Deterministic lateral separation of cells

by

Per Sommansson

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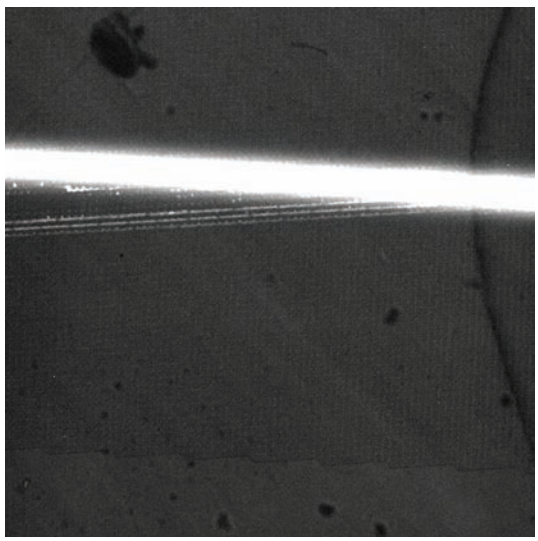

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Abstract

The separation of cells is an important technology in medicine. Every day millions of separations are conducted in hospitals all over the world. The most common separation might be the separation of whole blood into its different parts. This is one of the most challenging problems there is in separation science. The blood has a myriad of different constituents. Within each little drop there are more than hundreds, if not thousands, of different cells, proteins, markers and other biological data that can hold the key to the diagnosis of a particular disease. But, as can be imagined, it is not all that easy to find what you are looking for. Adequate separation techniques are essential for proper diagnostics. In this project an attempt to separate cells differing in size has been made. Separation of cells, and especially of whole blood, is an old scientific practice that has been in use for more than a century. Right now we are standing on the verge of a new revolution. The use of micro- and nanotechnology in separation shows great promise. This could make it possible to analyze minute samples of proteins and cells, and at the same time have a very high through-put, be cheap and easy to use. This project examines a novel separation technique that has shown very promising results on polystyrene beads, and could possibly be used to separate cells discriminating solely on size. The technique is based on the use of a device called "the bumper array". The device has been developed by Robert Austin's lab at Princeton University[7][23][24]. It relies on a simple geometric pattern that creates discrete flow lines in a microfluidic channel. Depending on the size of the particle being separated it will follow different flow lines. The small particles will go straight forward ignoring the geometric pattern and larger particles will be restricted from going forward by the posts and be bumped to the side creating a lateral separation. To date only a few promising results have been reported on the usage of the bumper to separate blood[57]. But the good results with beads make it attractive to try to overcome the problems with cell separation in the bumper. The biggest challenges with regards to the separation of cells are clogging and problems in defining the critical size of the cell. The clogging issue is an eminent problem. In an untreated bumper the time for clog development is only a few seconds. After clogging has occurred the bumper is useless for any quantitative measurements. To limit the clogging effect both the blood and the channel surface can be treated. The blood can be treated with Heparin, Aspirin and other antithrombtic drugs. The bumper surface can be treated with BSA, pluronics and PLL-Peg. The study made by Zheng used blood treatment to dicrease cloggong. In this study we compare blood treatment and surface treatment of the bumper. The advantage of using surface treatment instead of blood treatment is that you get a device that can be used without any nessecary laboratory work done on the blood, and the bumper therefore becomes eaiser to use and much more efficient. In this study we show results on clogging properties of bumpers with and without PLL-PEG. When the channels of the bumper were covered with PLL-PEG they showed very little clogging and could be run for a long time before clogging got too severe. Another way to improve the throughput is by design changes. The fibrin plug that causes greatest effects can be stopped from entering the bumper using a prechamber. The test herein are only simple and future studies should be done, especially the need for design improvements concerning the cutoff size and the period should be dealt with as well as usage of prechambers to decrease clogging.

Chapter 1

Acknowledgments

First of all I would like to thank Dr. Jonas Tegenfeldt and PhD-student Jason Beech for their support during this project. They have both been valueble with their knowledge and deep insight in the Bumper array. I aslo like to thank Linda Geironsson at the Division of Immunology at the Faculty of Medicine at Lund University for her help with lymphocyte isolation and lymphocyte staining. The help from Rodolph Marie should not go unnoticed. Thank you for helping me with the PLL-PEGs. Last I'd like to thank the whole Bio-group at the Solid State Physics Department at Lund University, Faculty of Engineering. The vibrant atmoshpere and the comrade within this group was a true pleasure experience. Thank you.

Contents

List of Figures

Chapter 2

Introduction

This Master Thesis was conducted during the spring of 2006 at the Biophysics group at Lund Institute of Technology under the supervision of Dr. Jonas Tegenfeldt and Jason Beech. The research of the group is focused on the development and application of novel techniques in micro- and nanotechnology with a clear focus on bio applications. As stated in the title the aim of the project was to investigate the possibility of separating cells in a device called the "Bumper Array". This device has been used in the group for a few years and recently Jason Beech did a Master Thesis on the device called "Elastic Deterministic Lateral Displacement Devices"[12]. The basic theory of the Bumper array is, as will be presented below, very simple and its ability to separate beads has been shown[7][23][24]. The step from separating "dead" plastic beads to living cells is quite natural and logical application of the device. But separating complex, living things such as the cell is very different compared to simple beads. The thesis is divided into two parts. In Part One the background theory of the project will be presented. The thesis starts with a short introduction to the biology of the cells used in the project. This in order to get a feeling for the cells being separated. The cells are not simple things to separate and in part one there is a short presentation of some of the problems one encounters when separating cells. Throughout the thesis there will be more added to this section and one can say that the main part of the project is to find the obstacles that lie ahead and give some comments on possible improvements. There is also a part about the general use of this kind of research and its motivation. The presentation of the biology and the direct applications in medicine is short and to get a deeper understanding of how the cells work one needs to go to other literature[13][5]. The thesis goes on to introduce the field of microfluidics. Microfluidics is important in many different fields and especially in medicine. The theory will start with the fabrication of the devices used. This part is aimed to give a more general presentation of the fabrication techniques used in microfluidics, and it will go outside the framework used in the project and give a short presentation of the most common techniques. In the final section of Part One the theory will focus more on the actual research field that this project lies in,

the field of blood-on-a-chip. This takes the theory from general microfluidics to the application in medicine. It is in this field that the project has its roots and it is a good link between the general theory of the cells and the microfluidics, as well as a good introduction to the actual project. This is presented in Part Two. Part Two starts with a description of the device used, the "Bumper Array". The physics of the bumper will be presented. A thorough examination of the problems of clotting and clogging will be given both theoretically and experimentally. Results from cell separation is presented. The project started as an initiative to look at the possibility of separating different sized lymphocytes from each other. The idea was that a patient who suffers from a form of breast cancer will have enlarged lymphocytes. The idea was that the bumper array would be able to separate these enlarged lymphocytes. This project was a little too ambitious and there was too little knowledge about the desired cut-off size and size difference made it not the perfect candidate for a master thesis, though future research on this project may prove very successfull. The second idea was to investigate the possibility to separate whole blood. This means to separate the cells from the plasma and the erythrocytes from the leukocytes. The results obtained show that the cells can be separated in the Bumper array. But the separation physics and conditions for the separation of cells differs from that of the separation of beads. Comments on needed theory will be given and examples of improved designs for cell separation will be presented. The ultimate goal of the project was being able to completely separate two, with respect of size, different populations of cells from each other. We did not reach this goal, but key findings will make it easier in future research to find the solution to cells separation in the Bumper Array. The report is divided into two parts. In part one, chapters 1-4, the main theory of the project is presented. The theory is broad but light. In part two, chapter 5, the research conducted is presented. There is some theory in this part, but it is more specific for the project and not as broad as the theory in part one. All the results are presented in part two.

Chapter 3

The Blood

Blood is one of the most important fluids in separation science. Few things are as complex in their composition and at the same time as important. In every drop of blood there are literally millions of different cells, proteins, ions and other components essential for mankind. The importance of the blood can not be over stated. The blood supplies the tissue of the body with oxygen and nutrients such as glucose, amino acids and fatty acids. It removes waste such as carbon dioxide, urea and lactic acid. The blood contains the cells and proteins handling the immunological functions of the body. It is also responsible for wound healing by coagulating when exposed to extravascular tissue, as well as regulating the pH and core temperature of the body. Research of the blood has been going on for quite some time. The first known experiments with blood to treat patients were made by the Egyptians in ca 2500 BC who bled people who were ill. The first documented study of the bloods constituents is believed to have been made by Jan Swammerdam, a 21-year-old Dutch microscopist, in 1658. He is thought to be the first person to observe and describe red blood cells, a remarkable feat considering the small size of the red blood cell. In 1874 Sir William Osler discovers the platelets. These two examples are just two of many in the process of finding all the ingredients of the blood[2].

3.1 The components of the blood

In the blood stream several different types of cells are circulating. Every different type has its own characteristics and function. In figure 3.1, you can see a list of the cells in the blood. The larger classification of the cells is into Erythrocytes, Leukocytes and Platelets.

3.1.1 The Erythrocytes

The Erythrocytes, also know as the red blood cells, are the carriers of oxygen to all the cells of the body. Hemoglobin contains Iron and is an excellent trans-

Cell type	Average/μL	Percent of WBC
Erythrocytes (RBC)	5,000,000	
Reticulocytes	30,000–70,000	
Platelets	200,000–500,000	
All leukocytes (total)	5000–10,000	100%
Neutrophils	4000–8000	40%–66%
Monocytes	200–800	4%–8%
Eosinophils	50–300	1%–3%
Basophils	0–100	0%–1%
Lymphocytes (total)	1000–4000	20%–40%
CD4 + T Cell	400–1600	15%–20%
CD8 + T Cell	200–800	7%–10%
B-Cell	200–800	8%–12%
NK	100–500	4%–6%

Figure 3.1: Blood count[44]

porter of oxygen; this hemoglobin has a distinctive red color. The cell has a biconcave shape and this shape renders quite interesting features for the red blood cell. Its dimensions can be seen in figure 3.2 and considering the fact that the capillaries of the body has a diameter of 5μm, one can draw the conclusion that the cell can squeeze to a large extent. There are several computer simulations made on the rigidity of the cell and Brody et al. have presented experimental results measuring their ability to squeeze in microfluidic channels. The biconcave shape result in 50% excess of surface to volume ratio compared to a sphere with the same volume. This excess surface area makes it geometrically possible for the cell to have iso-volume deformations as it goes through a tight channel. This is not possible for the sphere since the surface to volume ratio needs to be constant, and the sphere has the minimum surface to volume ratio. Brody et al[14] showed that when the red blood cell be squeezed through a channel with a width of 4μm the cell took the shape of a hot dog bun shape if the speed was slow and an elongated shape if the speed was fast, but other strange shape were also recorded. The results from Brody's study can be seen in figure 3.3.

3.1.2 The Leukocytes

The Leukocytes or White blood cells do not contain Hemoglobin. They are an important part of the body's own defense system called the immune system.

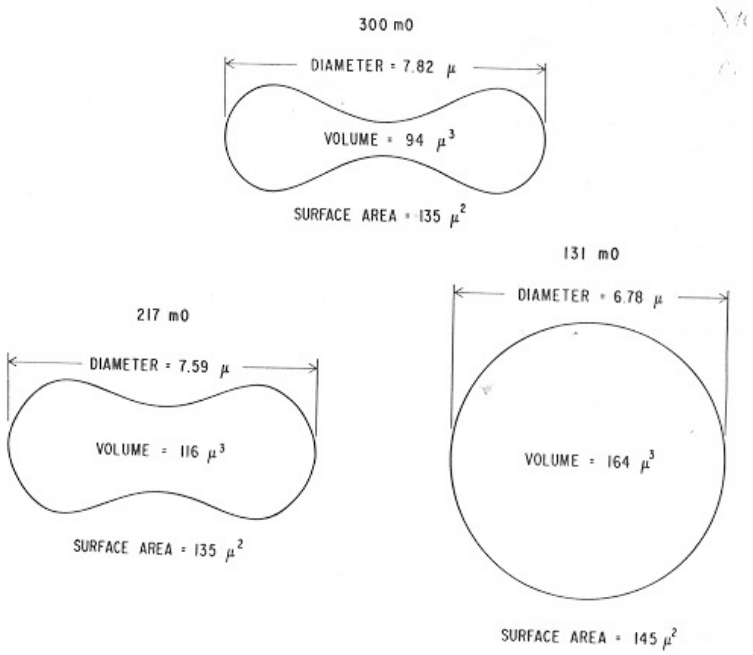


Figure 3.2: Dimensions of the red blood cell

The number of Leukocytes per volume of blood is much less than the Erythrocytes. As can be seen in figure 3.1, there are a thousand Erythrocytes to every Leukocyte, and within the population of Leukocytes there is a huge difference in numbers. The Leukocytes are divided into subclasses. These subclasses are historically defined as Lymphocytes, Monocytes(figure 3.4(a)), Eosinophils(figure 3.4(d)), Neutrophils(figure 3.4(c)) and Basophils(figure 3.4(b)), this classification is rather old and was developed using microscope techniques. In the last decades more information has been gained and the classification has changed to some extent. Today, apart from the classical classification, a nomenclature based on function is being used. In this classification different surface proteins are being used to distinguish, the cells. The most important of such a protein is a class called cluster of differentiation (CD) markers. It has been shown that these different CD markers are important when making diagnosis and treating patients. The Neutrophils, Eosinophils and Basophils are gathered under the common name Granular leukocytes. They are all approximately the same size - about 12-15 μm in diameter. Their nuclei form lobes, and nucleoli cannot be seen. The number of nuclear lobes varies according to cell type. All granulocytes are motile. The Monocytes migrate into the connective tissue and differentiate into Macrophage. They have a size distribution between 12 to 18 μm in diameter. The two subpopulation of Lymphocytes, B- and T-Lymphocytes can not be distinguished in the microscope before activation. They are the smallest in size

in the range of 7-10 μm in diameter, but upon activation the B-lymphocytes double there cell plasma and start producing antibodies. The Leukocytes have a natural tendency to adhere to surfaces. This is important in order to be able to migrate into the infected tissue. The adhesion is made possible by a surface protein called selectin. The leukocytes have a rolling motion in the blood vessels and when an infection occurs, thrombin and histamine is secreted into the blood stream by the endothelial cells and induce adhesion.

