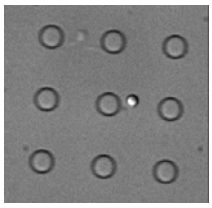
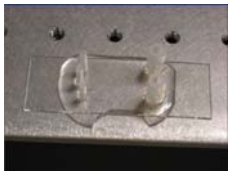
Cell synchronization using deterministic lateral displacement

Hanna Nicklasson

Bachelor Thesis
Spring 2007
Department of Solid State Physics
University of Lund

Supervisors (physics): Jonas Tegenfeldt and Jason Beech
Division of Solid State Physics
Supervisors (biology): Kersti Alm and Veronica Johansson
Department of Cell and organism Biology





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Acknowledgements

This project has been an exciting experience even though there have been several frustrating moments. I would like to take this opportunity to thank the people that have helped me in all kinds of ways.

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I would also like to thank my bio supervisor Kersti Alm and her doctoral candidate Veronica Johansson, both at the Department of Cell and Organism Biology, for providing me with cells and giving me instructions on how to handle and study them.

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At last I would like to thank all the nice people on the department who has been helpful with smaller things like showing me where to find different things in the laboratory.

Abstract

The point with this research was to synchronize cells with the help of a micro device called a bumper array. A bumper array is a micro channel with obstacles (posts) arranged in a certain pattern. This special arrangement of posts will make a fluid flowing through the micro channel divide into different paths. If particles of different sizes are suspended in the fluid they will separate according to their size because particles with different sizes take different paths as the fluid streams through the bumper.

Since this is a rather wide research area and the work was supposed to be done in 10 weeks, the focus was put on three experiments. The first one was to find out whether the cells would clog in the device or not. The second one was to test different flow rates to see how fast the separation could be done without harming the cells. The third one was to test the device and analyze the result to see if it really managed to separate the cells.

The results were not as expected, mainly because of problems that occurred during the experiments. There was a lot of clogging, especially in the tubes that led the cells into the device, which made the final cell concentration lower than expected. Some cells did survive the higher rates and they might be enough to culture some new colonies. But thread-like formations were accumulating in the device at higher flow rates and cells were caught in these threads. The threads were not studied further but it seems likely that the threads were DNA from burst cells. This would indicate that higher flow rates are not suitable for cells, unless something can be done to prevent the DNA from accumulating. The third experiment showed that cells with different sizes were actually separating in the device. However, no analysis could be made since one of the outlets had collapsed, which disturbed the flow at the end of the channel.

Populärvetenskaplig sammanfattning

Syftet med det här projektet var att synkronisera cellers tillväxt med hjälp av en specialdesignad mikrokanal som innehåller en massa små hinder. Hindren är arrangerade på ett speciellt sätt i ett mönster, vilket gör att vätska som flödar genom kanalen delas upp i skilda delflöden som tar olika vägar genom kanalen. Om partiklar med olika storlekar befinner sig i vätskan så separeras de efter storlek, beroende på att olika stora partiklar tar olika vägar.

Eftersom detta är ett ganska stort forskningsområde och arbetet skulle vara klart på 10 veckor, så lades fokus på tre experiment. Det första var att undersöka om cellerna fastnar i mikrokanalen eller inte. Det andra var att testa olika flödeshastigheter för att se hur fort separeringen kunde genomföras utan att skada cellerna. Det tredje var att testa själva mikrokanalens förmåga att separera celler och analysera resultatet.

Resultaten blev inte som förväntat. Huvudsakligen berodde detta på olika problem som uppstod under experimenten. Ganska många celler fastnade, speciellt i slangen som ledde cellerna in i kanalen, vilket gjorde att den slutliga koncentrationen blev lägre än avsett. Några celler överlevde de högre hastigheterna och dessa skulle kunna vara tillräckligt många för att odla nya kolonier. Men trådliknande formationer ansamlades i kanalen vid högre hastigheter och celler fastnade i dessa trådar. Trådarna undersöktes inte närmare men det verkar troligt att trådarna var DNA från spruckna celler. Det här skulle betyda att högre flödeshastigheter inte lämpar sig för celler, om inte något kan göras för att förhindra att DNA ansamlas i kanalen. Det tredje experimentet visade att celler med olika storlekar faktiskt separerades i kanalen men ingen analys kunde göras eftersom en av utgångarna hade kollapsat, vilket störde flödet i slutet av kanalen.

