velocity is observed.

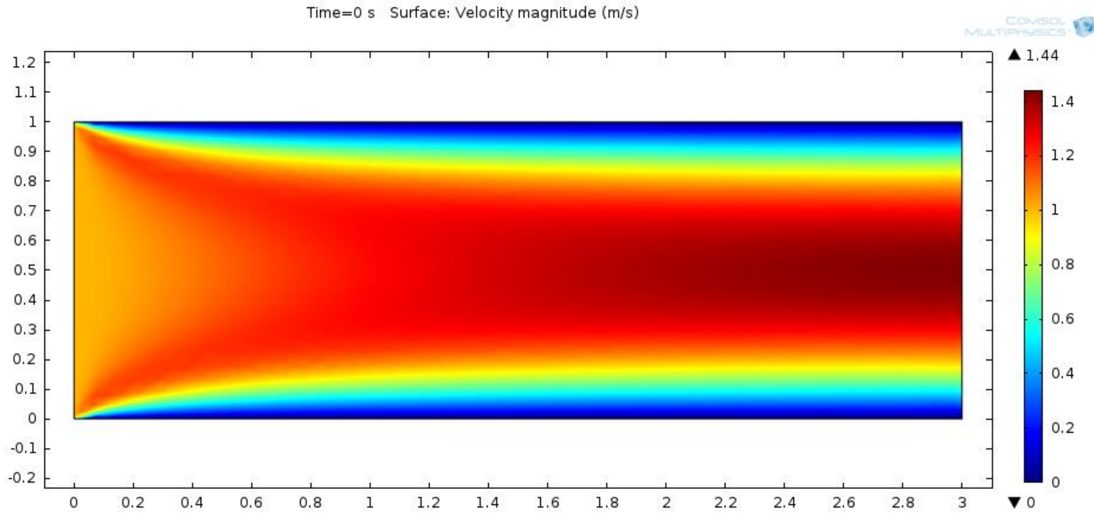

Figure 3.2: Parabolic profile in a fluid flow simulation (COMSOL).

This behaviour disrupts the way in which we calculate the speed of the flow. However through mathematical analysis we can extrapolate the equation that governs the flow.

$$u(r) = 2\,V_{avg}\left(1 - \frac{r^2}{R^2}\right), \tag{3.6}$$

$$V_{avg} = -\frac{R^2}{8\mu}\left(\frac{dP}{dx}\right), \tag{3.7}$$

where r equals the distance from the middle point and R is the radius of the pipe the fluid flows through.

3.6 Hydrodynamic Focusing

Hydrodynamic focusing is also called flow focusing or sheath flow, however many names it may have, they all have one underlining function: to force a fluid or cell to pass through a specified section. This technique has been used for many years in the realm of fluid dynamics to control the way we interact with the different phases of a fluid in a closed environment. Hydrodynamic focusing has

been widely used in many applications as in flow cytometry (Shuler, Aris and Tsuchiya 1972).

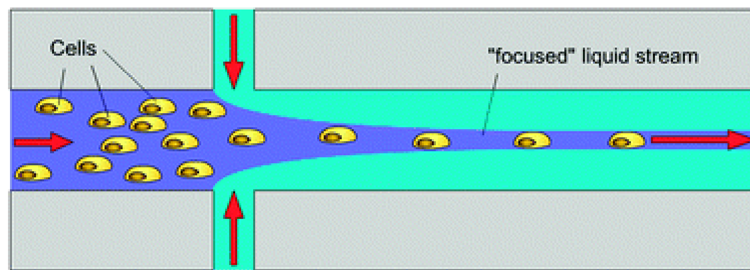

Figure 3.3: Flow focusing structure.

A flow focusing device is mainly composed of three inlets and one outlet as seen in Figure 3.3. The flow focusing method has the goal of placing a stream of fluid or solid phase materials in the central portion of the channel by the use of the auxiliary side channels. When the central channel which flows at a lower pressure, arrives at the junction point, the fluid that comes in from the auxiliary inlets pushes the central interface into a determined section, which will be fixed by the pressure at which the focusing fluid is flowing at.

This phenomenon and tool widely used in microfluidics is due to the conservation of Energy and Mass law.

3.6.1 Conservation of Energy

Energy within a fluidic system remains constant in such a way that energy acting on the system is continuously changed in to other forms such as work. For fluids the equation that governs this principle is very complicated, due to fact that is taken in consideration several forms of energy acting and dissipating out of a defined body. In its simplest form it can be described as:

$$\Delta E = constant \,. \tag{3.8}$$

This has certain ramification such as the conservation of volumetric flow rate.

$$Q(volumetric\ flow\ rate) = v \cdot A \,, \tag{3.9}$$

where v is the velocity of the flow in m/s and A is the cross sectional area.

This formula explains that in the presence of a bottleneck, the cross sectional area will decrease and therefore increase the speed at which the flow is flowing.

3.7 Lab on a Chip

Lab on a Chip or “LOC” is a term used to describe a specific use of microfluidics. As explained in the introductory section of this chapter, microfluidics came to be due to the need of having a quick and small field deployable device to detect certain chemical agents in the air. This initial goal of miniaturization has been extrapolated to other applications creating the field of LOC. LOC tries to synthesize all the necessary analytical tests that would be needed to perform certain laboratory analysis on a silicone chip the size of a coin as seen in Figure 3.4.

Lab on a chip microfluidic devices are being developed to be used in many different industries, with the goal of making chemical analysis much faster, cheaper and precise (Whitesides 2006). One of the said industries is healthcare. Hospitals perform thousands of tests on a daily basis, costing them a lot of resources and money on personnel and equipment. LOC devices offer a very promising future where certain clinical testing can be made accessible and affordable to those healthcare facilities that cannot afford traditional glassware laboratories.

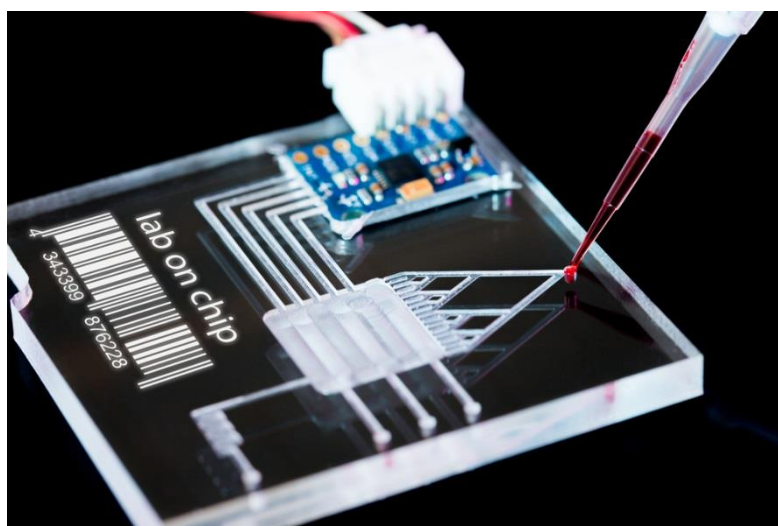

Figure 3.4: Example of microfluidic lab on a chip device. This device aims to perform chemical analysis using microfluidic and MEM technology (Shutterstock).

3.7.1 Organ on a chip

Another emerging area where microfluidics is being used would be biological emulation. Laboratories worldwide are trying to replicate the phenomena that occur in our bodies with the use of microfluidic technology (see Figure 3.5). These devices are referred to as “Organs on a chip”. With these organs on a chip, researchers aim to study how our body works and how they could behave under the use of certain drugs and diseases.

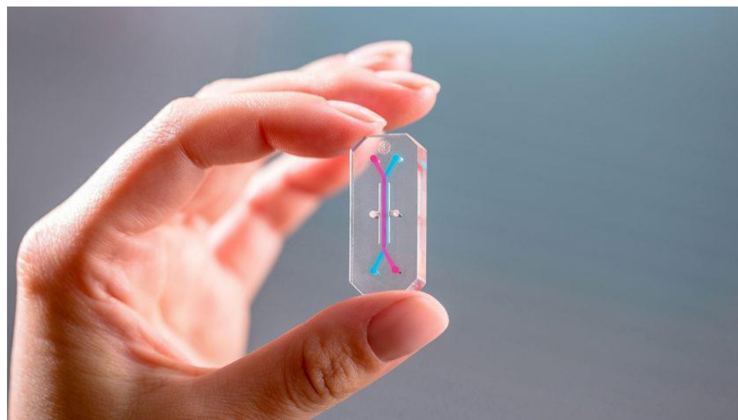

Figure 3.5: Emulate Lung on a chip microfluidic device. This device aims to provide a medium in which we test new therapy drugs.

Chapter 4: Simulations

4.1 Introduction

Computer simulations and CAD software has become an increasingly used technology in engineering, due to the advantages of working in a virtual environment. This chapter contains the simulations performed on COMSOL that were necessary for the development of this research. Simulations were used and are needed in this research because they pose a cheap and accessible environment where we can perform tests on our prototypes before building the physical models; these models give a deep understanding of the possible behaviour of our device.

The software of choice was COMSOL, a Multiphysics modelling software which allows simulating microfluidic and creeping flows.

4.2 Simulations

4.2.1 Fluid Flow Simulation

This section corresponds to the simulation of a viscous fluid along a microfluidic channel in a laminar flow. The cross section of the channel is 50 μm x 100 μm.