3.1.3 Platelets

Platelets or thrombocytes are the cell fragments circulating in the blood that are involved in the cellular mechanisms of primary haemostasis leading to the formation of blood clots. Dysfunction or low levels of platelets can lead to bleeding, while high levels, although usually asymptomatic, may increase the risk of thrombosis. Like red blood cells, platelets are anuclear (no cell nucleus) and discoid (disc shaped); they measure 1.5-3.0 μm in diameter. The body has a very limited reserve of platelets, so they can be rapidly depleted. They contain RNA, a canalicular system, and several different types of granules; lysosomes (containing acid hydrolases), dense bodies (containing ADP, ATP serotonin and calcium) and alpha granules (containing fibrinogen, factor V, vitronectin, thrombospondin and von Willebrand factor), the contents of which are released upon activation of the platelet. These granule contents play an important role in both hemostasis and in the inflammatory response.

3.2 Problems with the blood

The classic that blood is thicker than water is in some sense true when it comes to the separation of blood. It is a fluid with so many different cells and proteins that it is a challenging task to separate it into to its components. The amount of cells makes the blood appear like a thick smear. The active components of the blood, that is the cells and the proteins, do interact and change the outcome. The cells can adhere, form clusters and lyse. These actions must be taken into account. The cells size and rigidity will change with salinity, pH and other buffer factors. The activation of the lymphocytes will give a different composition in the leukocyte population. There are so many effects that interfere with the result of the separation. In order to control this, one needs to limit the number of changing variables between experiments. It is also important to acknowledge the most influential effects, such as salinity, and limit there variance. Swift and fast handling of the blood, from the time it is drawn to the time it is analyzed, is also important.

3.3 Why study blood?

The blood is a treasure of markers stating the condition of the patient. Through the blood you can diagnose several different diseases. Malaria, Sickel cell anemi,

cancers and other blood diseases are just a few examples of diseases that can be discovered by studying blood. Malaria alone kills approximately 1.5-3 million per year[38] and it is approximated that there are 300-500 million clinical cases per year[35]. In order to improve treatment and survival rate, new methods needs to be developed. Many patients with these diseases lives in underdeveloped countries where there are no means for the doctors to make the right diagnosis. The focus of the health industry has been to supply the world with cheap medicine but the progress of cheap diagnostic tools has been much slower. Today the most effective tool for blood diagnosis is the FACS machine, but the cost of the machine is about a quarter of a million dollars. This is of course too much for the local physician in the rural areas of Africa. The development of low cost point-of-care devices will make it possible to produce lab-on-chip devices running as low as 10 cents per device. The Bumper Array is an example of possible point-of-care device, although far from any clinical application it shows promising results.

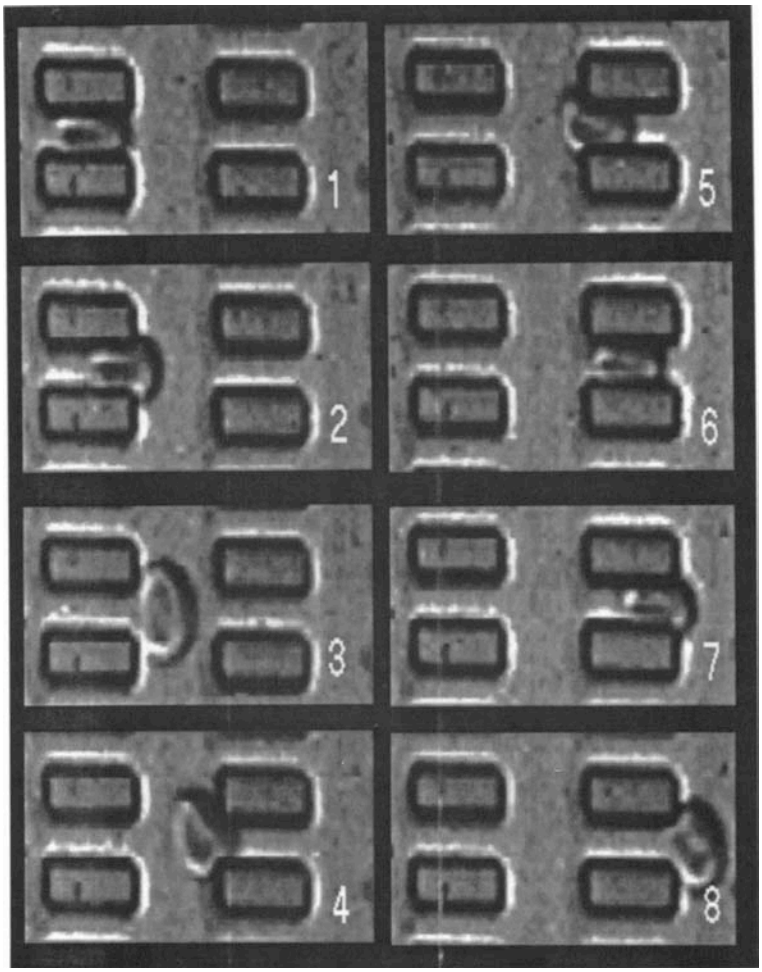
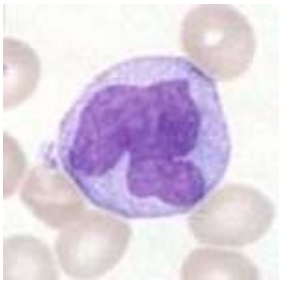
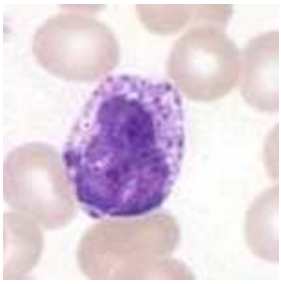


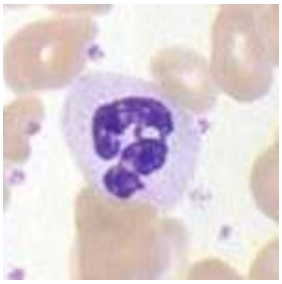
Figure 3.3: The motion of the Erythrocyte captured by Brody et al.



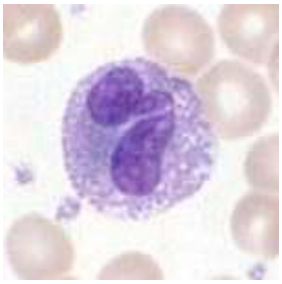
(a) Monocyte



(b) Basophil



(c) Neutrophil



(d) Eosinophil

Figure 3.4: Granular leukocytes and Monocyte. Images are from Wikipedia[1]
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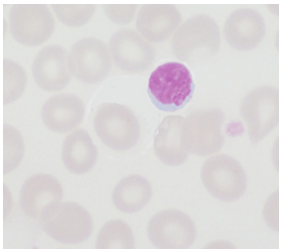
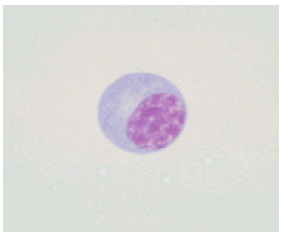


(a) Resting lymphocyte



(b) Active B-lymphocyte, Plasma cell

Figure 3.5: Images of lymphocytes. In a) the resting lymphocyte is pictured. In this stage one can not distinguish betwen T- and B-lymphocytes. Once activated the B-lymphocyte will form into a plasma cell dispalyed in b)3.5(a)

Chapter 4

The Chip

In this section the science of lab-on-a-chip will be presented. The term covers devices that integrate laboratory functions on a single chip. A very important part of a lab-on-a-chip device is its microfluidic components. The research on microfluidics took its first stumbling steps from four different fields; molecular analysis, biodefense, molecular biology and microelectronics. The contribution from molecular analysis was the first to be made to microfluidics. Gas-phase chromatography (GPC), high-pressure liquid chromatography (HPLC) and capillary electrophoresis (CE). These methods made it possible to simultaneously achieve high sensitivity and high resolution using very small amounts of sample. With the success of these methods the path was paved for more compact and versatile formats of them leading the way for microscale methods in chemistry and biochemistry. The contribution from biodefense is, as sad as it may be, an important one. In the wake of the cold war more threats against the US came from chemical and biological terrorist attacks. As a precaution the US through its DARPA (Defense Advanced Research Project Agency)project poured money into the development of small and cheap sensors and analysis methods. The explosion of genomics in the 80s, followed by the advent of high-throughput DNA sequencing, required methods to analyze the rich amount of data. This drove the development toward more and more throughput, higher sensitivity and resolution in the devices. The fourth contribution comes from microelectronics. The huge success of the semiconductor industry with its methods of fabricating microelectronic mechanical systems or MEMS, made scientists think of a use for direct implementation of the techniques to construct microfluidic systems. Though the electrical industry mostly uses silicon the field of microfluidic has moved toward other materials, from silicon to glass and then polymers. Today the most important material in microfluidics is a polymer, poly(dimethylsiloxane) or PDMS.

4.1 Fabrication

As mentioned above a lot of the techniques and methods for fabrication were derivates of the techniques used in the semiconductor industry. The methods used to make integrated circuits were adopted for the fabrication of fluidic channels. Microfabricated devices have been around from more than 30 years with several applications attaining commercial or scientific success. The evolution of microfabricated devices towards medical application may not have reached the same success as some other areas but only in the last year more and more application of the methods have found there way into the labs of physicians and biologists. The fabrication process, although there are an enormous amount of variations, follows a few standard steps. Some of these are taken from the semiconductor industry and some are developed especially for microfluidics. The process can roughly be divided into four different steps. Transferring the pattern into the material. Thin-film growth/deposition, in which thin films (usually on the order of micrometers in thickness) are grown or deposited onto a substrate. Etching, the third process, creates features by selectively removing material. The final kind of process is bonding. This process bond two substrates to each other one way or another to form closed channels.

4.1.1 Substrate material

Many different substrates have been tested through the years, here are those that have been most widely used.

Silicon

Silicon is a truly interesting material. Its electronic properties have been one of the big reasons for making the semiconductor success so big. But the material also has great mechanical properties, making it possible to design micromechanical structures. Silicon can be micromachined in a number of ways. But for biological and medical applications, the fact that it is opaque to lightwaves and high fabrication cost makes it not the perfect candidate for microfabrication.

Glass

Glass was introduced to overcome the problem of transparency in silicon, but it lacks some of the range in micromachining of the silicon. Glass for micromachining comes in many different forms. Most notably are fused silica and borosilicate. Fused silica wafers are pure amorphous silicon dioxide (SO_2). It can withstand high temperatures and is transparent down to short wavelengths. Borosilicate wafers, of which Pyrex is the most common, are much less expensive then fused silica. It can be easily bonded to silicon, but lacks some of the resistance to high temperatures compared to fused silica, as well as a higher autofluorescence.

Plastics

The introduction of plastics has revolutionized the field of microfabrication. Plastic is often the least expensive substrate material. The ability to mass produce devices in plastic through methods such as injection molding[29][31] and hot embossing[30][11], is a great advantage of plastics. This means that plastic devices can be produced in mass production at very low costs. This allows for disposable devices. The most important of the plastics is the polymer PDMS[42][49][32].