Contents

1 Introduction

When cells grow they pass through the cell cycle. A cell cycle has different phases in which different events of the cell development take place [1]. Unfortunately, it is difficult to measure chemical events in single cells. Therefore it is important to be able to synchronize cells so that they grow simultaneously. This makes it possible to study a whole cell culture in the same phase, which is much easier [2].

The purpose of this work is to synchronize cells mechanically with the help of a device called a bumper array. Bumper arrays are micro channels that contain small obstacles (posts) arranged in a certain way so that laminar flow around them will separate particles of different sizes.

This report includes a presentation of the fabrication of bumper arrays, the experiments, the results and some discussion. To begin with, some background theory will be presented.

2 Theory

2.1 The Cell Cycle

To understand why it is important to synchronize cells and why it is so problematic, it is important to know a little about the cell cycle.

The cell cycle starts with a "newborn" cell right after a cell division and ends with another cell division of the fully grown cell, see figure 1. The cell cycle is divided into two main phases; the interphase and the mitotic phase (also called the M phase) [1].

The interphase is the phase between cell divisions and is divided into three phases called G1, S and G2. The "S" stands for synthesis of DNA, which means that this is the phase where DNA replicates. The "G" stands for gap. That is, the G-phases are time gaps between the M and the S phase. The cell continues to grow in size throughout the whole interphase [1].

The M phase consists of two overlapping events; mitosis, which is the division of the nucleus and cytokinesis, which is the division of the cytoplasm. The mitosis is, in turn, divided into different phases [1], but that is not essential for the understanding of this report.

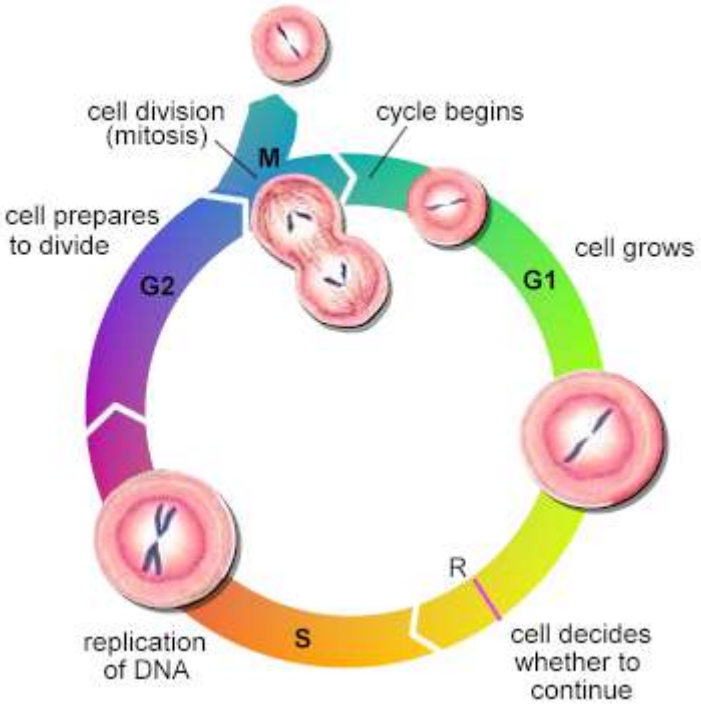

Figure 1. *The eucaryotic cell cycle. Image from http://teachline.ls.huji.ac.il/72373/substance_x/q2a.html*

2.2 Synchronization of Cells

For many years now, scientists have tried to find ways to synchronize the growth of cells to make it possible to study cells in a particular phase. Many different methods have been tested and some have appeared to work better than others.

For example, a chemical substance called Nocodazole has been used to arrest cells at a certain point in the cell cycle. When the Nocodazole inhibitor is knocked out the cells are supposed to be synchronized and continue throughout the cell cycle together. This is a method called a whole-culture method, which means that all the cells in a culture are treated [2]. However, a couple of years ago Stephen Cooper and his co-workers implied in an article [2] that Nocodazole does not synchronize cells. In fact they implied that none of the whole-culture methods synchronize cells. According to Cooper the cells might get a certain property in common, such as DNA content, when synchronizing with a whole-culture method but they will not be synchronized since they do not have all their properties in common. They might for instance have very different sizes [2].

Clearly, these whole-culture methods are not that good for pure synchronization. For that purpose, the methods that Cooper calls selective methods [2] seem better. Selective methods are those where a certain part of the cell culture are sorted out. The method that has been used in this work is a selective method.