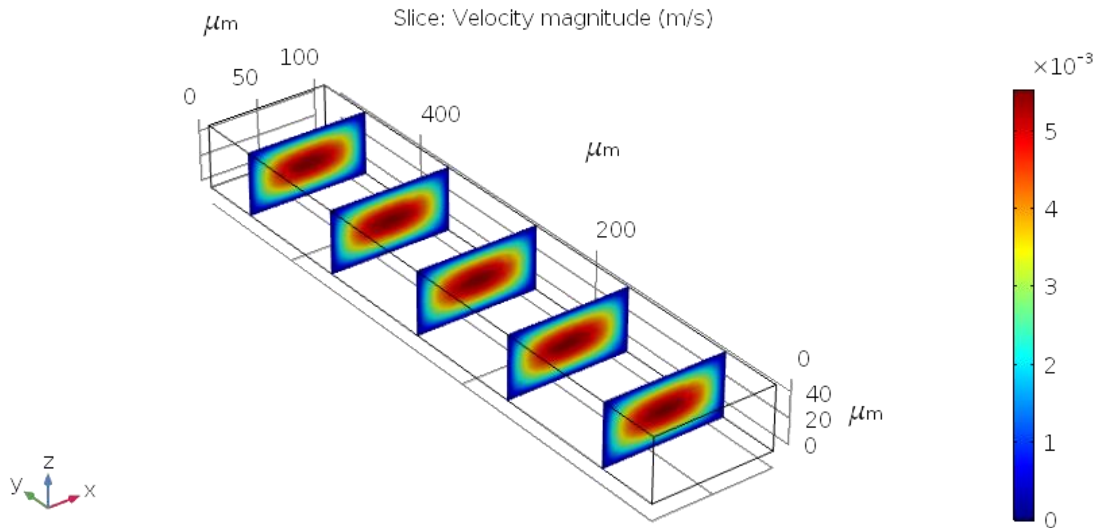

Figure 4.1: Fluid Flow simulation 50 μL/h XZ plane visualization. The units expressed in the legend are m/s.

As we can observe in Figure 4.1, where velocity contours at different longitudinal locations are show, the flow is faster in the central portion rather than on the sides, this is the well-known effect of the symmetrical parabolic fluid profile. The average velocity is given as:

$$v_m = \frac{1}{4\eta} \frac{\Delta P}{\Delta x} R^2 , \quad (4.1)$$

where, η is the dynamic viscosity, ΔP the difference of fluid pressure, Δx the distance covered and R half the width of the channel.

While the velocity at a given point is defined by the following formula:

$$v(x) = v_m \left[1 - \frac{x^2}{R^2}\right], \quad (4.2)$$

where v_m is calculated in Formula 4.1, R is half of the width of the channel and x is the position along the perpendicular axis to the flow of the fluid. This effect of faster fluid at the centre of the geometry is given by the wall friction with the flowing fluid.

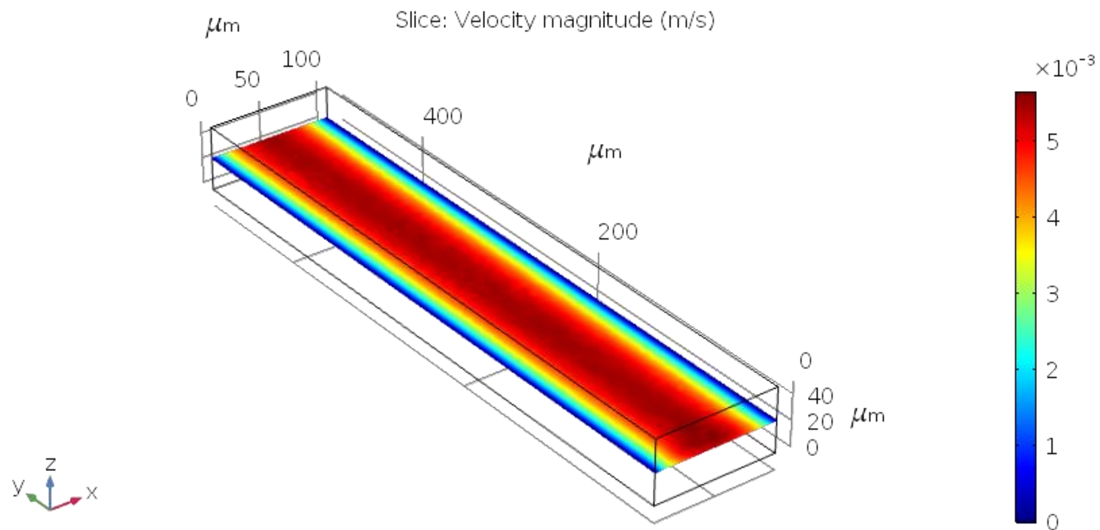

Figure 4.2: Fluid Flow simulation 50 µL/h YX plane visualization. The units expressed in the legend are m/s.

As we can observe in Figure 4.2 and Figure 4.1 where the flow is faster in the central section, this effect is given in both the x and the z direction, this is due to the presence of side walls in both these axes. In the following figure (see Figure 4.3) we can perfectly observe how the speed varies in the different x, y and z coordinates. Due to the laminar flow and dynamic viscosity the profile of our flow will be parabolic. For this simulation we used particle tracing physics to clearly represent the different speed and their distribution along the channel.

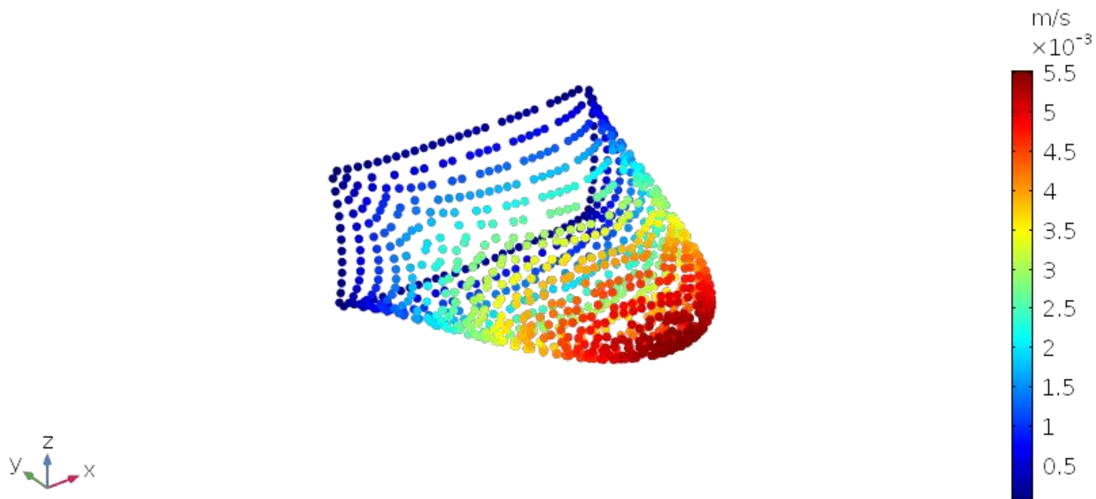

Figure 4.3: Parabolic profile of a laminar flow in a rectangular channel.

4.2.2 Sheath Flow Simulation

These following simulations try to show the effect of the sheath flow in a microfluidic channel.

In Figure 4, the total flow in the central, post focusing stage is 50 µL/h; in this plot all three inlets have the same flow rate: 16.67 µL/h, as given by the following formula.

$$Q_{total} = Q_1 + Q_2 + Q_3\,. \tag{4.3}$$

Q_1, Q_2 and Q_3 correspond to the different inlets of the microfluidic hydrodynamic focusing device; by altering the different inflow rates of these inlets we will have different focusing widths in the post-focusing section.

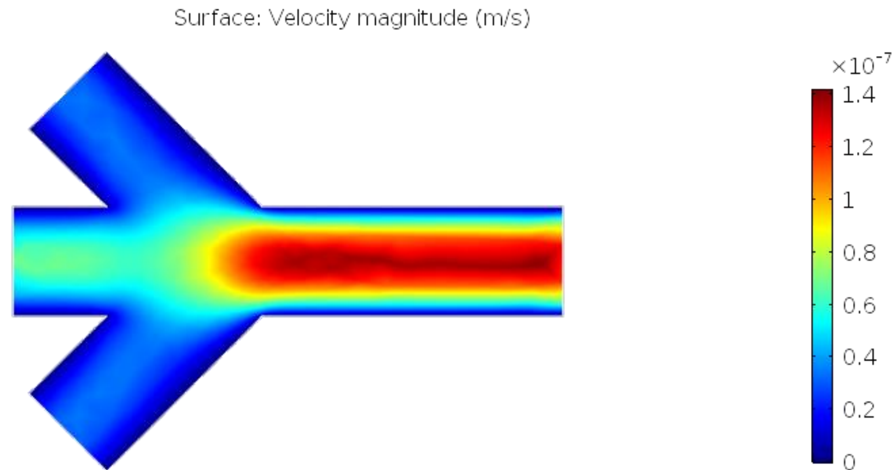

Figure 4.4: Surface plot of the fluid velocity. Speed measured in m/s.

In Figure 4, we can observe the surface plot of the fluid velocity. Each of the inlets have the same flow rate: 4.63×10^{-12} m^3/s or 16.67 μL/h. The total flow rate in the central channel is 50 μL/h. We immediately see that in this central channel, the velocity magnitude is much greater. Flow rate in a pipe is given by fluid speed multiplied by the area of the channel cross-section:

$$Q = v_{average} \times A_{cross-section} \,. \tag{4.4}$$

$$v_{average} = \frac{Q}{A_{cross-section}} \,. \tag{4.5}$$

Therefore, while the cross sectional area remains constant we see a threefold increase in volumetric fluid flow rate.