4.1.2 Pattern transfer

Photolithography

The photolithography step is the step that transfers the pattern made by the designer into the material. There is again several different ways to do photolithography but most of them follow a schedule as seen in figure 4.1. The design is made on a computer aided design (CAD) program and transferred to a mask. The mask is usually a glass plate with opaque features defining the design. After the mask is done a layer of photoresist is applied to the substrate, usually by spin-coating it to the substrate. The photoresist is a photosensitive polymer and can work in two different ways, positive or negative. A positive photoresist is made soluble when exposed to UV-light. This makes it possible to remove the unwanted photoresist with a developer. A negative resist works in the opposite fashion, it is made insoluble by UV-light and the unexposed regions are removed.

Other methods for pattern transfer

Although photolithography is the most important pattern transfer there are others. Microcontact printing[53][47] is a method that uses a soft polymeric stamp, usually PDMS, to "stamp out" structures. The stamp is made by conventional methods. The stamp is "inked" with alkanethiols or alkylsilanes and placed on a gold- or silicon dioxide-coated surface, respectively. This transfers the molecules from the stamp to the substrate, where they form a self-assembled monolayer.

4.1.3 Thin-Film Growth/Deposition

The usage of thin-films in fabrication varies. They are used as mask making material, structural material, sacrificial material and electrical devices. They are formed on the substrate either by a chemical reaction-driven process or a physical process.

Dielectrics

For the purpose of electronic isolation or etch mask, silicon dioxide and silicon nitride are commonly used. Thermal silicon dioxide is grown by placing a silicon

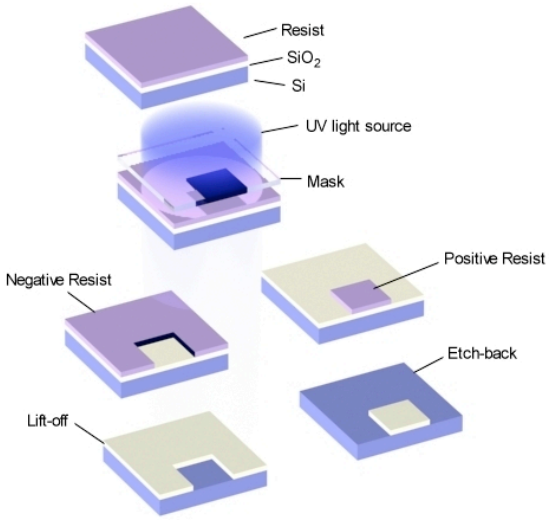


Figure 4.1: The principle of photolithography

substrate in a high temperature (900-1200° C) oxidizing ambient. Growth is limited to roughly 1 μm due to the slow process and diffusion. Thicker films (10-20 μm) can be obtained with chemically deposited oxides, although these films lack some of the robustness in the thermally grown films. Silicon nitride is always deposited.

Silicon

For structure material, polycrystalline and amorphous silicon thin-films are often used[15]. These films are deposited by chemical reaction-driven processes. There is also a possibility to dope the surface by adding dopant atoms into the surface of the silicon wafer. Generating a thin doped film of single-crystal silicon that can be used as etch stop for wet etching[47][40].

Metals

Physically deposited metals or electroplated metals are usually used for electrical interconnects and electrodes. Another application of thin film metals is as a surface for self-assembled monolayers. For these purposes several different metals have been reported to work. Gold, Aluminum and Platinum are just a few of the metals used.

Plastics

The usage of plastics to form a layer on the substrate has many different purposes. They are used as mechanical structures, thick structural layer for molding, or as chemically sensitive films. One example is polymides, these have been utilized for many years in microfabrication, as have spin-on silicon-rubber and photoresist. SU8, an epoxy-type photoresist that can be spun on into thick layers (more than 100μm thick) and can make anisotropic structures has been used as a mold for PDMS casting and as structural layer for microchannels. Another type of plastic thin film is transparent thin films (e.g. parylene C), these can be vapor deposited to the substrate.

4.2 Etching

Etching is divided into wet-etching and dry-etching[47]. These classes are then divided into isotropic etching and anisotropic etching. The isotropic etching etches with the same degree in every direction, generating a rounded etch profile. This etch profile of isotropic etching leads to an underetch of the mask. This effect can be unwanted and then anisotropic etching might work. Anisotropic etching is a directional etch, it can etch straight down or at an angle. Wet etch is a method were the layer being etched is exposed to a chemical liquid. Dry etch can be either chemical or physical. A chemical dry etch uses gas-phase chemistry to perform the etching. Physical etch is made by sputtering the surface. In general chemical etches are isotropic and physical etches are anisotropic. These differences can be used to get the desired result on the thin film you want to etch. When etching silicon both isotropic and anisotropic etching is used. Wet etching can be performed with solutions of hydrofluoric acid and nitric acid. Wet anisotropic etchants on silicon will exploit the cristallinity of silicon by etching the {111} crystal plane slower than the {100}, leaving a characteristic 54.7° sidewall on a [100]-oriented wafer. Primary wet anisotropic etchants are potassium hydroxide, tetramethylammonium and ethylenediaminepyrocatechol. Standard dry etching can be used to etch silicon to depths ranging from submicron to roughly 10 μm. In figure 4.2 you see examples of isotropic and two anisotropic etch profiles.

4.3 Bonding

This step seals the device and is a very important part of the fabrication. The microfabricated channels in the substrate are given a top lid so that the cavity is confined by four walls. Different substrates use different techniques to bond. When bonding PDMS to glass oxygen plasma can be used. The exposure of the PDMS and glass to the oxygen plasma will induce a formation of silanol groups on the surface. The two treated surfaces, that of the PDMS and the glass, will react in a condensation reaction to covalent Si-O-Si crosslink under production of water.

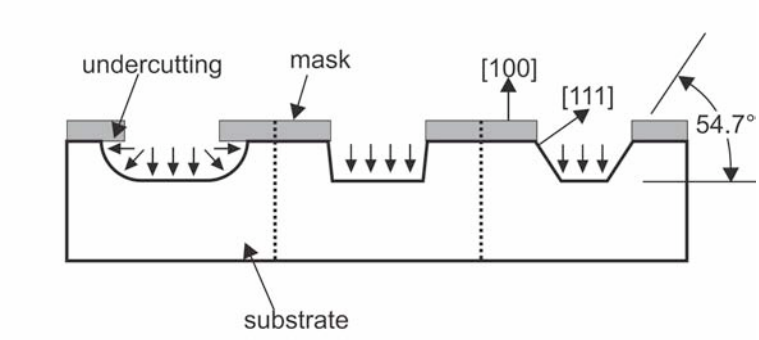


Figure 4.2: Etch profiles[47] Overview of isotropic(left) and anisotropic(middle and right). The middle figure is an example of dry anisotropic etching and the figure on the right is wet anisotropic etching

4.4 Anti-sticking

When PDMS fabrication is used. The uncured PDMS is poured on to the substrate(master) with the structures. The master with the PDMS is then baked at 80° for 40 minutes. The PDMS will then harden and can be teared off with the intact structeres still present. But adhesion of residues on the master is a big problem in all fabrications using a master-polymer system[9]. It is desirable to reduce this effect and for this the addition of an anti-adhesion layer is often added to the master's surface. Different molecules with desired functions have been available for a long time. Most widely used are fluorinated alkyl derivates or fluorinated polymers such as polytetrafluoroethylene (PTFE), which is well known as an anti-adhesion layer in frying pans, see [6] for a nice review on the most common anti-adhesion molecules used microtechnology fabrication. In this study though we used the molecule tridecafluoro-(1,1,2,2)-tetrahydrooctyl-trichlorosilane ($F_{13}-TCS$). In the process the chlorosilanes react spontaneously with the hydroxylated silicon or silicon-dioxide on the surface under the elimination of hydrochloric acid, as can be seen in figure 4.3. For a complete description on how the monolayer is formed and a detailed explanation of the set-up see [9]. In theory the silane layer should remain intact during the fabrication process, due to the covalent bonding between the silicon surface and the $F_{13} - TCS$ molecules. In theory there should not be any residues left over from the polymers either. But experimental usage shows that there are individual differences between two samples prepared in the same way as described in [9].

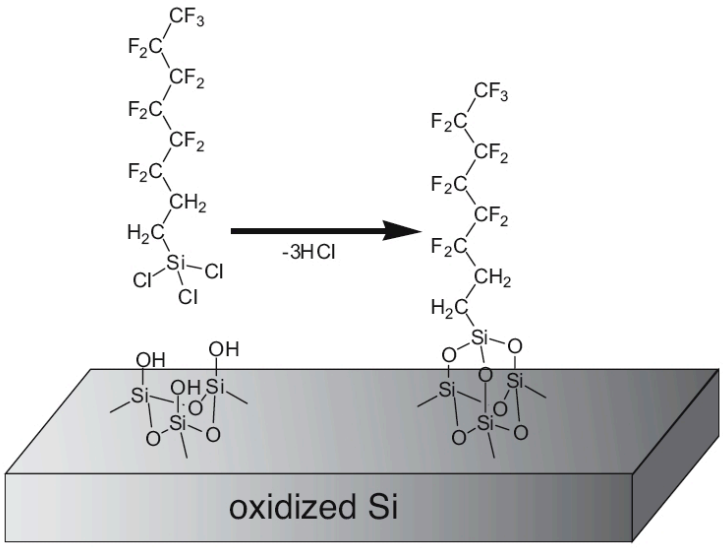


Figure 4.3: Anti-sticking procedure

Chapter 5

Blood-on-a-chip

The analysis of the cellular components has developed to a science limited to highly specialized and strictly regulated laboratories, at the cost of point-of-care diagnosis. But even in the present day, with twentieth century technology and a lot of advanced automation, a significant portion of blood handling is still performed manually or in conditions that may seriously affect the results of subsequent analysis. So there is still a great need in reducing the occurrence of such errors. Limiting the time from the collecting of the blood sample to the analysis of it is one example. This step, to take the analysis technique from the high-end laboratory to where the blood is drawn, would require faster, cheaper, and more comprehensive approaches. One of the most promising techniques for such an impact in biology is microfluidics and miniaturized lab-on-a-chip-type devices. These are extremely attractive for blood analysis and there is great anticipation of their future. The ability to assemble complete labs for blood analysis on one chip in point-of-care analyzers capable of complete diagnostic is poised to revolution the industry of patient health care. The doctor could perform the diagnostic of an infectious disease, cancer or inflammatory disease in his office or even in the patient's home. This would take a huge burden off the hospitals. They may also allow better matching between drugs and patient pathophysiology, reducing side effects and improving efficiency of therapy. The development of new medicine may be redefined by microfluidics, making it easier to perform clinical trials[18]. In small-animal studies, microfabricated devices would only use minute amounts of blood for analysis, allowing for repetitive sampling at multiple time points and minimizing the adverse effects of blood drawing. Even more ambitious, in the discovery mode research, microfabricated devices for sample preparation would open new possibilities by allowing comprehensive genomic and proteomic analysis from small homogenous subpopulations down to single cells. On the whole, on-chip blood sample preparation would lead to more gentle, fast, and consistent manipulation of the living cells, and therefore more accurate and better quality of extracted information.

5.1 Separation of cells on a chip

The blood cells have natural heterogeneities in their physical properties. This can be used to separate the cells by a physical field. Different fields affect different features of the cell and differences in size, density, electrical permittivity, dielectric characteristics, or adhesiveness among cells can be revealed in the form of forces differentially acting on cells of a particular type. In a group of cells with different behavior upon field exposure, a subgroup of cells affected in the same way by the field can be isolated. One great advantage of most uniform field separations is that it can be achieved without the need of additional cell-labeling or cell-modification steps. The fact that these devices may also perform the steps of identification and separation simultaneously results in less complex and more accurate measurements. In microscale world, strong fields can be created over small distances and small displacements on the scale of cell size can be used for effective separation. Also the fact that microscale allows the manipulation of single cells and particles in suspension, with the potential for extreme purity or efficiency of the separation. Since the principles of separation of particles from solutions by using fields were outlined by Giddings[21], a variety of fields have been employed in small-scale applications. Those relevant to blood separation are presented in the following sections.