2.3 Microfluidics

Microfluidics is: "The science and engineering of systems in which fluid behavior differs from conventional flow theory primarily due to the small length scale of the system." [3].

2.3.1 Laminar and Turbulent Flow

In microfluidics there are two possible modes of flow, namely laminar flow and turbulent flow, which occurs under different circumstances [4]. When the liquid follows distinct paths, streamlines, through a micro system the flow is said to be laminar. All fluid elements that enter the micro channel at the same point will follow the same streamline through the whole system. When the flow velocity is raised enough the streamlines start to alter and turbulence is introduced into the system. The flow is turbulent. At what point the fluid reaches turbulent flow depends on different parameters like the flow rate, the design of the micro channel, the fluid density and the fluid viscosity. To determine whether the flow is going to be dominated by laminar or turbulent flow in a certain micro channel, the ratio between the inertial and viscous effects can be considered. This ratio is called Reynolds number.

$$Re = \frac{\text{Inertial effects}}{\text{Viscous effects}} = \frac{D_H \rho < v >}{\eta}$$

where Re is the Reynolds number, D_H is the hydraulic diameter of the channel, ρ is the fluid density, $<v>$ is the flow velocity and η is the viscosity [4].

When the inertial effects dominate over the viscous effects the flow is turbulent [4]. As a rule of thumb, if Reynolds number is higher than 2000 the flow will be turbulent. When it is lower than 30 the flow is laminar. If the Reynolds number would lie between 30 and

2000 the flow can be either laminar or turbulent depending on the geometry of the micro channel [4].

2.3.2 Diffusion

When the flow in a system is laminar, the only mixing that occurs between streamlines is that due to diffusion [4]. Diffusion is a relatively slow process where the molecules move randomly due to collisions between them. The collisions are caused by their thermal energy, which makes them move. The mean square of the distance d a particle can travel in one dimension in time t is dependent on the diffusion coefficient, D.

$$< d^2 >= 2Dt$$

The diffusion coefficient can be calculated with the Stokes-Einstein equation.

$$D = \frac{k_B T}{6\pi\eta R_H}$$

where k_B is Boltzmann's constant, T is the temperature, η is the viscosity and R_H is the hydrodynamic radius of the particle. The Stokes-Einstein equation also indicates that larger particles have a slower diffusion rate. For the purpose of this work diffusion between streamlines is not a desirable phenomenon since it will lower the resolution. A good thing is that eukaryotic cells are relatively large particles, which means that they have a low diffusion coefficient. Nevertheless, the diffusion has to be minimized. To judge the effect of diffusion the Péclet number could be of good use.

$$Pe = \frac{\text{Advective rate}}{\text{Diffusive rate}} = \frac{\frac{v}{d}}{\frac{D}{d^2}} = \frac{vd}{D} = \frac{< d^2 >}{\Delta x^2}$$

where v is the flow rate, d the characteristic dimension of the array, and D the diffusion coefficient [4]. A high Péclet number indicates low diffusion. In our case a Péclet number as high as possible is desirable.

2.3.3 Parabolic Flow Profile

Due to stick-boundary conditions the flow in micro channels is parabolic, see figure 2. The fluid velocity is zero by the channel walls and it is at its maximum in the channel center [4].

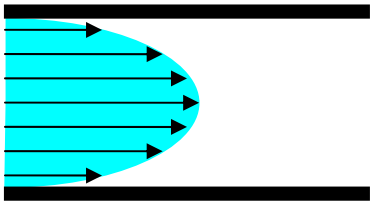

***Figure 2**. A schematic picture of the parabolic flow in a micro channel.*

The reason for this is the polar nature of the molecules in the wall and in the liquid. If both the wall molecules and the liquid molecules are of a polar nature, the molecules closest to the walls will stick to the walls due to polar forces. These forces may however be of different sizes depending on the molecules. This means that the velocity is not necessarily zero at the channel walls. The flow profile is still parabolic, but the "edges" of the parabola lie outside the channel walls, see figure 3.