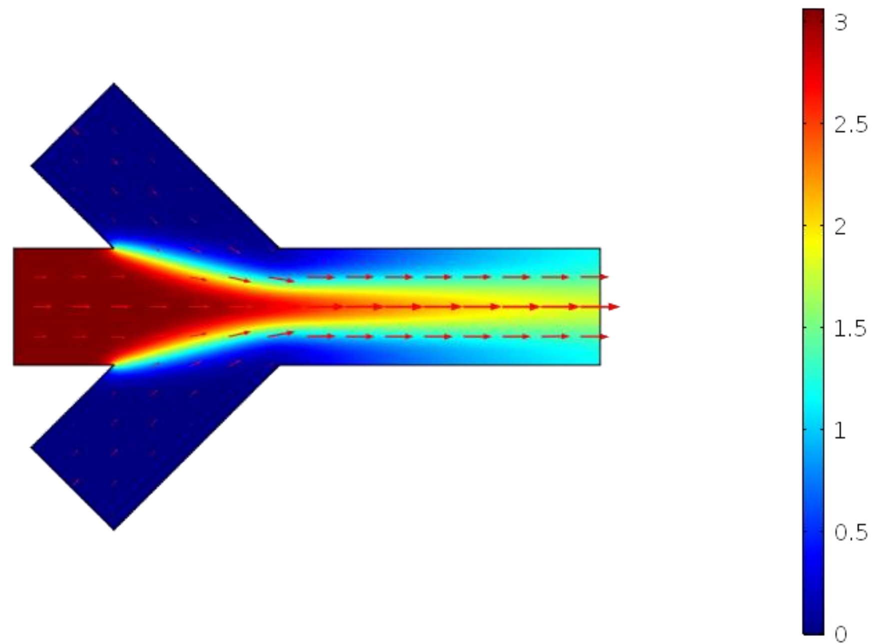

Figure 4.5: Sheath flow visualized. In this simulation Transport of Diluted Species was used as a form of simulating dye. The inlet speeds are all equal.

In the simulation viewed in Figure 4.5, COMSOL was used to show a representation of the future sheath flow developed. For this image I used the Transport of Diluted Species physics. Applying a fine meshing and a low diffusion coefficient we obtain this image where we can clearly observe the distribution of chemicals and therefore estimate the future behaviour of our sheath flow.

The same method was used to simulate the configurations shown in Figure 4.5 and 4.6. The speeds were varied in both simulations to show the way the sheath flow will behave with a higher flow rate through the side inlets. As we can see in Figure 4.6 and Figure 4.7, the higher the speeds through the sheath flow, the smaller the flow focusing area. Therefore, we will be able to focus leukocytes with a higher precision.

Using this information, we can measure approximately the width of the sheath flow and select a few promising data sets for further experimentation.

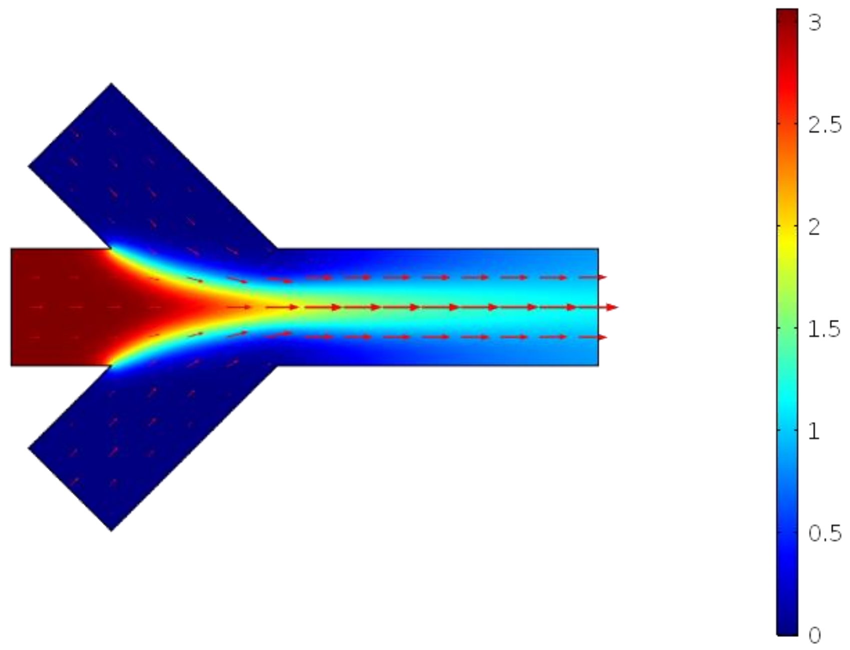

Figure 4.6: Sheath flow visualized. The inlet flow ratio is 1:2. Legend expresses concentration.

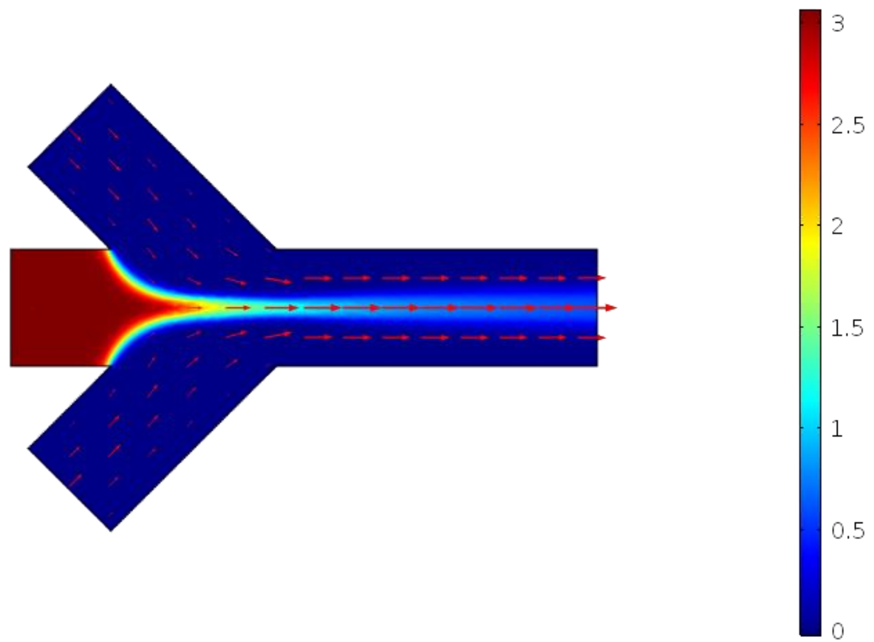

Figure 4.7: Sheath flow visualized. Flow ratio 1:12. Legend expresses concentration.

4.2.3 Particle tracing sheath flow Simulation

In this section, a new set of simulations were designed with the goal of verifying the information obtained in the previous section. Particle tracing physics was used to track the possible flow of leukocytes along my device. COMSOL was the software of choice due to its many possibilities. This software enables to recreate the physics governed in the flow of particles with the possibility of changing diverse parameters such as particle diameter, particle density, particle release, forces acting on the particles, etc.

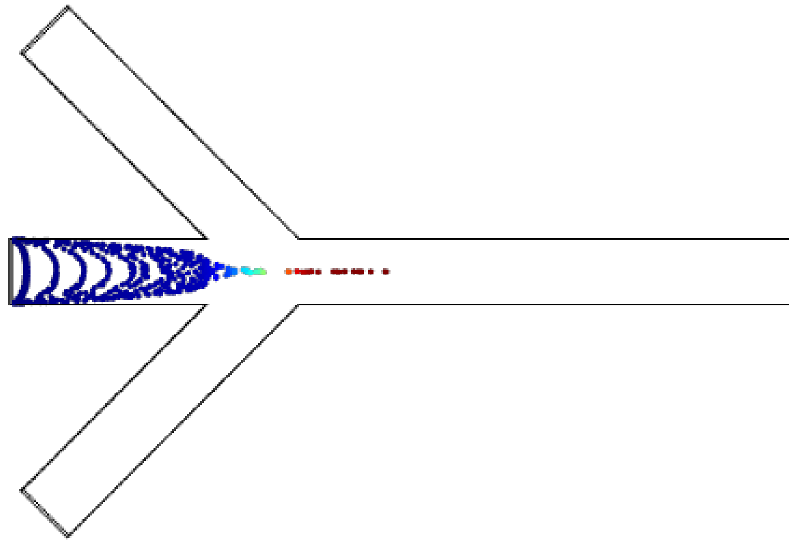

Figure 4.8: Sheath flow visualized with particle tracing physics. Flow rate 1:4. Max flow rate 45.5 µL/h.

In Figure 4.8 we can observe a simulation showing the effect on particles when using a hydrodynamic flow focusing section. The particles will flow with a parabolic profile as observed previously and once they are focused with a sheath flow, the particles will be located in the central section of the channel. It was estimated that with a total flow of 45.5 µL/h in the central channel and a 1:4 flow rate (central inlet: 5.05 µL/h), we can achieve a 15 µm centre sheath that will satisfy the needs of our experiment.