5.1.1 Mechanical force separation

Differences in cell size are easy to observe in a microscope[52]. A modern method to observe the difference in size is common centrifugation methods. These rely on differences in size and density among cells for blood separation. Geometrical differences between blood cells can also be exploited in microfabricated devices that achieve cell separation based on the mechanical restriction of cell displacement. However such methods have limitations to adjust to changes in the properties of the sample. When a new cell is targeted a completely new design is in the worst cases needed, or at least adjustments to the old design is required. Another drawback of microfabricated devices working solely on the principle of size separation is the, to date, limited efficiency or poor purity of the final sample. Several different designs of devices, using this method, have been published. Wilding et al[50] made a design of dense arrays of posts spaced 5 μm or 7 μm apart, and weir-type structures of 3.5 μm heights were employed for the separation of leukocytes from whole blood, see figure 5.1. Another design made by Mohamed et al[34] consists of successive arrays of narrow channels with sizes decreasing from 20x10 μm to 5x2.5 μm were also tested for separation of tumor cells from blood samples spiked with cultured tumor cells. The separation relied on the idea that Leukocytes and other larger cells would get trapped in the spacing between the post, while Erythrocytes and other small cells would easily pass through. However, experiments showed that Leukocytes, although bigger in size, could also s̈queezeẗhrough the same narrow spaces, limiting the overall efficiency of capture to values between 7% for the weir and 35% for the 5 μm spaced posts. Another big problem for the group was that the cells even after a

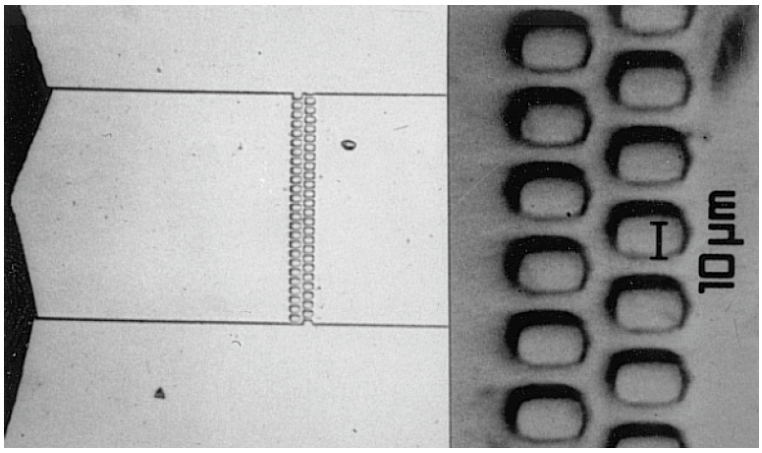


Figure 5.1: The Design used by Wilding et al. In the figure to the right there is a zoom in on the interesting structures[50]

rather short time period clogged the device to such severe extent that the flow was altered. This is a serious problem when dealing with blood samples were the sample contains millions maybe billion of cells, see figure 5.2.

Working with a slightly different device featuring a 5 μm lattice of posts with a polar urethane coating, Carlsson et al[16] observed an interesting phenomenon during the passage of cells. They could see a difference between the cells trapped at the entrance array and those further in. Granulocytes and T lymphocytes were differentially trapped at different distances into the array. Trapping occurred in the absence of adhesive proteins on the posts, and it has been speculated that differences in viscosity between the two cell types or differences in nonspecific stickiness following the interaction between granulocytes and T lymphocytes during the separation process may be responsible. The limited success of these early approaches only underlines the challenges in understanding and exploiting the interaction between mammalian cells and mechanical structures for separation purposes.
In yet another approach, made by Yamada et al[54], a channel with the width comparable to the width of the cells was allowed to merge with another channel of the same size but with no cells, see figure 5.3. Working in the laminar flow limit made the cell align along on of the channel walls. This pinched flow resulted in a separation between cells of different size. This separation is caused by the fact that larger cells will have a geometrical center closer to the center of the channel than smaller cells and the parabolic flow profile will make the larger cells move faster in the channel. Although several steps remain before these devices can give any clinical contributions, it is becoming evident that control of the microscale flow in the vicinity of cells is one critical element for the success of separation. The challenges on separating cell on size are tricky to overcome. The fact that the cells deform make it hard to define the cell size.

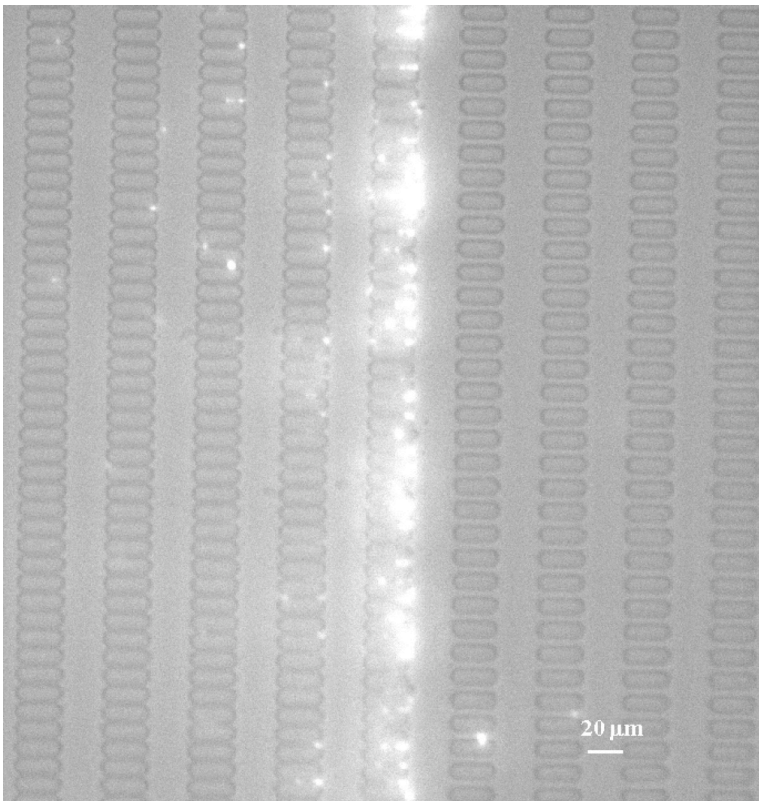


Figure 5.2: In this figure one clearly sees the clogging in Mohameds device[34]

But there are other examples of separation of mechanical rigidity. One example of this is a technique to discover Malaria. Malaria affects the erythrocytes by increasing their rigidity[17]. Shelby et al[41] have microfabricated a device simulating capillary vessels as microchannels with sizes from 2 μm up to 6 μm. This device has been used to effectively separate more rigid, infected erythrocytes from normal, uninfected erythrocytes. Increased rigidity of the infected erythrocytes inhibits their passage, even through large capillaries, and infected cells accumulate at the entrance to the channel. The crucial point in the separation is that even when infected cells block the entrance to a channel, normal healthy cells can still pass through to some extent. Efforts to develop cheap and reliable diagnostic devices for malaria are not limited to mechanical separation of infected cells. Other principles have emerged, underlying the flexibility of microfabricated approaches. Another important task for separation science is effective separation of plasma and cellular fractions from whole blood. This has also been done using mechanical forces in micro devices. In general, the purpose of such a procedure is to prepare plasma for subsequent analysis by eliminating the potential confounding effects of cellular components on plasma biochemical

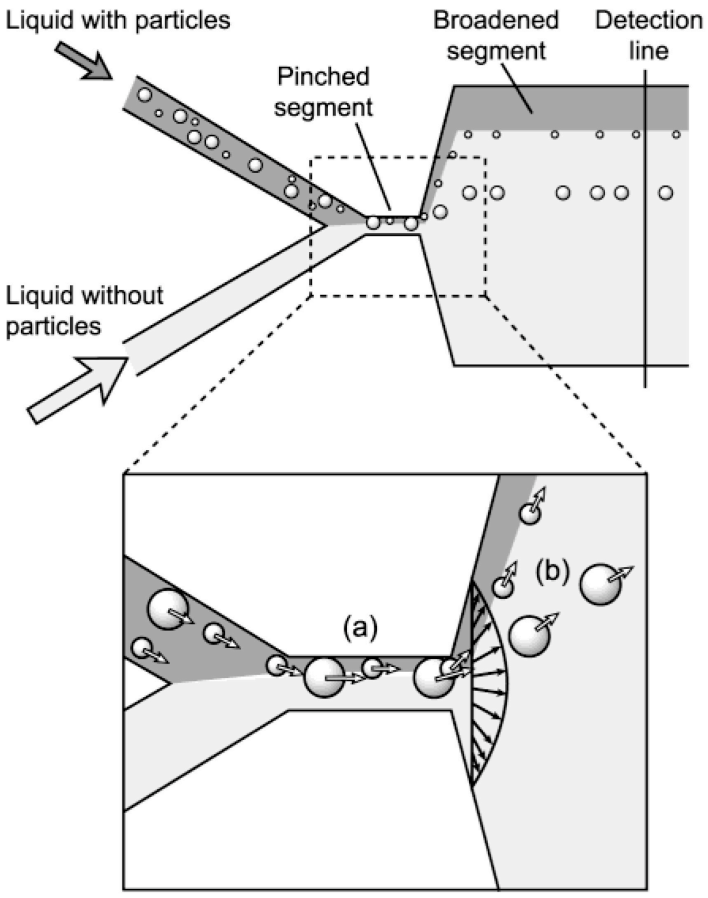


Figure 5.3: A schematic picture of the theory of Yamada et al's device[54]

assays. Both the groups of Lauks[27] and Ahn[4] have designed microfabricated devices for this purpose. The bench top centrifugation technique used today is anticipated to be replaced by small, single-use, lab-on-a-chip-type devices for plasma analysis. Other solutions include separating plasma by flowing whole blood through microfilter-like parallel arrays of shallow channels, microchannel bent structures, or by centrifugation of 5 μL of blood in lab-on-a-disk-type devices. Gentle handling of cells in many of these devices helps reduce the potential for the release of cellular factors in the plasma samples during the separation procedure. Conversely, target cells may be washed from plasma proteins, which may interfere with subsequent cell analysis. Overall, better understanding of the micromechanical interactions between cells and microstructures is poised to lead to very simple devices for cell separation, with a great impact on reducing

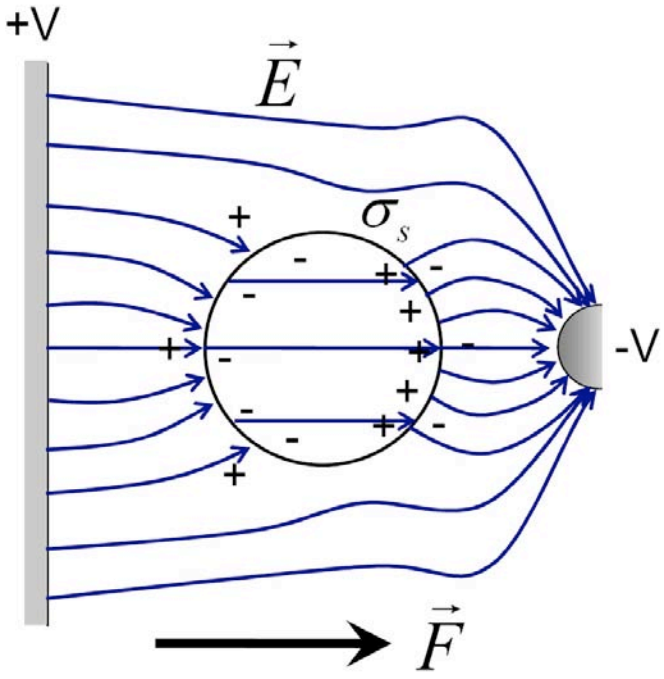


Figure 5.4: Principle for positive DEP

the costs and increasing the accessibility of diagnostic and monitoring tests. The Bumper array can also be added to this group of separation techniques.

5.1.2 Dielectrophoresis

Dielectrophoresis(DEP) relies on the fact that a non-uniform electric field will induce a polarization of the cells. This polarization makes it possible to contol their motion and position in the device. The advantage of using DEP on controling and separating cells is that the cells remain intact to a large extent in the electric filed. This applies to several physical properties of the cell such as the bilipid membrane, internal structure, or size of the nucleus[20]. Depending on the cell and its surrounding medium, the frequency of the electric field will induce a polarization either in the direction of the field vector, positive DEP, or in the opposite direction, negative DEP. Using this fact a correcly designed electric field can be used to separate two populations of cells that under the influence of the electric field induce different polarization.

But it is not as easy as it might seem. The fact the outcome of DEP is affected by so many factors makes it hard to make any comparison between different studies and tests[44]. The great advantage of using microscale system instead of macroscale when using DEP is that low voltages are enough to

produce intense electrical fields. This makes the use of microscale systems for DEP very favorable. For example, alternate or direct electric fields are relatively easy to implement and control in microdevices. Thermal and hydrolysis effects that are detrimental for living cells, and that plagued early large-scale DEP devices, can be avoided. Exquisite control over the characteristics of the electric fields at length scales comparable to cell size can be achieved by the use of microfabricated electrodes and features. Because cells respond differently depending on the frequency of the applied alternate electric fields, it may be possible to adjust DEP devices for separating different cell subtypes just by changing the field frequency or amplitude. As a consequence of this display of favorable factors, a large number of microscale devices have been demonstrated for different applications involving the manipulation of cells and particles in suspensions. Among the applications of DEP of the microscale that has been developed for non-biological use can be extended to cell separation. An important factor behind the choice of DEP solution is the sample size. The standard way to implement this is by using positive DEP to create traps to separate cells from a flowing mixture[44][48]. When the electric field is turned off the cells are released and can be collected. Another way to implement DEP is by using arrays of insulators to create perturbation of the electrical field[26].