Finally to visualize the results of our design a 3D time dependant simulation was performed. In Figure 4.9 we can see a time lapse where the movement of particles in our device will behave. As we can clearly see, the particles will be focused in the central section. In this figure, each figure represents a 0.5 second increment

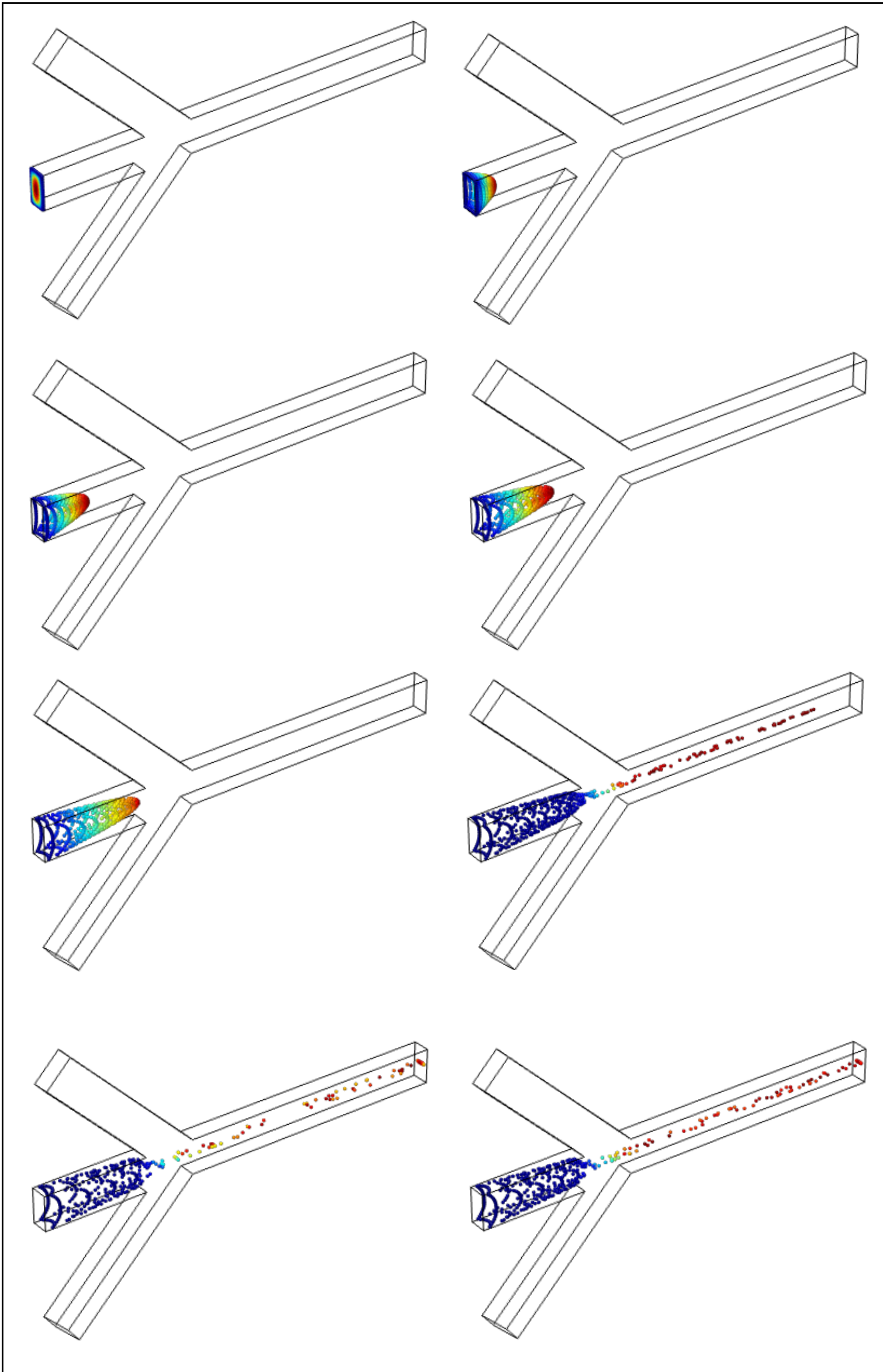

Figure 4.9: Time lapse, simulated on COMSOL with the aid of particle tracing physics configuration. The increments represented are of 0.5 s.

4.2.3.1 Particle parameters

Particle type	Solid particles
Particle density	1077 kg/m^3
Particle diameter	10 μm
Number of particles per Release	500

Table 4.1: Particle parameters for COMSOL Particle tracing for fluid flow physics **(Zipurski, et al. 1976)**.

In Table 4.1 we can observe the parameters used in the COMSOL particle tracing physics for fluid flow. All the data used to mimic the properties of leukocytes in our simulation was extracted from Zipurski's paper (Zipurski, et al. 1976).

4.3 Discussion

By conducting these simulations we have observed the flow of a fluid in our squared channel, we have studied the way particles will behave and interact and finally proved that our design is feasible and would give us the results we desire. However, we must never forget that simulations are always ideal situations, in real experimentation many interferences and errors might occur, such as the existence of bubbles in the microfluidic device. These results give a firm ground on which to lay our thoughts and future experimentation. In chapter 6 we will discuss the experimental results and compare them to the ones obtained in this chapter.

Chapter 5: Materials & Method

5.1 Rolling Cell Adhesion Microfluidic Device

Microfluidics can be used in a variety of fields as previously mentioned; their usability extends to many areas in science. In our case the use of the microfluidic channel will be used to help us understand a little bit further about the rolling cell adhesion phenomenon therefore, our microfluidic channel must simulate a blood vessel. For this we have built several designs we will now demonstrate.

5.2 Channel Geometry

For us to observe the rolling cell adhesion phenomenon, we shall pump a fluid containing a mixture of lymphocytes and Phosphate Buffered Saline (PBS) Solution into the microfluidic device. Our device has been built in such a way that due to the coating of the substrate and the geometric shape of the channel we will be able to see and study the rolling of lymphocytes through the microscope.

The channel has a squared section to easily control the wall shear stress that each section will be subject to. The channel has been designed with an absence of 90^{0} corners to prevent flow alteration so that there is no induced mixing throughout the length of the channel, as seen in Figure 5.1. The key feature of the device was the use of a sheath flow or flow focusing technique, in order to control the interface of cells in the centre portion of the channel.

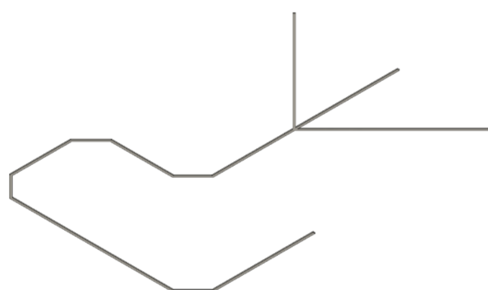

Figure 5.1: AutoCAD render of the microfluidic device.

5.3 Microfabrication Technology

For the development of this microfluidic device, the standard microfluidic protocol was used. Firstly, a photomask was developed using microfabrication technology, then several Silicon wafers were produced by photolithography, lastly, PDMS was poured on onto the Silicon wafers and cured to produce the resulting microfluidic channel.

5.3.1 Electron Beam Lithography

As it is the case with almost all of microfluidic technology, this technology was firstly developed for electronics applications, however due to the capacity of achieving very high resolutions it may be used in several different applications. This technique consists in performing patterns on a substrate using an electron beam. This photomask is then used in the successive stages of the development of microfluidic channels. The photomask was developed at the University of Tokyo, Takeda Tipezaki Building Clean Room by the student Sayaka Nomura of the Tokyo University of Science.

5.3.1.1 Photomask Creation

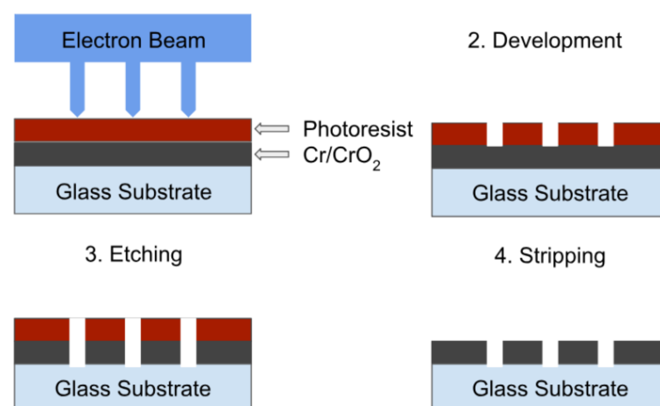

Figure 5.2: Electron Beam Schematic showing the process of characterization of a photomask.

As illustrated in Figure 5.2, the different steps to making the photomask are:

1. Initially, the photomask (see Figure 5.2) is covered in a special photoresist that when in contact with the electron beam will draw the desired pattern onto the mask (see Table 5.1). This part is critical in the development of the mask,

an incorrect exposure to the photoresist will conclude in an inaccurate master photomask that will then influence in a negative manner the rest of the process. For this section of our process the machine shown in Figure 5.3 was used, with the parameters shown in Table 5.2, respectively.

Each resist has specific curing parameters and a required energy per unit area, therefore the amount of light and exposure time must be adjusted accordingly to the resist used. An incorrect adjustment of these specifications could result in an insufficient chromium removal of the underlying layers.

Manufacturer	Toppan
Model	ST-TLR6-TQZ-5009(5T)
Mask depth	2.3 mm
Resist	ZEP7000
Resist depth	3000 Å
Prebaking	190 ºC for 30 minutes
Metal layer	Cr / CrO_2
Metal Layer depth	100 nm

Table 5.1: Photomask Specification.

Figure 5.3: Electron Beam Lithography equipment used for the creation of the photomask. This system is located in Tokyo University.

Manufacturer	Advantest
Model	F5112
Smallest dimension possible	100 nm
Dimension deviation	$3\sigma \leq 15$ nm
Superposition precision	$\lvert$mean value$\rvert + 3\sigma \leq 40$ nm

Table 5.2: Specification of the Advantest Electron Beam System.

2. Once the resist has been exposed to the electron beam, it has to be developed and then rinsed off. Isopropyl alcohol (IPA) and methyl isobutyl ketone (MIBK) are the chemicals used in this process. The machine EVG 101D Automatic Developer was used for this step (see Figure 5.4) (see Table 5.3).

Figure 5.4: Automatic photomask developing system. Located in Tokyo University.

Manufacturer	EVG
Model	101D
Spin module max. deployment num.	1
Types of Development	Pressure tank, flow control, nitrogen nozzle for spay developing, paddle developing, rinse developing

Table 5.3: Automatic Photomask Developer technical Specifications.

3. This step is called Etching. Etching consists in using strong acids and chemicals to cut or produce a pattern into the unprotected parts of a metal surface. In our case, we immerse the mask into a chemical solution with the goal of removing the exposed metal and thus forming our pattern. In some cases Plasma Ashing is used as a method to pre-treat the surface for a better etching process. Using a plasma source, Plasma Ashing is performed onto the photoresist to enable a better penetration of the chemicals used in the later etching process. The ashing apparatus used was the FA-1 manufactured by Samco (see Figure 5.5) (see Table 5.4). The etching apparatus used was the Fairchild 1140 etching machine (see Figure 5.7) (see Table 5.5)

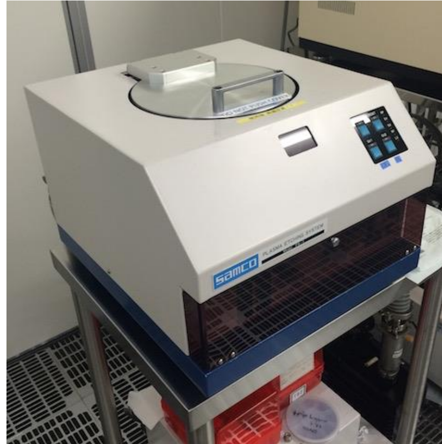

Figure 5.5: Ashing apparatus located in Tokyo University.

Manufacturer	Samco
Model	FA-1
Gas	CF_4 (50 sccm) O_2 (50 sccm)
Maximum RF	200 W

Table 5.4: Ashing apparatus technical specifications.

4. Finally, we wash off the resist with an ashing device (see Figure 5.6); consequently, all the resist is removed from our mask leaving behind a Chromium Oxide layer on top of a glass substrate, with a pattern that will then be used throughout the whole microfluidic device fabrication.

This process previously explained will lead to the creation of a photomask (see Figure 5.6). The photomask has a series of patterns etched into the glass-metal surface which are transparent, this will enable light to pass through and thus create the shape of the device we desire.

Figure 5.6: Photomask used in the microfluidic channel development, created by Sayaka Nomura in 2015.

Figure 5.7: Etching apparatus, located in Tokyo University.

Manufacturer	Fairchild
Model	1140
Output Power	3.6 kW
Supply Pressure	CDA 80PSI N_2 60 PSI D1water 30PSI
Discharge Condition	80SCFM 20SCFM

Table 5.5: Technical specifications of the etching machine.

5.3.2 Photolithography

We call photolithography the process in which we use UV light to cure photosensitive chemicals in order to make patters on a wafer. Just like the previous technology, this proceeding was borrowed from the electronics field. In electric and electronic engineering, photolithography is used in the manufacturing of transistors and complex integrated circuits.

In this process, we shall use the photomask fabricated in the previous section and use it for patterning of a silicon wafer, this silicon wafer will be the master mould we will then pour our PDMS solution on to build the channel.

5.3.2.1 Silicon patterning procedure

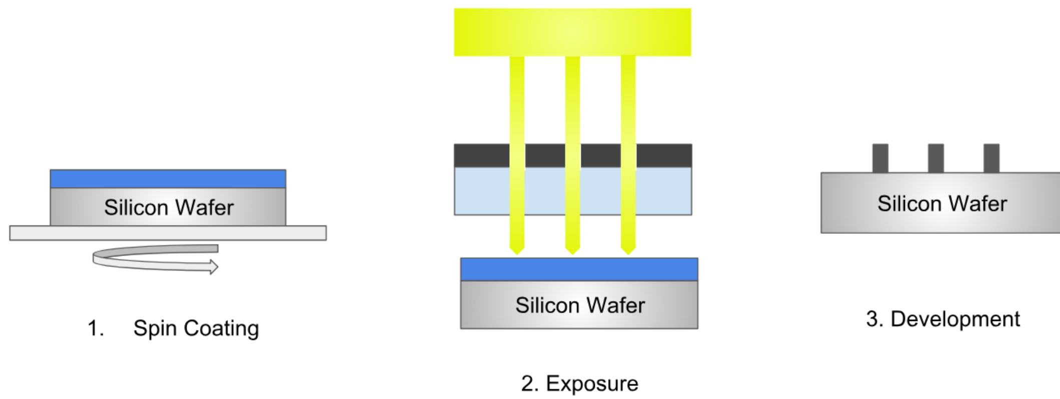

Figure 5.8: Master mould lithography process.

As illustrated in Figure 5.8, the different steps to manufacturing the silicon wafer microfluidic channel master mould:

1. The first step in photolithography is to apply a coating of photoresist to our silicon wafer. In our case we used SU-8 photoresist (see Figure 5.9) due to its chemical attributes (see Table 5.6).

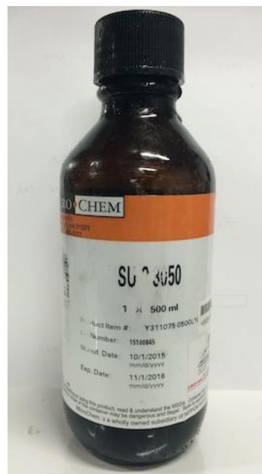

Figure 5.9: SU-8 photoresist.

Manufacturer	Micro Chem.
Model	SU-8 3000
Adhesive strength	71 MPa
Glass transition point	198 ºC, DMA tan δ
Volume Resistivity	1.8 x 10^{16} Ω·cm
Water Absorption	0.5 % 85 ºC / 85 %RH, 120h

Table 5.6: Chemical Specifications of Su-8 photoresist.

In order to apply the photoresist we first pour the solution onto a silicon wafer and we spin it at a rate of 2250 rpm (see Table 5.7) with the use of the spin coater (see Figure 5.10), this spinning will extend the SU-8 over the entire silicon wafer achieving a thickness of 50 μm. We then prebake the silicon wafer using a hot plate at 60 ^{o}C for 1 minute and then at 90 ^{o}C for 15 minutes. After heating, we leave to cool down in room temperature.

Figure 5.10: Spin coating apparatus located in Motlab, Tokyo University of Science.

Manufacturer	Active
Model	ACT-220D
Rotation range	200 ~ 8000 rpm
Rotation revolutions	± 3 rpm
Vacuum	350 Torr
Steps / patterns	10 steps / 100 patterns

Table 5.7: Technical specifications for spin coater.

2. The next step consists of exposing the spin coated silicon wafer to a UV light to generate the pattern on our wafer. For this we use the photomask aligner (see Figure 5.11). This machine will expose our silicon wafer to a UV source with the shape of the photomask, for this to happen UV light is emitted through the source (see Table 5.8) and only passes through the photomask in the areas where the Chromium Oxide has been etched out in the previous process. Once the photoresist is cured with UV light, we can proceed to the development of the SU-8. After the exposure, we perform a post-bake by heating on a hot plate the Si wafer at 60 ^{o}C for 1 minute and 90 ^{o}C for 15 minutes.

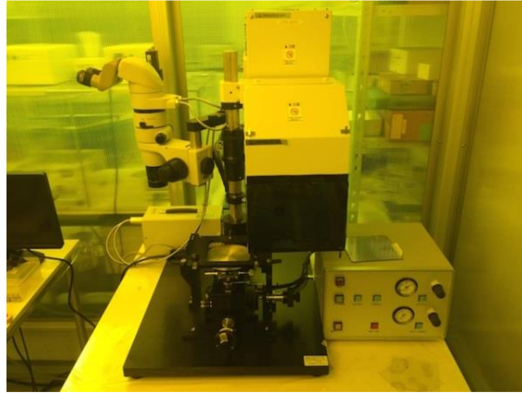

Figure 5.11: Mask Aligner apparatus, located in Tokyo University of Science.

Manufacturer	Nanotec
Model	ES20tr
Exposure area	ϕ105 mm
Illumination uniformity	±5 %
Illumination distance	200 mm
Dominant Wavelength	365, 405, 436 nm
UV irradiation strength	More than 35m W/cm^2 (at 365 nm)

Table 5.8: Technical specifications of Nanotec mask aligner.

3. Finally, we proceed to develop the silicon wafer by rinsing and immersing the wafer into SU-8 developer. In some cases, SU-8 will remain; therefore, we will wash the wafer with isopropanol, acetone, ethanol and distilled water.

Once we have completed all this process we will have correctly developed a silicon wafer with our pattern drawn on it (see Figure 5.12).

Figure 5.12: Silicon wafer microfluidic channel master mould.

5.3.3 PDMS Channel

For the characterisation of the microfluidic channel, we used PDMS (Figure 5.13). PDMS is a widely used silicon-based organic polymer in microfluidics; this polymer is used due to its many advantages. As a result of its flexibility and its mechanical properties this type of technological procedure is also referred to as soft lithography.

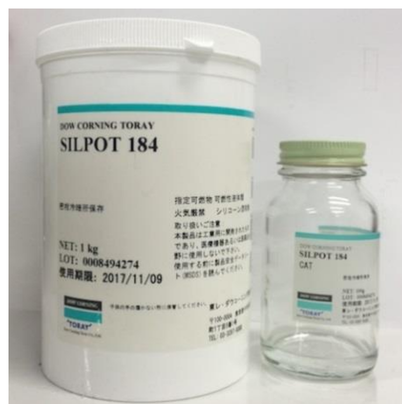

Figure 5.13: PDMS.