In the absence of flow, discrimination may be improved by dielectric levitation and concentration of target cells on the surface of electrode arrays. For example, malaria-infected erythrocytes that have different electrical properties compared with normal erythrocytes owing to changes in their ionic content were concentrated at the center of a spiral array of electrodes. Gascoyne et al[19] have shown a sensitivity of 20 infected cells separated from 10^6 total cells have been reported by the use of simple, four-pole, planar devices. Separated cells can be examined further by different detection and imaging systems (fluorescence, cell morphometry, etc.), and some of them selectively released to improve the purity of the final sample. When it comes to separating larger samples, containing millions of cells, a technique using hyperlayers can be used. This technique uses DEP forces in the bottom of the device to control all the cells flowing in a microchannel. Each cells motion is decided by three forces. These are sedimentation forces that are proportional to particle weight, DEP forces that falls off exponentially with the distance between the cells and the electrodes, and drag forces that are proportional to fluid velocity in the channel[55]. Equilibrium positions at different heights inside the channel for different cells were the result of the balance between changing levitation and constant sedimentation forces. For each cell, different velocities along the channel were the consequence of the parabolic velocity flow profile across the channel. As a result, cells from a heterogeneous mixture were separated along the channel and captured at different time intervals at the outlet. Differences between normal and cancer cells were exploited for hyperlayer separation of whole blood samples. Cells of the metastatic human breast cancer cell line MDA231 were selectively captured from whole blood by balancing the hydrodynamic and dielectrophoretic forces acting on cells upon their passage over a microelectrode array[10]. Interdigitated electrodes patterned on the bottom of thin flow chambers were used to

levitate normal and cancerous cells at different levels, depending on their different dielectric properties. Separation of 50,000 tumor cells from a mixture of 10 μL of blood was achieved under the influence of a parabolic flow profile in less than 5 min[55]. Although impressive, the efficacy of separation in these devices would need to be improved even further for applications such as the detection of cancerous cells in clinical samples of blood. Especially in the early stages of cancer, when only a few tumor cells are present in blood, larger volumes of blood, of the order of milliliters, would need to be processed before one or more cancer cells could be detected. Physical separation of cells in distinct streams using DEP has also been demonstrated. A complex system where several steps of using DEP forces to funnel cells into narrow streams, break aggregates, and trap or separate cells along the stream has been used to separate individual cells. Separation of cells from the stream has been demonstrated for macrophages, T cells, and B cells. Upon passage over an array of interdigitated electrodes, at 400 μm from the edge of the array, the cell stream was separated into three distinct 150-μm-wide bands. More recent experiments from the same group, using a leukemia cell line, showed that further increases in selectivity could be achieved by focusing the cells in a narrow stream on top of the electrodes. Following a different approach, tumor cells from a Jurkat cell line were separated by deflection through negative DEP from erythrocytes in phosphate buffered solution. Other tumor cell lines mixed with whole blood were also separated by the use of dielectrophoretic forces in devices with arrays of microelectrodes. When the contrast between the electrical properties of cells is less clear, cells can still be enriched in complex mixtures. Fivefold enrichment of hematopoietic cells (CD34+) in whole blood samples was observed in fractions collected after cell passage over a dielectrophoretic electrode array. Stem cells remained viable, were not altered by this enrichment method, and were capable of forming colonies in culture. Thirty-fivefold enrichment of leukocytes from whole blood diluted 1:1000 in sucrose buffer has been reported by the use of the hyperlayer DEP separation method. Optimization of the separation conditions led to relatively high performance separation of T and B lymphocytes from monocytes, T (or B) lymphocytes from granulocytes, and monocytes from granulocytes. Because DEP devices are usually not complicated and do not involve cell-labeling or cell-modification steps, they are always a serious candidate for any on-chip hematological analysis. The use of electric fields for cell separation is not limited to dielectrophoresis devices. Selective destruction of cells in a mixture has been shown by the use of pulsed electric fields of up to 2 kV/cm. Although not implemented in a microdevice, but nonetheless using microelectrodes spaced 400 μm apart, this method is based on the fact that the strength of the electric fields can be adjusted such that pores can be opened in larger cells before smaller cells are affected. Exposure of blood (mixture of peripheral blood mononuclear cells) to pulsed electric fields caused stepwise elimination of large monocytes without affecting smaller lymphocytes or stem cells. Through the same method, peripheral blood has been enriched in hematopoietic stem cells (CD34+/CD38-) and stem cell function preserved.

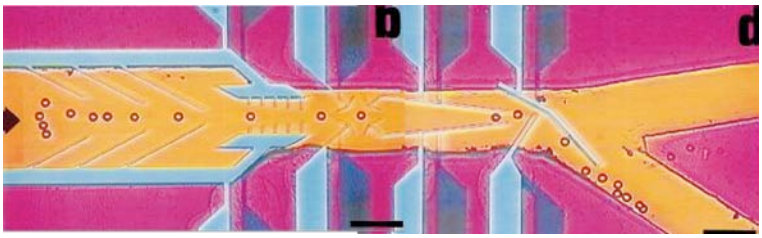


Figure 5.5: Example of a device using DEP for separation

5.1.3 Optical

The introduction of handling the separation by means of light is particularly interesting since approaches using light to separate cells from complex mixtures are particularly attractive because they avoid mechanical contact between cells and surfaces. The most common way to implement optical separation is by laser tweezers type devices. Optical trapping for manipulation and sorting of individual cells can be readily achieved by the use of laser tweezer-type devices. The limitating factor in tweezer type separation is that, Although cell handling can be very precise, only one cell at a time can be manipulated[36]. Scaling up the separation for millions of cells at a time, like those in small blood samples, would be very challenging using such systems. Thus, devices for parallel processing of a large number of cells in parallel have been tested. A microfluidic system using a diode laser bar has been demonstrated for the separation of cells based on their size. While larger cells are deviated when passing through the laser enlarged beam, smaller cells are not, and consequently, separation of large and small particles can be achieved. A dynamically reconfigurable optical lattice, produced as an interference pattern, was also used to separate particles cells from mixtures based on size and refractive index[28]. More recently, the same principles of tunable optical lattices have been used to separate erythrocytes from leukocytes with more than 95% efficiency[44]. This type of device is easily reconfigurable by adjusting the interference pattern, and because it has no narrow channels it is less prone to clogging during its use. The increased availability and continuously decreasing costs of solid-state coherent light sources are additional arguments for the use of microscale optical interactions for the separation of cells from blood samples.

5.1.4 Magnetic separation

Intuitively, the iron-bearing hemoglobin molecules contained only by erythrocytes would lead to differences in the magnetic properties between erythrocytes and other blood cells. However, practical measurements showed that, in spite of the higher iron content, erythrocytes have, at least in oxygenated blood, the same diamagnetic behavior as Leukocytes. A weak paramagnetic behavior has been observed for erythrocytes in deoxygenated blood. Only reduced forms

of hemoglobin, deoxihemoglobin and methemoglobin, and pathologic hemozoin in erythrocytes infested with malaria are paramagnetic[37]. In the presence of high magnetic fields and gradients, such differences can be used to separate leukocytes from erythrocytes. This was first demonstrated by Melville et al.[33] through the capture of erythrocytes from whole blood on a metal mesh in the vicinity of a powerful magnet. Although large magnets are still needed to produce the intense magnetic fields, microscale devices have the advantage of producing high magnetic field gradients in the proximity of microscopic magnetic features. Analytical magnetopheresis has been demonstrated in experimental settings involving microscopic features. Particles and cells with various magnetic susceptibilities were selectively deposited and separated from suspensions flowing in a thin layer under a magnetic field applied perpendicular to the flow. The selectivity of magnetic separation of cells can be tremendously enhanced by coupling magnetic beads decorated with antibodies against proteins on the surface of the cells, a process generally known as magnetic cell sorting (MACS). Because in this process the identification of cells is based on protein recognition rather than intrinsic magnetic properties of the cells, devices using these principles are discussed separately in the next section.

5.1.5 Biochemical

Biochemical differences among cells can be effectively used to selectively destroy some cell subpopulations after uniform exposure of the whole sample to a selectively toxic environment. It has been known, for example, for almost a century, that leukocytes are more resistant to solutions of ammonium chloride than erythrocytes, which are readily lysed within tens of seconds after exposure[44]. In regular separation procedures, bulk volumes of blood are mixed with bulk volumes of the lysing agent for periods of time long enough to account for diffusion and erythrocyte lyses. At the same time, leukocytes are also exposed and may be affected by the lysing solution; shortening the exposure time is of interest for minimizing the chance for leukocyte alteration. Although calculations and experiments show that lysing agent diffusion is usually the limiting factor for speeding up the reaction, it is evident that shortening the diffusion distance would provide significant advantages. This can be easily achieved in microfluidic devices, and complete lyses of erythrocytes and approximately 100% recovery of leukocytes were accomplished after exposure to an isotonic lysis buffer for less than 30 s[44]. Alternatively, complete lysis of erythrocytes in hypotonic deionized water occur two times faster, in only 15 s, whereas the leukocytes appeared to be intact. Following erythrocyte lyses, leukocytes are immediately returned to isotonic physiological conditions by the addition of buffer. Microfluidic selective cell lyses not only results in improved yield and viability of the Leukocytes compared with bulk methods, but by assuring equal treatment for all cells in the sample it may also have a favorable effect on subsequent analysis by reducing yet another source of variability.

Chapter 6

Blood-in-a-bumper

This part of the project concerns the research conducted in the project. The device used is the Bumper array or simply the Bumper. The Bumper is a microfluidic device developed at Robert Austin's lab at Princeton University. Its geniality lies in its simple construction. Results of the device have been published several times and Zheng et al. [57] have shown initial results on separation of blood. They treated the blood with Ficoll-Paque to reduce the clogging they experienced. In this project another method is approched to reduce the clogging. To get a sustainable and non-invasive method the bumper surface is treated instead of the blood. This is done with the idea to design a device that can run on untreated blood and hence be more efficient and more robust. The project is more of an observational project than trying to quantify anything. Documentation of how cells and blood behave in the bumper is the first step towards a functional bumper in clinical applications.

6.1 The Bumper

The Bumper array relies on particles restricted by certain streams. The geometric pattern of the post and the laminar flow creates certain flow-lines. A bifurcation is created by shifting the alignment of the posts from row to row and hence creating a separation among different sized objects.

6.1.1 The design

The bumper has a simple design. It can be made in any material suitable for microfabrication, and for biological separation PDMS is the material to prefer. The device is made up by two plates. One plate, the lid, has inlet holes, outlet holes and reservoirs. No microfabrication is needed on the lid. The bottom contains the actual channels. The pattern of the devices needs to have inlet and outlet channels, and between these there is a main channel, see figure 6.1. The placement of the inlet and outlet channels is not so important, though there

are a few points to consider when designing them. They need to have long enough dimensions to have a sufficiently high flow-resistance, higher that the resistance of the main channel, to prevent backflow. The inlet channels should be constructed in such a way so that the sample channel is centered and the buffer channels are giving a laminar flow. The design of the outlet channel is not so important. Another thing to keep in mind when designing the bumper is to place the inlet and outlet holes so that the assembly of the device is facilitated. The core part of the bumper is the main channel. It consists of a wide channel filled with evenly spaced posts. This is where the actual separation takes place. Depending on the different size of the cells, they will be restricted to follow different streams.

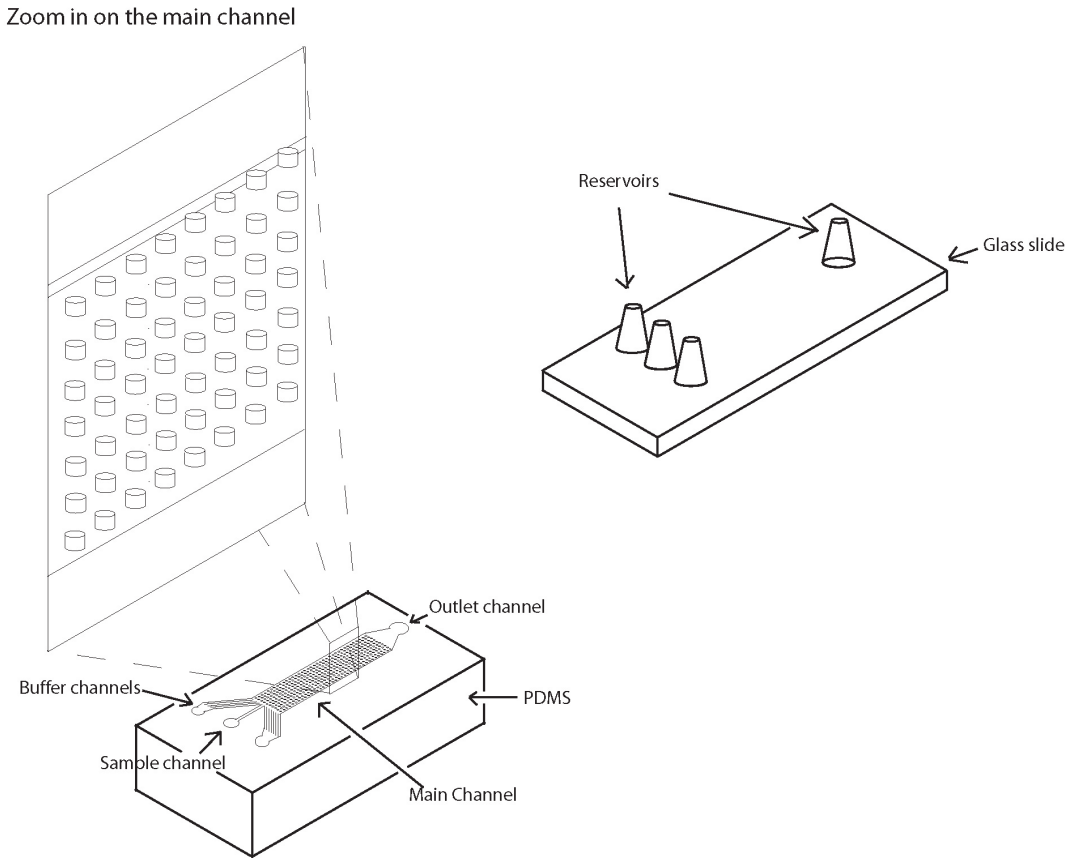


Figure 6.1: A schematic drawing of the bumper. Here you can see the glass lid with the reservoir and the PDMS slab with the channels

6.1.2 The deterministic lateral separation

The physics behind the separation follows the general physics of microfluidics. The flow is limited to laminar flow and the separation is solely determined by the size of the particles.

Geometry of the separation

The laminar flow will give a characteristic flow scheme in the Bumper. The periodicity of the row shift will induce certain streams to form. The geometrical constraint together with the laminar flow will lead to bifurcating streams. If N is the periodicity, defined as the total lateral shift after one period divided by the lateral shift after one row, the gap can be divided into N streams. Each stream takes it start in the center of a post and after one period end up at the center of another post. At each new row the stream position will have shifted one step to the side in the opposite direction of the shift. If we define the stream 1 to be the stream closes to the post, it will after one row have position N and at the next row have position N-1. After N rows, or one period, it will be back at position 1. General fluidics gives that the fluid flux is equal in each such stream. But since of the parabolic flow profile, there will be a variable width of each stream. The reason behind the separation is that, larger particles are restricted by their size. When a particle is in a stream it wants to follow the stream's fluidic path. Say a particle in stream position 1 will want to go to stream position N after one row and to stream position N-1 after two rows, and so on. Ending up in stream position 1 after one period, without any lateral displacement. But if a particle has a radius larger than the width of the first stream it will be forced to be follow to the stream 2. So for a large particle in stream position 1 it will follow stream position 2 instead of 1. But at the next row stream position 2 has moved to stream position 1 and we are back to were we started. The particle will never change stream position and hence be shifted at each row. The effective lateral displacement is after one period equals the total shift of the rows. This displacement is called "bumped" and from here comes the name bumper array.

Fluid flow in a bumper

The most important feature of the flow in the Bumper is that it is laminar. Without the laminar flow no quantitative separation can be made. The principle behind the turbulence is of extreme complexity but thanks to the dimensions of the device, there is no concern of giving turbulence. A god measure of turbulence is the Reynolds number. It serves to measure the ratio of the inertial forces to the viscous forces. At low Reynolds numbers the fluid dynamics are dominated by viscous drag rather than inertia, this is to say more laminar flow. If one wants to work in the laminar region a Reynolds number below 30 is desirable, turbulent flow is achieved with a Reynolds number above 2300, although laminar flow have been reported in as high Reynolds numbers as 10^5. In the design used

in the project the Reynolds number is typically around 1[12]. The flow in a microfluidic device is governed by the Navier-Stokes equation[43].

$$\rho\frac{\partial v}{\partial t} = \nabla P + \rho f \tag{6.1}$$

This is the general form of the Navier-Stokes equation, here ρ is the fluid density, v is the velocity and f is the body force vector. The tensor P represents the surface forces applied on a fluid particle[43]. The Navier-Stokes equation is basically Newton's law for a fluid, with forces per unit volume on the right hand side due to pressure gradient and viscosity. The left hand side gives mass per unit volume, ρ, times the acceleration of the fluid, expressed in terms of connective derivate in a Eulerian representation. The Navier-Stokes equation is a powerful equation, it determines the fate of every fluid-component. Though the equation can be simplified by making some cleaver assumption, the equation holds an enormous amount of complex information in it. The fact that there is a prize for a million dollar for the one who can prove the existence of its solution and its smoothness, shows you how complicated it is(and this is just the existence of a solution, not the solution itself)[3]. But as said one can make some assumptions and use it in practice. There are also a lot of people making computer simulations on the microfluidics using the Navier-Stokes equations. Graduate student Martin Heller at DTU in Bruus Group has made simulations of the Bumper array using FEMLAB[22]. These simulations are made for a 2D bumper array with solid spheres, see figure 6.2. Though the real situation is a complicated 3D world and the cells are not perfect spheres, some information can be drawn from the results.

Particle separation

The critical size of the separation is given by the width of the first stream position. It was derived in a recent paper by Inglis[24]. It assumed that the flow will have a parabolic flow profile. The flow as a function of x can be written as,

$$u(x) = \left(\frac{g^2}{4} - \left(x - \frac{g}{2}\right)^2\right) \tag{6.2}$$

where g is the distance between the posts, see figure 6.3. Taking the starting point in the fact that the fluid flux is equal in all segments gives the condition

$$\int_0^\beta u(x)dx = \epsilon\int_0^g u(x)dx \tag{6.3}$$

where β is the distance to the first stream from the post and ϵ is the shift in every row. With the assumption that the flow at the post wall is zero, it is possible to solve the integral expression for β as a function of ϵ. One gets

$$D_c = g\left(1 + 2w + \frac{1}{2w}\right) \tag{6.4}$$

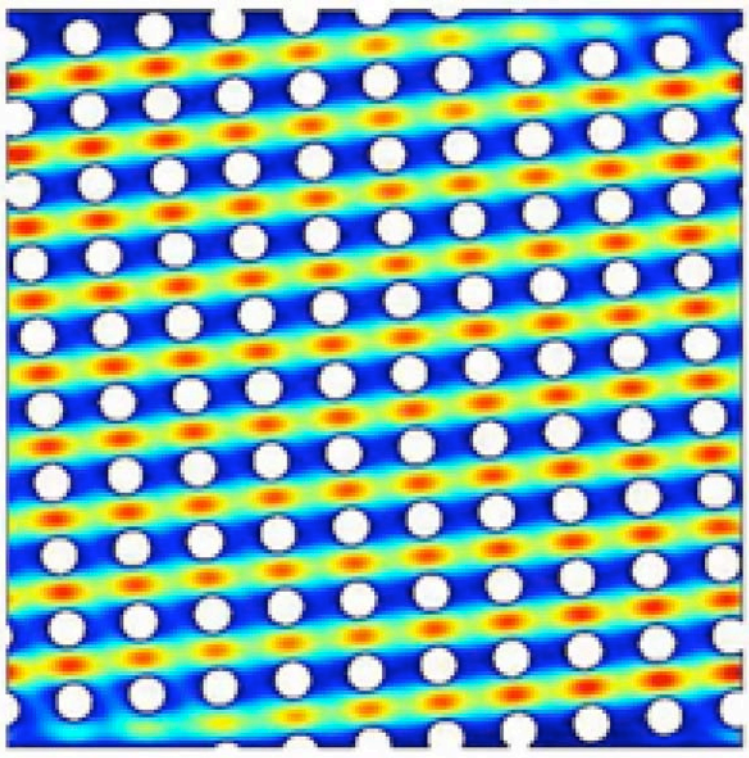


Figure 6.2: Femlab simulation of the bumper array made by Martin Heller[22]

where w is given by

$$w^3 = \frac{1}{8} - \frac{\epsilon}{4} \pm \sqrt{\frac{\epsilon}{16}(\epsilon - 1)} \tag{6.5}$$

This gives the width of the first stream position. The desired critical radius is then 2β. A somewhat simpler calculation of the critical radius is given by Tegenfeldt[12]. He determines the critical size to be

$$D_c = \frac{2g\alpha}{n} \tag{6.6}$$

where α is a correction factor approximated to

$$\alpha \approx \sqrt{\frac{n}{3}} \tag{6.7}$$

The fact that the cut-off is inversly proportional to the period length can be used to increase the gap size without altering the critical radius. This has been confirmed by unpublished results made by Asker Vig Larsen and Jason Beech.

6.2 The bumper without the blood

The major work done on the bumper has been made by two groups. First Robert H. Austin's group at Princeton University, who developed the method, and by Jonas Tegenfeldt's group at Lund University were this thesis was made. Austin's group has published two papers on separation using the Bumper Array. They have focused on latex beads trying to decrease the sizes of the particles being separated. The results published have shown remarkable sensitivity. They were able to separated microspheres of 0.8, 0.9 and 1.0 μm in 40 s with a resolution of roughly 10 nm. They also managed to separate bacterial artificial chromosomes in 10 minutes with a resolution of roughly 12%[7][23][**?**].

6.3 Blood-in-a-bumper experiment

When choosing a design for the bumper, see figure **??**, the goal was to separate erythrocytes from leukocytes and separate activated lymphocytes from inactive lymphocytes. With this in mind the design was chosen such that the critical diameter would be 6 μm, 10μm and 16 μm. The 6 μm bumper was made to be able to separate the erythrocytes and the leukocytes. The erythrocytes have an average diameter of 7 μm but previous results have shown that the effective diameter is less, though the exact effective diameter is not know. The leukocytes have different diameters but the smallest leukocyte are the lymphocytes with a diameter of roughly 10 μm, the leukocytes are also anticipated to have a somewhat smaller diameter but the actual number is not known. The 10 μm bumper is designed to separate the population of leukocytes. The diameter of the leukocytes ranges from 10 μm for the lymphocytes to roughly 20μm for the Monocytes. The third design, the 16μm bumper, is design to separate activated lymphocytes from inactivated. As stated in the chapter on leukocytes, the lymphocytes will upon activation increase their cell plasma to twice the normal volume, from 10 μm to 20 μm. The bumpers were meant to be fabricated at Riso in PMMA with injection molding. The blueprint of the bumper arrays were made to fit on an area of 3 x 3.5 cm, so fit into their injection molding machine. A silicon wafer negative was also produced to be able to make PDMS replica molding. The bumpers were bonded to a glass slide with reservoir holes made by sandblasting. The reservoirs were made out of pipette tips. There was also a lymphocyte isolation experiment, to get a higher concentration of lymphocytes. When trying to separate cells in general and blood in particular, you introduce some new problems to the experiment. Instead of having a nice set of beads with two distinct sizes, you now have to deal with a thick smear of cells in many different shapes and sizes. Another problem is that even though the cells you want to separate differ by a significant amount in size, other effects can make the cells indistinguishable when run through the Bumper. Such effects can be surface effects, rigidity effects and shape effects. Cells are not a solid spheres nor are they homogeneous lipid vesicles, it is a living unit. The cell can actively adhere to surfaces, by either surface proteins or by making an adhesion

cup, much like a suction cup. The rigidity of the cells differs a lot between different cells. The rigidity how the cell is governed by the internal cytoskeleton and different cells has different cytoskeleton. For example the flexibility of the erythrocyte is much higher than the leukocytes. The theory of the separation is based on the assumption of spherical particles. The cells need not be spherical, a good example of this is the erythrocytes bifocal disk shape. Also the fact that in the blood there are a lot of extra cellular proteins. These proteins function in the body is to create a web for the platelets to adhere to when stopping a wound.