In order to create our PDMS channel, we will simply pour the mixture of PDMS on our SU-8 mould and leave to dry up until forming a solid block. The mixture we pour is a 10:1 PDMS to curing agent ratio (Table 5.9). Note that before pouring the PDMS onto the wafer we should place the mixture into a vacuum for 40 minutes to remove all bubbles.

Manufacturer	Dow Corning Toray
Model	Silpot 184
Viscosity	5000 cSt
Density	1.11 g/cm^3
Mixing Ratio	Main Agent: Curing Agent 10:1
Heating temperature and time	60 ^{o}C 80 minutes, 150 ^{o}C 30 minutes
Refractive Index	1.41

Table 5.9: PDMS chemical specifications.

Once we pour the PDMS we heat at 60°C for 80 minutes and 150 ^{o}C for 30 minutes respectively. When dry (see Figure 5.14), we can simply cut with a razor blade, punch holes into the inlet and outlets and place on a clean glass slide to perform our experiments and tests.

Figure 5.14: Final microfluidic PDMS channel before assembly.

5.4 E-Selectin substrate preparation

The cell adhesion molecule used in this work is the E-selectin (see Figure 5.15) therefore we must coat the substrate of the channel for our setup to work properly. The preparation method is fairly simple due to the characteristics of the materials used (see Table 5.10).

Figure 5.15: E-selectin.

First we coat our glass slide with a fine layer of PDMS, for this we must spin coat the PDMS on the glass substrate. Thanks to the functional amino group of the E-selectin, we can easily bond the CAM to the PDMS forming a peptide bond, which strongly binds the molecule to the substrate of out microfluidic channel. Therefore, we must simply pour the E-selectin (500 mg) dissolved in

1mL of PBS onto the PDMS and leave to react at 37.5 ºC overnight (see Figure 5.16).

Manufacturer	R&D Systems
Product	Recombinant human E-selectin / CD62E
Source	Chinese Hamster Ovary cell line, CHO-derived
Molecular Mass	58.6 kDa

Table 5.10: E-selectin chemical characteristics.

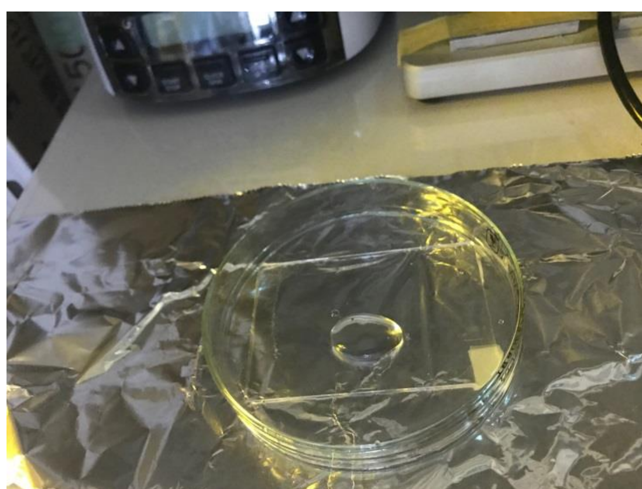

Figure 5.16: Preparation of E-selectin coated substrate for microfluidic channel.

5.5 Leukocyte culture

In order to have a continuous storage of cells for our experiments, we use cell cultures. Live leukocytes were acquired and performed continuous medium change with the goal of growing the cells and keeping their viability.

Periodic medium change and cell counting is needed to assess the progress of the cells. The following protocol was followed in order to concentrate cells from a suspension culture:

1. Transfer the cell suspension to a sterile centrifuge tube of appropriate size and centrifuge for at 3700 rpm for 7 minutes.
2. Carefully remove the supernatant without disturbing the cell pellet.

3. Add the desired volume of fresh medium gently to the side of the tube and slowly pipette up and down 2 to 3 times to resuspend the cell pellet.
4. Transfer the cells to the desired cell container.

The following protocol was followed as indicated by the Thermo Fisher Scientific protocol website (Thermo Fisher Scientific 2016).

Once we performed the periodic medium change, we counted the cells with the use of a haemocytometer following the protocol stated underneath.

1. Clean the haemocytometer chamber and cover slip with alcohol. Dry and fix the coverslip back in position.
2. Harvest the cells. Add 10 µL of the cells with Trypan Blue to the haemocytometer. Do not overfill.
3. Place the chamber in the inverted microscope under a 20X objective.
4. Count the cells in the large, central gridded square (1 mm^2). The gridded square is circled in Figure 17. Multiply by 10^4 to estimate the number of cells per millilitre. Prepare duplicate samples and average the count.

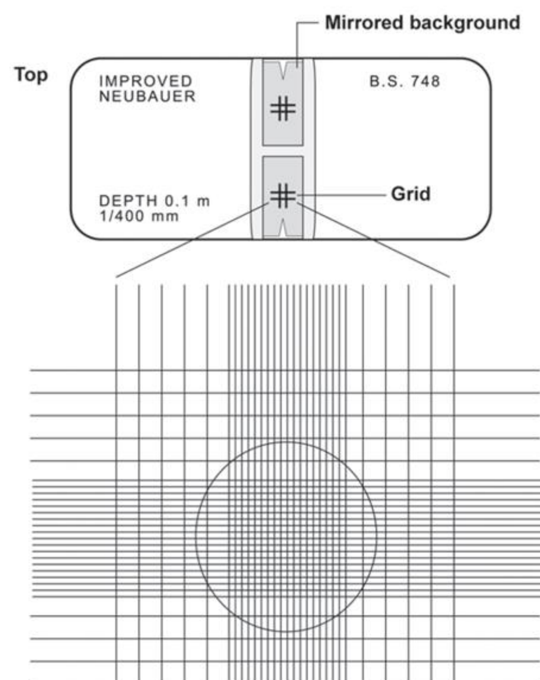

Figure 5.17: Haemocytometer grid for cell counting **(Thermo Fisher Scientific 2016)**.

With this method we can observe the size and number of cells being produce, to assess the development our cells exhibit.

Chapter 6: Experimental Results

6.1 Sheath Flow Experiment

This experiment was made in order to evaluate the suitability of our hydrodynamic focusing technique, its implications and the relevancy in our final experiment. We aimed to measure the width of our focused flow. For this we experimented with different flows and ratios to assess the optimal condition for our posterior experiment.

6.1.1 Experimental Setup

For this experiment, fluorescein and distilled water was used. We introduced fluorescein in the central channel and used water as the sheath fluid. These were the chosen fluids due to their similar properties and to the fact that fluorescein emits luminescence, which represents an advantage because it is easy to capture with fluorescence microscopy and the posterior analysing of our images are both easy and intuitive.

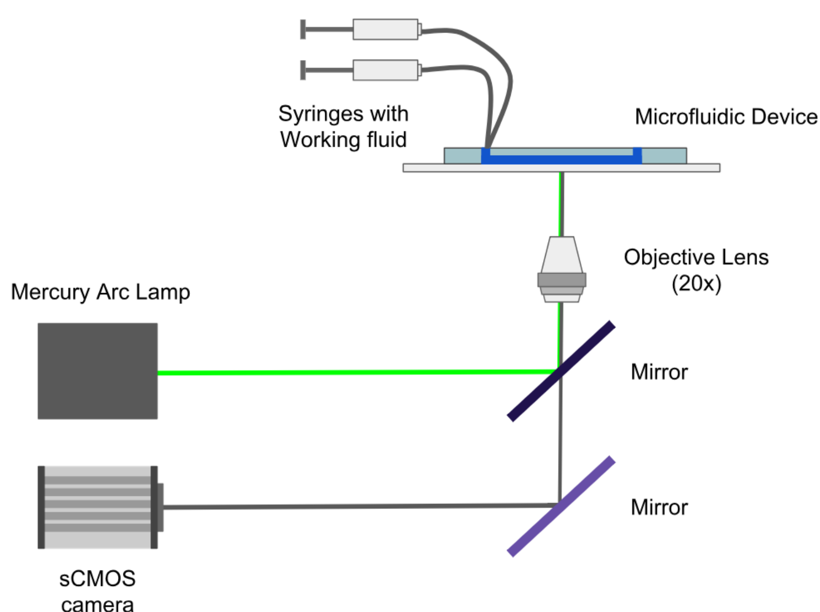

Figure 6.1: Schematic image illustrating the experimental setup for this assay.

The microfluidic channel was prepared as described in the previous chapter. Then it was connected to syringe pumps (Harvard Apparatus Pump 11 Elite)

through 0.5 mm diameter tubes to the inlets of the microfluidic device as shown in Figure 6.1. The central channel's syringe was filled with fluorescein, an organic dye used in fluorescence microscopy, and the side syringes were filled with distilled water. The syringe pumps were programmed at different flow rates as described in Tables 6.1 and 6.2. In Figure 6.2 we can see the final setup.

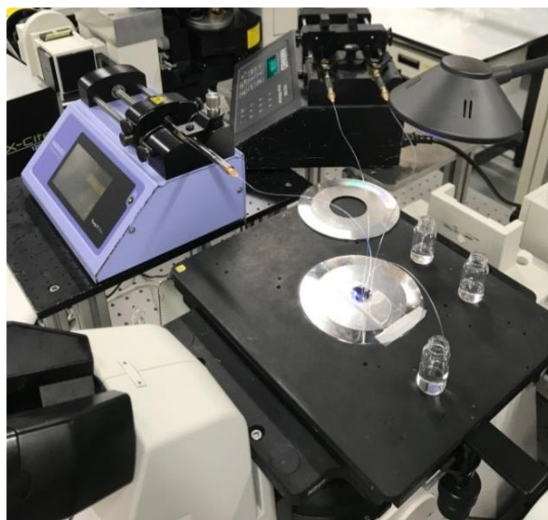

Figure 6.2: Experimental setup of the hydrodynamic flow focusing essay.