6.4 Blood clot formation

When running blood through the bumper there is an extensive formation of blood clots. The physiological reason behind the clot formation has two separate explanations. There is a passive creation of clots and an active. These two phenomena are treated separately. Clot formation is an important feature of the blood. Without it we would die from bleeding. The clotting process in vivo is a complex system of protein interactions. Though the knowledge of blood clots is old, the exact process of their formation in vivo is not fully understood. Blood clotting and dysfunctioning blood coagulation lead to many disease, and the research of blood coagulation is a very hot topic.

6.4.1 Active clot formation

The clot formation in vivo or thrombosis as it is also called is a common disorder which effects society with health and economic issues. There are several different types of thrombosis, but the three most important are coronary arterial, cerebrovascular and deep venous thrombosis (DVT). Coronary artery thrombosis results in myocardial infarction, sudden death and other acute coronary events. Cerebrovascular thrombosis causes ischemic stroke and the devastating squeal of stroke. DVT may lead to life-threatening pulmonary embolization (PE) and infarction. Collectively, arterial and venous thromboses have the highest morbidity and mortality. Thrombosis was discovered as early as the beginning of the 19th century. Virchow postulated that thrombosis is initiated by blood stasis, vascular wall damage and blood cell activation. The discovery of the Platelets function in thrombosis came in the end of the same century. In the 20th century a lot of progress was made in the understanding of the molecular aspects of thrombosis. Importantly, the tissue factor pathway of coagulation was identified as the physiological pathway leading to hemostatic plug formation following blood vessel damage. Today the concept of atheromatous plaque rupture being the mechanism behind arterial thrombosis has become the accepted general theory. For the study of the factors behind all different thrombosis the usage of molecular biology and recombinant DNA techniques have provided clinical investigators powerful tools. Mutations or polymorphisms of several genes have been unequivocally established as risk factors for DVT and PE. Results from

genetic analysis provide valuable information regarding the crucial role that naturally occurring inhibitors play in defense against thrombus formation. The explanation of the reason for arterial thrombosis has not progress as fast as it has been for DVT and PE, this is due to the fact of the complex mechanism behind the conditions.

Molecular aspects of active clot formation

Arterial Thrombosis

When platelets are activated a chain of reactions begins. The initiator for this event is the exposure of the blood to extravascular tissue. In the extravascular tissue there are collagen and von Willebrand factors(vWF). The coagulation is initiated by monocyte surface TF. The surface TF comes in contact with factor VIIa following the rupture of the endothelium. After this several steps are followed and in figure 6.5 the schematics of the process is displayed. On the left in the figure is the process of platelet aggregation and on the right is the coagulation process. In short the process of platelet aggregation is begun with the platelet adhesion. This is the process when collagen attracts the platelets to the site of the ruptured plaque. The platelet has a surface glycoprotein complex, GPI_b-IX-V, the interaction between this protein and vWF results in attatchment of single layer of platelets to the plaque surface. This process is called platelet aggregation. This inding of platelets to collagen results in platelet activation. The platelet activation starts with the contraction of the intracellular cytoskeleton and release of intrcellular granules, a process called release reaction. This is lead by an activation of arachidonic acid metabolism with the generation of thromboxane $A_2(TXA_2)$. Next, the glycoprotein makes a conformational change, making them receptive to fibrogen binding. Also, the membrane phospholipids makes a rearrangement to facilitate binding of coagulation factors such as Va and VIIa.

Deep Venous Thrombosis

The underlying reason for Deep Vein Thrombosis(DVT) is not fully understood. The most accepted guess is that it is related to blood stasis. Studies on the actual thromos reveals a predominant composition of fibrin, suggesting that coagulation activation and fibrin formation are largely responsible for DVT. Unlike Arterial thrombosis, is there no evidence for a TF-mediated initiation. There are theories that the endothelial cell in DVT express TF in respons to blood stasis. Another explanation could be activation through the contact of coagulation factor, i.e. factor XII. Factor XII initiates factor XI that activates factor IX via factor XIa. This sets off a coagulation cascade seen in figure 6.6. Platelet aggregation plays a secondary role in DVT.

6.4.2 Passive clot formation

Blockage of the channel can also occur due to other effects than the mechanisms behind thrombosis. The cells and especially the lymphocytes are capable to adhere to the surface. The process of cell adhesion is complexed and not fully understood. It has been showed[39] that the cells can actively form binding with a ridgid surface. This is mediated through actions of the cytoskeleton. The contractile machinery of the cell give rise to a large stable junction, termed focal adhesions(FA)[39]. These sites are formed when a focal complex(FX) present at the cell membrane is subjected to stress. This has been proved in vitro[56]. These FA function as anchor sites and can endure physical stress[39]. The time developement of the FA sites are not fully determined, but the cell need a adhesion time to be able to stick to a surface. Another effect in flow is the random formation of blockage in a channel with flow. Several reports have published results that blockage can form spontaneously in the channel by particles lining up in the flow profile and getting stuck. Much like clogs is formed in a silo even though the grains are as tiny as a few millimeters in a container with a diameter of several meters. Sharp et al have even reported the surprising result that these clogs can form even if the concentration of particles is low. For the Bumper array both these effects are probably present. The time formation of the FA's are important. In the pressure driven bumper the slow velocity at the walls will give the cells time to form these adhesion sites. This could be solved by using electroosmosis as the driving force.

6.4.3 PLL-PEG

The PLL-PEG coating material consists of two distict components. A poly-L-lysine anchor and PEG chains[46], see figure 6.7(a) for the molecular structure of the complex. It has been shown before[25][51] a PLL-PEG coated surface inhibits activation of platelets as well as imporved antiadhesion properties.

6.4.4 Results from clogging experiments

In the initial test of the bumper with whole blood, the time for clog formation was in the order of seconds, see figure 6.8. To improve this we treated the surface of the bumper with PLL-PEG. A complete study of the importance of surface treatments as well as blood treatments were conducted. The experiment was designed to be able to compare blood treatments and surface treatments. We tested blood treated with Heparin compared to untreated blood in bumpers with untreated surface compared to PLL-PEG treated surface. To treat the blood with heparin the blood was directley dispensed into a vacuum tube with heparin coated walls. This is standard procedures used in the medicine to take blood from patient without coagulation in the tube[45]. There was no significant difference from these measurements as compared with the untreated blood. The difference was that it took a little longer time for the clog to form but over a long period of time the same results were obtained as for the untreated blood. For

the PLL-PEG treated blood the results can be seen in figure 6.9(a)and 6.9(b). As can be seen a significant decrease in clog formation and an increased device life-time can be seen when comparing PLL-PEG treated bumpers to untreated bumpers.

6.4.5 Results of separation

In the untreated bumper no separation results were obtained due to the severe clogging as described in the previous paragraph. In the PLL-PEG treated bumper there was a separation of lymphocytes from erythrocytes. In the PLL-PEG bumper the running time was long enough to detect a separation. In figure 6.10 one such separation were the bumper was run with untreated blood for 10 min. As can be seen the two streams that develop, one bright with the erythrocytes and one with three bands of lymphocytes. The reason for the broadening is due to an small initial plug at the first row. The count was that during these 10 min, 13 lymphocytes were separated from the erythrocytes. Two lymphocytes were not bumped and hence and a size below the cut-off and 4 showed a mixed behavior, their size is probably right at the cut-off. In figure 6.10 1000 frames have been collected and stacked using ImageJ. The max intensity of this stack is pictured.