6.1.2 Results

In Figure 6.3 the results of the experiments are shown. The images were taken with fluorescence microscopy and show the width of the focused flow. In the first image to the left (see Figure 6.3(a)) we see that the flow has been reduced to 36.3 μm in width due to a 1:2 side inlet flow ratio (see Table 6.1). Consequently, in the next figures (see Figure 6.3(b)-(c)) the flow ratio was altered to a 1:6 ratio and a 1:10 ratio. Table 6.1 show the results depicted in Figure 6.3.

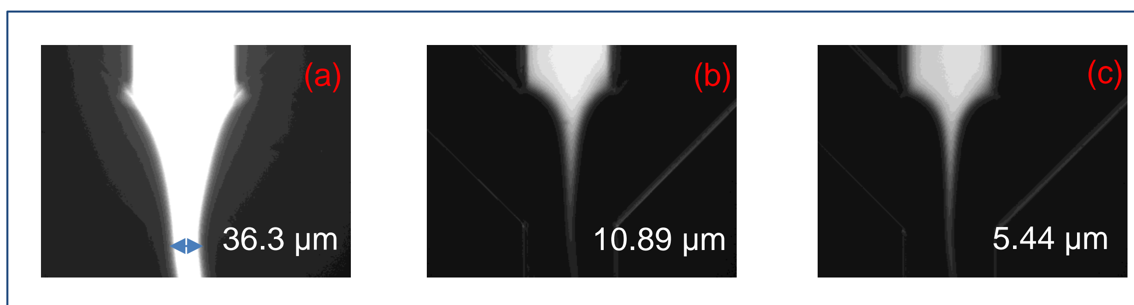

Figure 6.3: Hydrodynamic flow focusing visualization on microfluidic channel. Fluorescence microscopy 20x objective.

	Image 1	Image 2	Image 3
Sheath Flow Ratio	1:2	1:6	1:10
H_2O Flow Rate	20 µL/h	60 µL/h	100 µL/h
Fluorescein Flow Rate	10 µL/h	10 µL/h	10 µL/h
Total Flow Rate	50 µL/h	130 µL/h	210 µL/h

Table 6.1: Sheath flow ratios used in hydrodynamic flow focusing experiment. See also Figure 6.3

Constant Sample Flow			
Fluorine Rate (µL/h)	Sheath Rate (µL/h)	Pixels	Size (µm)
10	20	100	36,3
10	40	50	18,15
10	60	30	10,89
10	80	20	7,26
10	100	15	5,445
Constant Sheath Flow			
20	100	29	10,527
30	100	33	11,979
40	100	40	14,52
50	100	41	14,883

Table 6.2: Hydrodynamic focused flow under different sample flows and sheath flows. First the central channel remained constant while the side channel's flow rate was increased. On the second iteration of the experiment, the sheath channels remained constant while the central channel's flow was increased in increments of 10 µL/h.

In order to measure the width of the hydrodynamic flow the following formula was used:

$$n^{\underline{o}}\ of\ pixels\ \times \frac{3.63 \times binning}{lens\ magnification} = \mu m\ , \tag{5.1}$$

where binning represents the number of cluster of pixels into a single pixel. In these images this number is equal to 2. Lens magnification, on the other hand, is the magnification used in the lens in the fluorescence microscopy. To take these images we used a 20x magnification.

$$n^{\underline{o}}\ of\ pixels \times \frac{3.63 \times 2}{20} = \mu m\ . \tag{5.2}$$

6.1.3 Discussion

As we've seen previously, the setup and execution of this experiment show a promising hydrodynamic focusing. We have demonstrated that by varying the flow rates in each of the inputs we can modify the width of out focused channel. This is a fundamental first step in the development of our device, considering that we can now control the amount of fluid and cells that will pass through the central portion of our channel.

We must take a minute to point out the possible errors in calculating the size of the sheath. The error in the calculations may be of ±1 μm; this is not significant as the size of the leukocyte is generally 10 μm.

When using the final iteration of our design, the user may want to change the conditions of flow in the channel therefore we can make many combinations of flow rates to fit our needs. In our case the since we want a single cell focused flow, we aim to have dimensions ranging from 11 μm to 15 μm therefore we could use the above tested combinations. When choosing said flow rates, we must elect appropriate rates bearing in mind the total flow downstream from focusing point. Once the three flows meet in the channel the total flow rate would be the sum of the three flow rates, as indicated in the following equation:

$$Q = Q_{central\ channel} + 2 \times Q_{side\ channels}\ . \tag{5.3}$$

As we know, cells will behave differently with different flows; therefore, we must adjust these parameters accordingly.

6.2 Rolling Cell Experiment

In this second experiment, we recreate the rolling cell experiment on a simple straight channel microfluidic device. The goal of this experiment is to test the rolling conditions in which the last experiment will take place.

We shall experiment under the same conditions as in the last experiment and expose the problems we face when working with the absence of a hydrodynamic focusing module.

6.2.1 Experimental Setup

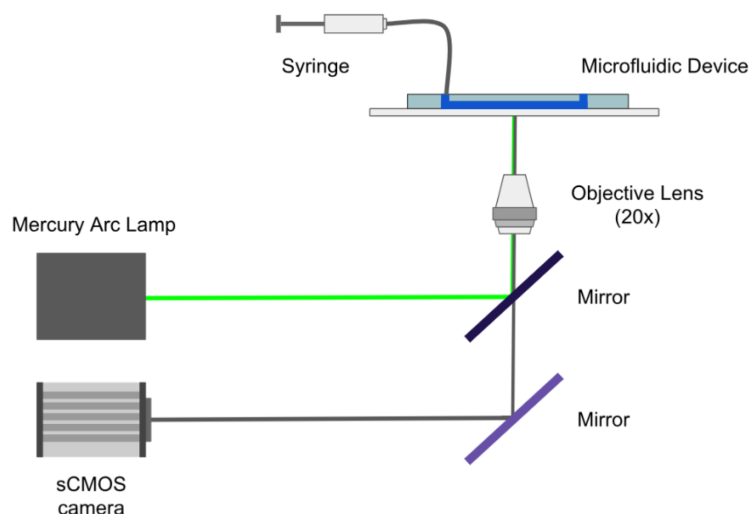

Figure 6.4: Schematic image showing the experimental setup for this assay.

The setup is very similar to the previous experiment, changing in aspects such as the number of pumps used and the nature of the fluid used. Due to the fact that we are testing live leukocytes on this assay, we used PBS as working fluid in order to keep the medium as stable as possible.

As explained in chapter 6.4, this experiment needs an E-selectin coated surface for the leukocytes to bind correctly; therefore a previously treated substrate was used for this experiment. The conditions, as explained previously, do not change much from the previous experiment; however we used Calcein AM for this experiment to stain the cytoplasm of the cell.

Calcein AM is a fluorescent dye with emission wavelengths of 495/515 nm. This dye is used in biology due to its ability to penetrate the cellular membrane in live cells, making it a very useful tool for testing cell viability and for short term cell labelling.

6.2.2 Results

The following results were obtained at 0.2 Pa of wall shear stress and are depicted in Figure 6.5 and Figure 6.6. As we can observe only leukocytes on the side of the channels attach, where the fluid flow is slower, leaving the central section completely barren.

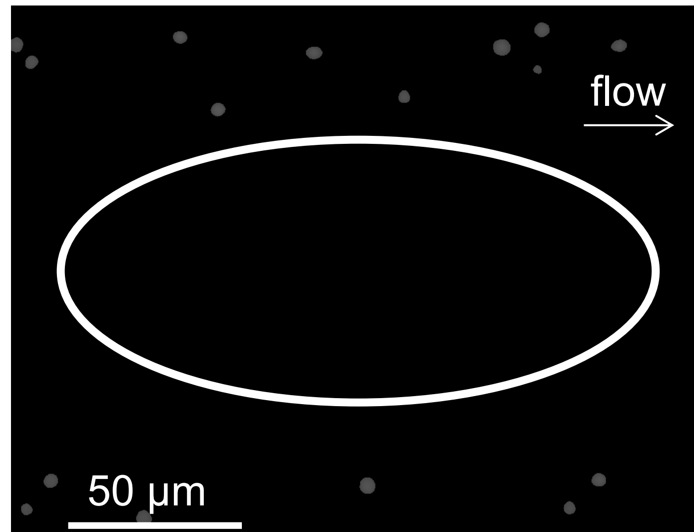

Figure 6.5: Top view of microfluidic channel. Leukocytes flow on the edges of the channel as opposed to the central area where we would expect them.

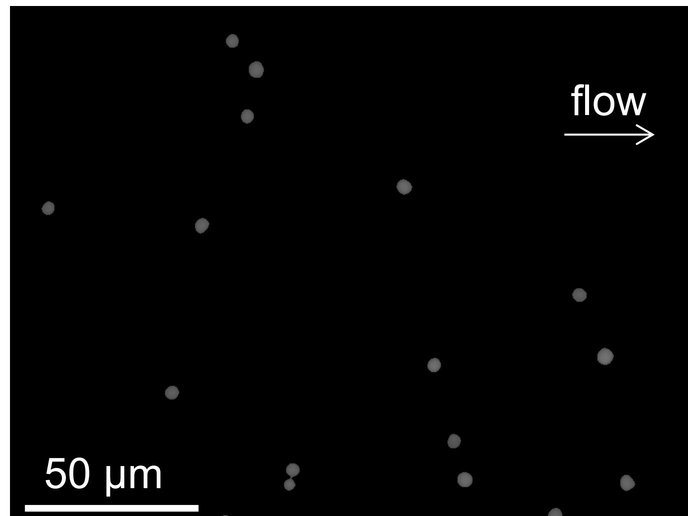

Figure 6.6: Top view of microfluidic channel. Leukocytes flow path is scattered throughout the whole domain of the channel.

6.2.3 Discussion

As we can see in the Figures 6.5 and 6.6, the conditions in which the measurements take place aren't the ideal position for us to have an accurate reading on the results of the experiment. This is the reason we need a sheath flow, in order to focus the lymphocytes in the central domain to control all the variables in our future tests.

Besides this inconvenience, the setup works as expected: the leukocytes bind to the substrate and roll at the appropriate speeds.

6.3 Rolling Cell Sheath Flow Experiment

In this experiment we test the definitive version of our experiment in order to validate the prototype we have created. We shall introduce a syringe of PBS with live lymphocytes suspended and produce a sheath flow to centre the immune cells in the channel to have more controllable interface.

6.3.1 Experimental Setup

The experimental setup is similar to those explained in the above experiments. The substrate was prepared the day before and left overnight in the incubator at 37.5 ^{0}C, as explained in chapter 5.

The channel used was prepared on the day of the experiment following the procedure of soft lithography explained in chapter 5. Once the channel was prepared it was sterilized accordingly with ethanol and distilled water following the same protocol as the experiments explained previously.

The syringes used for the experiments contained PBS alone and PBS with lymphocytes suspended in them, respectively, prepared following the same steps as the previous experiments. The lymphocytes dyed with Calcein AM, in the same way we performed in the previous experiment.

6.3.2 Results

The results observed in this experiment were as expected. When the lymphocytes were introduced into the microfluidic channel, the cells were focused into the central section of the posterior channel. This is due to the action of the hydrodynamic focusing module we have built.

As we can see in the previous experiment and on Figure 6.5, we can observe that the flow of the leukocytes is random, the same happens in Figure 6.7(a) and Figure 6.7(b). However once the cells have been focused by the action of the sheath flow they circulate through the centre of the channel as observed in the following images of Figure 6.7(c)-(h).

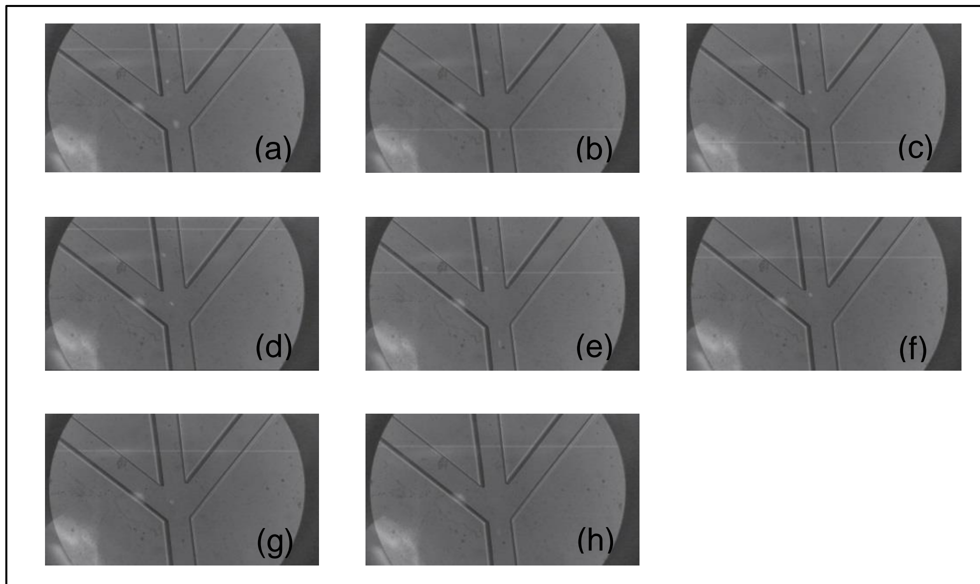

Figure 6.7: Time-lapse visualizing the flow of the lymphocytes through the flow focusing module. The total time of this video lasts 1 second. The leukocyte can be observed as a white dot over a grey background. This image was extracted using fluorescence microscopy.

We have to bear in mind, that although we are focusing the flow of the lymphocytes in the centre of the channel, due to the physics of the hydrodynamic focusing section, the speed at which these cells move along the channel will also increase. In this said section, we are pumping three times the volume of fluid as pumped in the initial section therefore as we explained in chapter 3, the increase of volume being pumped under the constant width of the channel will cause the speed of the particles to be increased.

6.3.3 Discussion

The initial results were positive, and we managed to obtain the results we were looking for. The ultimate goal was to create a stream of focused cells in order to analyse them in a more controlled environment. However good the result may be, there are certain aspects we must talk about regarding this experiment.

Firstly, as we have observed in the results, the speed of the cells increases by a certain factor depending on the flow in the inlets. The speed of the particle will see itself increased depending on how focused the flow of cells are. Meaning that the more focused we desire our model to be the higher the speed will be in our channel, due to the flow of higher volumes per unit of time pumped into the

system. This may pose certain problems, due to the limitations of the equipment used for the experiment.

If we desire a certain low particle speed (where $Q \leq 20$ μL/h) where we aim to perform an experiment with a specific pressure factor and we want a high focusing rate, the central inlets must pump the experimental fluid at very low flows, meaning that the microfluidic pumps used might have certain limitations.

When performing high focusing rates and lower particle speed experiments, the microfluidic pumps exhibited technical limitations and generated a pulsation effect in the fluid. This pulsation is a very negative effect that we want to eradicate at all costs.

Secondly, we observed that the binding with the E-selectin substrate was not always achieved and suspect this is due to the absence of contact with the substrate of the channel. Due to this most of the cells will flow along the channel never attaching to the substrate. However nothing can be done, because in the event of forcing the cells to attach we must exert a force onto the lymphocyte that would modify the behaviour of the cell significantly.

All in all, one thing is clear, although the experiment has given us positive results, luck is also a factor when performing experiments because lymphocytes can fail to attach as discussed previously.

6.4 Pulsation Experiment

In this experiment we aim to observe at what flow the microfluidic pump, Harvard Apparatus Pump 11 Elite, exhibits mechanical limitation and thus affect the flow of our fluid causing pulsation.

6.4.1 Experimental Setup

For this experiment we fabricated a simple straight channel microfluidic chip and pumped into the channel micro particles suspended in a distilled water solution at flows under 5 μL/h as seen in Figure 6.8. We then observed the flow of the fluid under the fluorescence microscope and analysed the results.

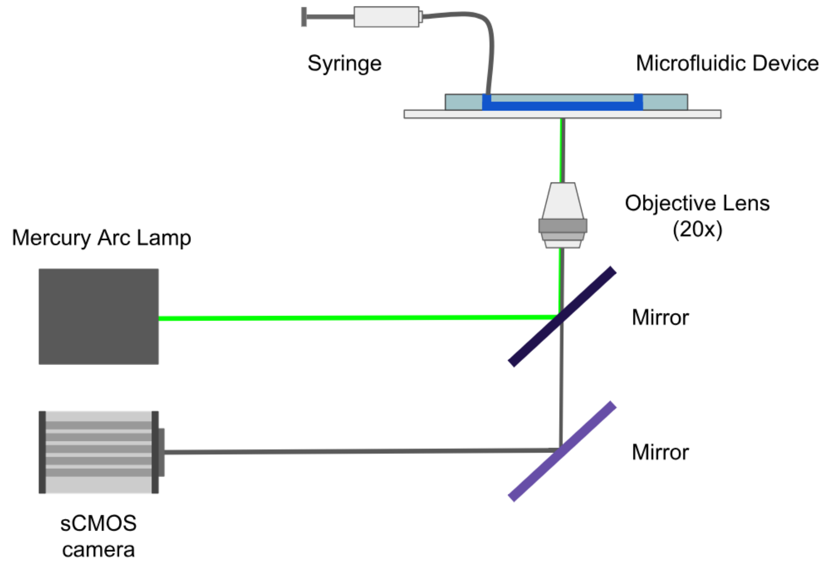

Figure 6.8: Schematic image showing experimental setup for this section.

6.4.2 Results

After testing a range of different flows, we observed pulsation in flows under 5 μL/h.

6.4.3 Discussion

As pointed out in the results section, pulsation occurs in flows under 5 μL/h, indicating a limitation in the apparatus used in our experimentation. Therefore, when performing experiments that require low flow rates, we must be very careful not to surpass this value because it could introduce undesired alterations to our experiment that could change the results of our tests.

Chapter 7: Conclusion

7.1 Conclusions

After analysing all the relevant results from the different experiments we can conclude the following:

- The potentiality of microfluidics for biomedical research has been proved. Microfluidics poses as an excellent technology that is widely used in many labs around the world.
- The importance of multiphysics simulation software in microfluidic research has been confirmed.
- A microfluidic system for leukocyte experimentation or Lab on a Chip that overcomes the initial non central rolling problem has been successfully developed.
- Although the system solves many problems, in cases of low flow rates, the system presents limitations due to the equipment used.

7.2 Future Work

Although the system that has been designed is fully functional and could be used in future research, there is still room for improvement. The following advances are proposed as a way for improving the capabilities of the system.

- Continue performing tests to evaluate functionality.
- Perform rolling cell adhesion analysis via Nomura´s software to compare the numerical data.
- Experiment with different leukocytes and cells.
- Experiment with circulating cancer cells.
- Develop a new substrate with live epithelial human cells for future experiments.

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