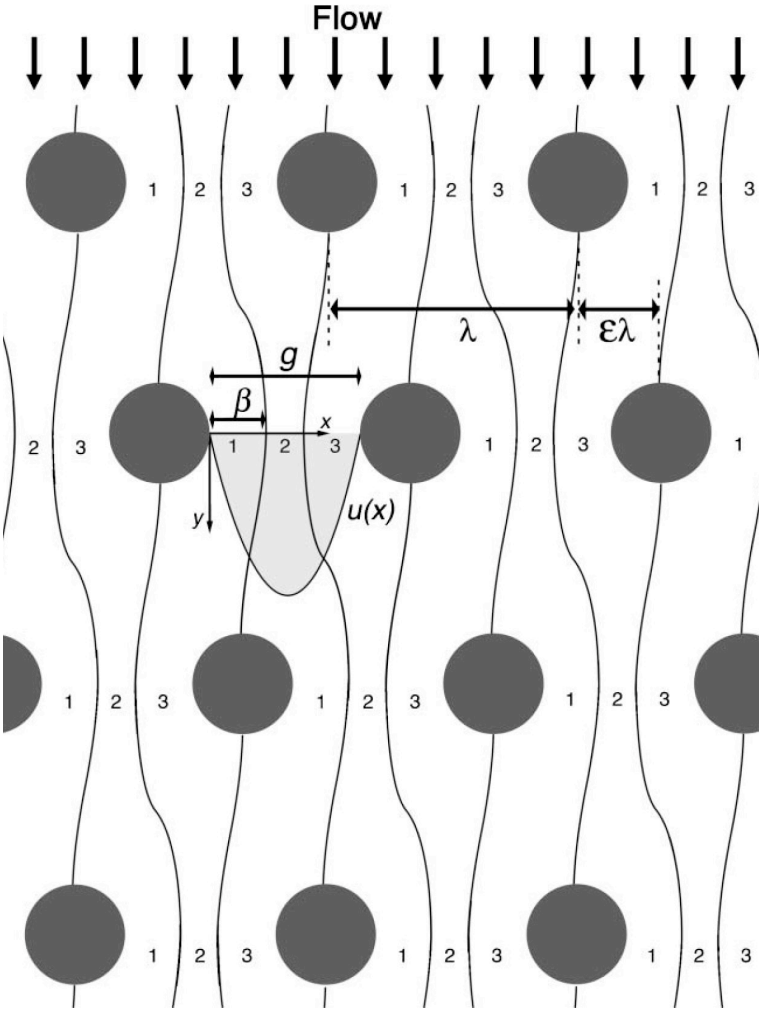


Figure 6.3: Stramlines in low Reynolds number flow

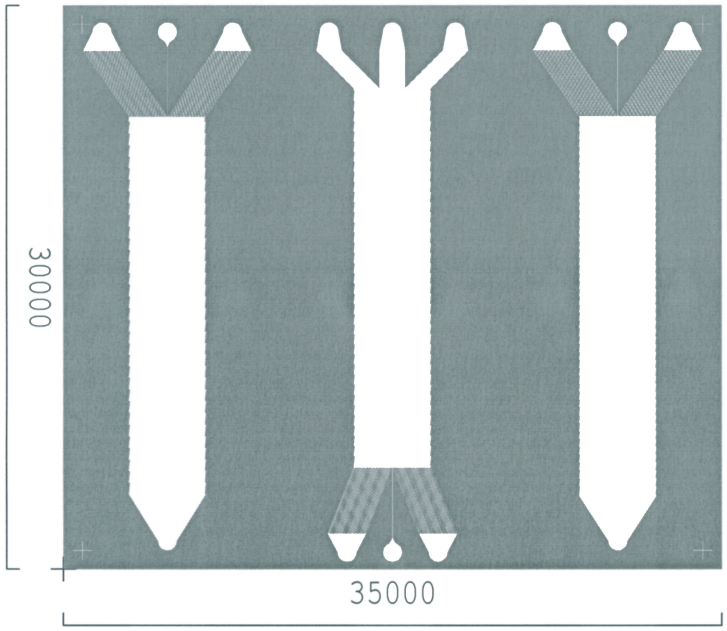


Figure 6.4: The layout of the bumpers on the master

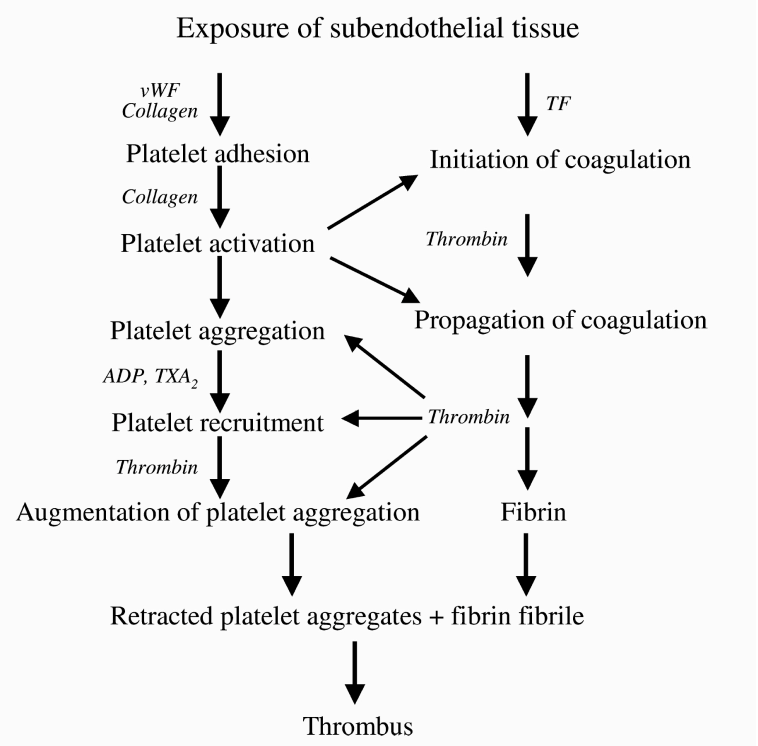


Figure 6.5: The reaction path of the arterial thrombosis

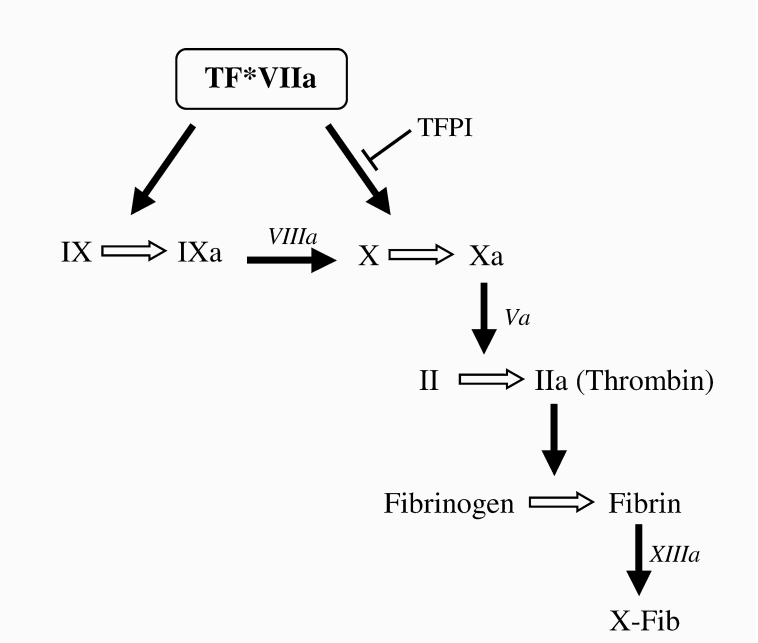
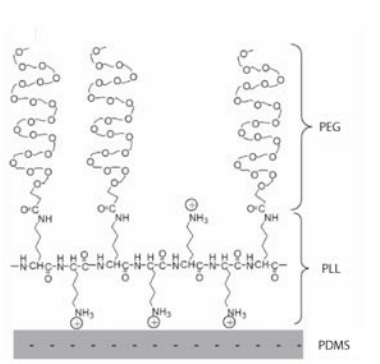
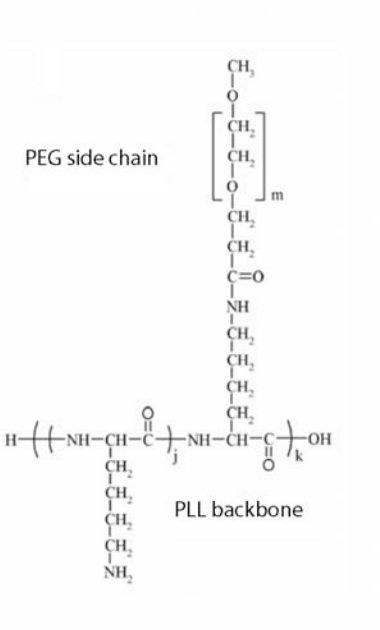


Figure 6.6: The coagulation cascade in thrombosis



(a) Molecular structure of PLL-g-PEG copolymer



(b) scheme

Figure 6.7: a)Molecular structure of PLL-g-PEG copolymer. b)Idealized scheme of the interfacial structure of a monolayer of PLL-g-PEG adsorbed on a PDMS surface via electrostatic interactions[8]

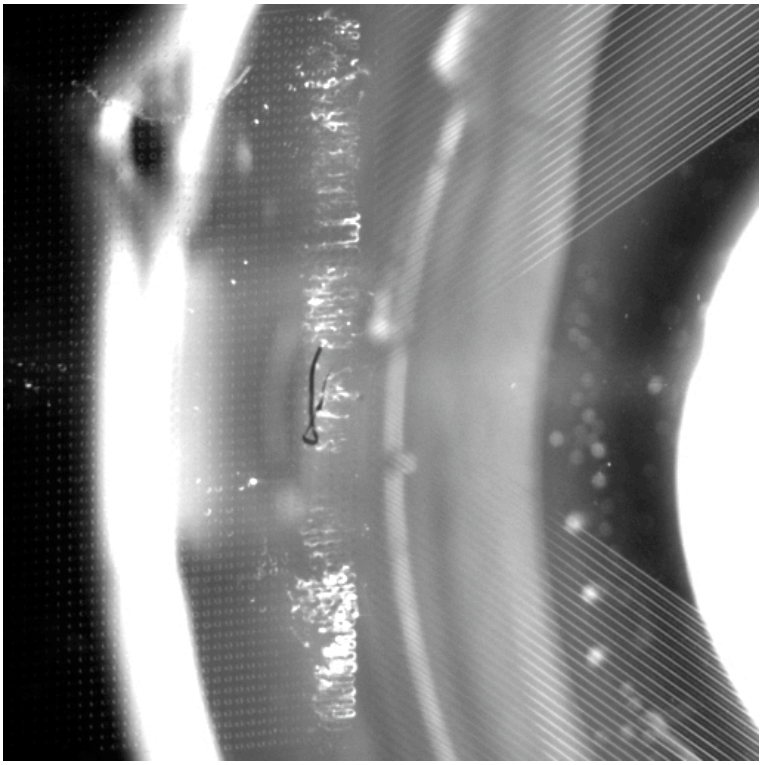
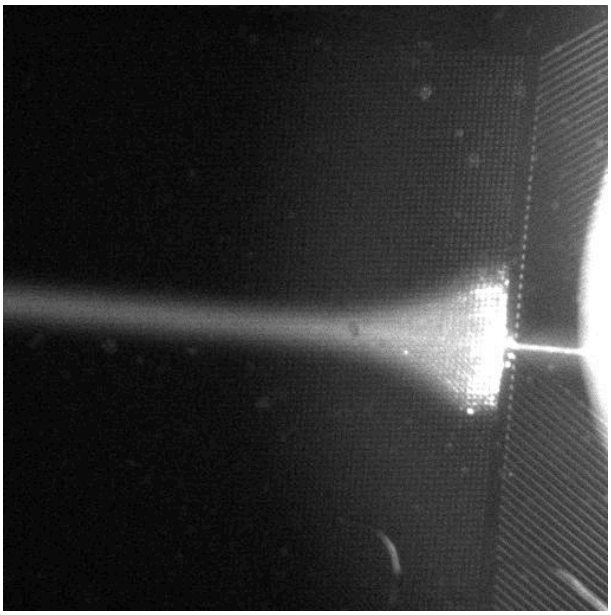
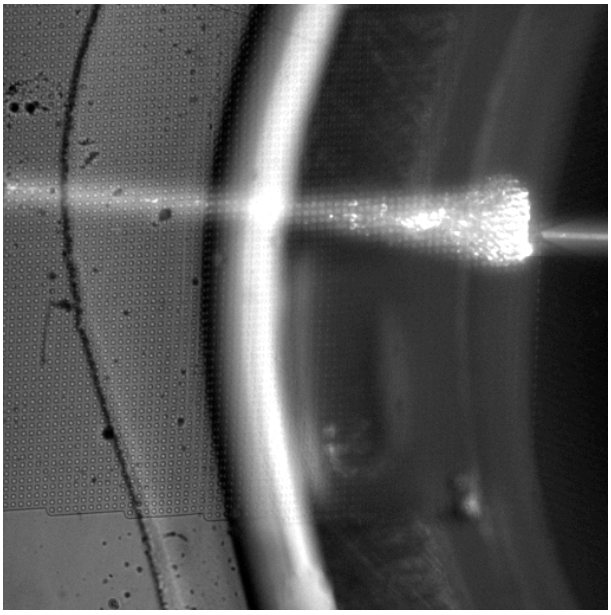


Figure 6.8: Untreated bumper with untreated blood 10x, The bumper is the 16 um bumper with lymphocyte enriched blood and 28 nm beads to show fluorescens. The picture is taken 5 min after start.



(a) Untreated Blood



(b) Heparin treated blood

Figure 6.9: a) Picture of a 6 um bumper treated with PLL-PEG with untreated blood. b)Picture of a 6 um bumper treated with PLL-PEG with heparin treated blood. Both pictures were taken after 5 min and with 4x

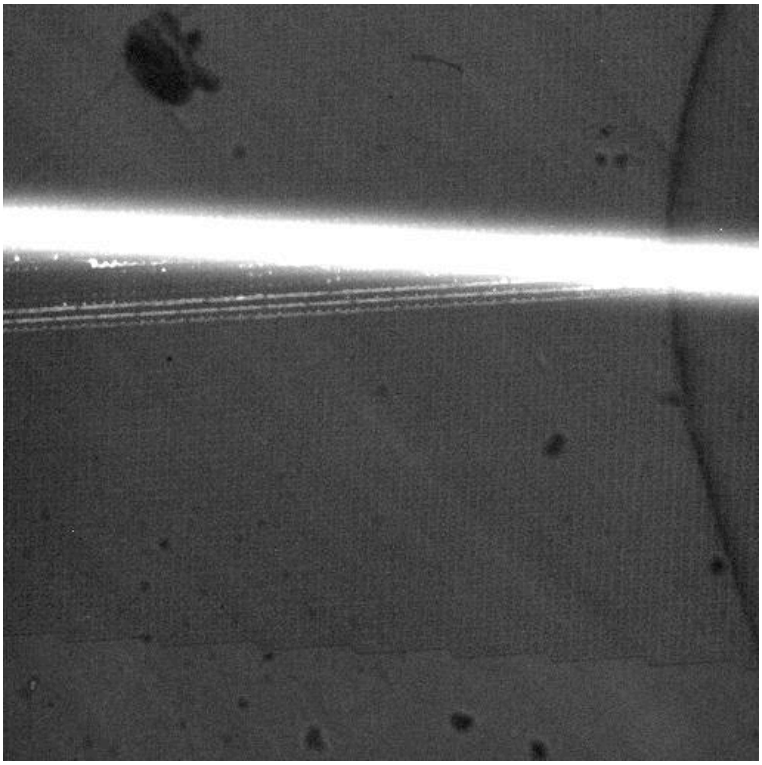


Figure 6.10: This picture shows the separation of lymphocytes from erythrocytes. The image is a fusion of 1000 frames and shows the max intestiy. Here the cells are stained with 28 nm beads

Chapter 7

Conclusion and final remarks

During this project new light has been shed on the difficulties on separating cells. There is a desire among researchers and physicians a like to develop new devices and techniques to be able to analyze biological matter in a swift and precise matter that is cost efficient and easy to use. Today's techniques, foremost the FACS, can analyze a sample with a enormous speed and accuracy. The FACS short having is the fact that it requires highly trained personal and is very expensive. The prospect of a point-of-care device that can be used with no specific training and at the same time capable of making the most advanced analyzes, could revolutionize health care in the third world countries. In this project a device called the bumper array has been tested for separation of biological matter. The device has shown promising results on non-biological particles. There are also published data on intial separation of cells. In order to apply the device on cells, tests needs to be taken to overcome the problems introduced by the cells compared to beads. First of all understanding and controlling of the clot formation needs to be achieved. In this project a study of the influence antithrombotic drugs and surface treatment have been studied. Some promising result, show less clotting when treatments are applied. More investigation needs to be done in order to optimize the conditions. But PPL-Peg and heparin combination seems promising. Other improvements that needs to be done concern the design of the device. The theory of the critical size is established for beads and solids spheres, in order to be able to make any qualitative research the theory needs to be extended to include soft condensed matter such as cells. The radius of the cell is not constant, contrary to the beads. The fact that the cells can squeeze makes important to design the device to increase the period of the post to be able to decrease the space between the posts and at the same time decrease the critical size of the bumper. The increase in space between the post will render in less clotting. The clotting is a severe problem for the bumper. In order to be able to separate whole blood it needs to be able to have a long running time, the clot formation will set the limit for the running time. For this study the clot formation appeared instantly for the non-treated bumper and after a minute for the bumper treated with heparin and

PLL-Peg. There are great advantages of this method compared to the previos method reported to overcome the problems of sticking. By treating the bumper you remove the need of blood handeling. An important factor considering the forseen use of the bumper as a point-of-care device that can be handled by an untrained person with no necessary extra lab equipemnt. Other factors are brought up such as design alterations. The increase of the period would lead to larger gap size with out altering the critical radius and could thus reduce the clogging.

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