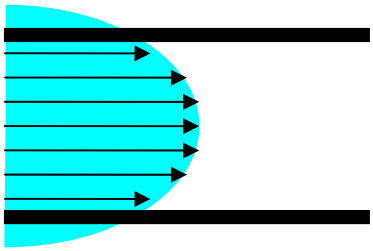

***Figure 3**. A schematic picture of the parabolic flow in a micro channel where the flow rate is not zero at the channel walls.*

2.4 Microfluidics in Bumper Arrays

2.4.1 Laminar Flow in Bumper Arrays

The purpose of this work was to try to synchronize cells with bumper arrays. A bumper array is a device that can separate small particles like micro beeds, molecules and cells with the help of laminar flow. A bumper array consists of many small obstacles (posts) in a micro channel. The posts are arranged in rows in the channel and each row is laterally displaced with respect to the previous row [5]. When liquid flows with laminar flow through the channel it will follow different streamlines. What streamline it follows depends on where it flows into the channel, see figure 4.

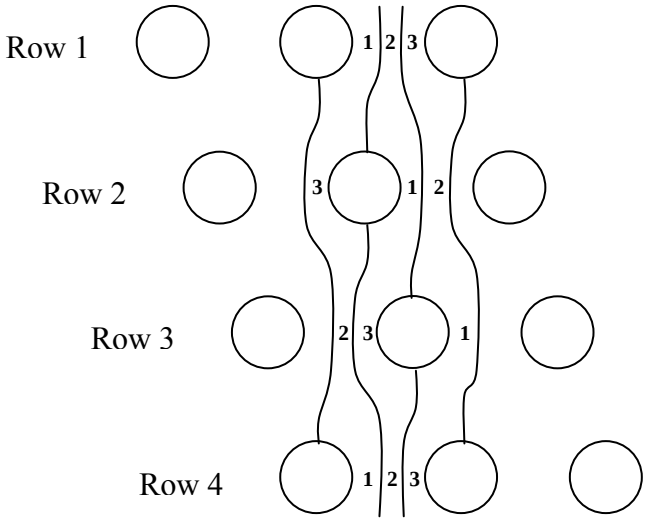

Figure 4. *A schematic picture of laminar flow in a bumper array.*

The flows are laminar so there is no turbulence. This means that if the liquid contains a particle small enough the particle will follow the same streamline throughout the whole bumper array, unless it diffuses into the path next to it and follow that streamline instead [4]. Diffusion is, however, a rather slow process compared to the flow rates used in the experiments, that is the Péclet number is high, so the diffusion does not disturb the separation of cells enough to be a problem [4].

A particle large enough will not be able to follow the same path throughout the bumper array since its center point will be bumped into the flow path next to it, see figure 5.

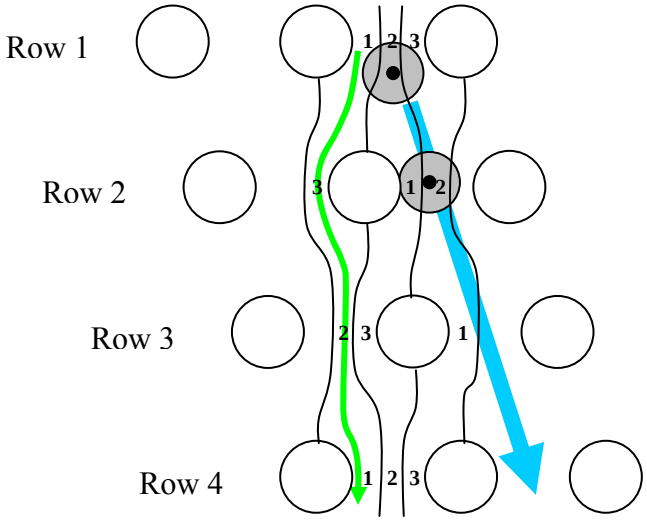

Figure 5. *A schematic picture of how a large particle becomes bumped in a bumper array.*

This is what makes the size separation work. Larger particles get bumped, which means that they are pushed into the next streamline by each new post it passes [5].

2.4.2 Geometry of the bumper array

The geometry of the bumper used during the experiments could be explained by the schematic picture below, see figure 6. The posts are separated with a center-to-center distance λ and the spacing between them is d [4]. The array is tilted at an angel θ in relation to the channel wall, which defines the direction of flow. Due to the tilting there is a displacement, $\Delta\lambda$, of each row in a perpendicular direction to the flow direction. There is a relationship between λ, $\Delta\lambda$ and θ.

$$\tan\theta = \frac{\Delta\lambda}{\lambda} = \frac{1}{N}$$

Here, N is the period of the array. N is defined as

$$N = \frac{\lambda}{\Delta\lambda}$$

This means that after N rows, counted along the wall, the posts in the next row will have the same positions as the posts in the first row had. Due to symmetry N also gives the number of streamlines between two posts [4].

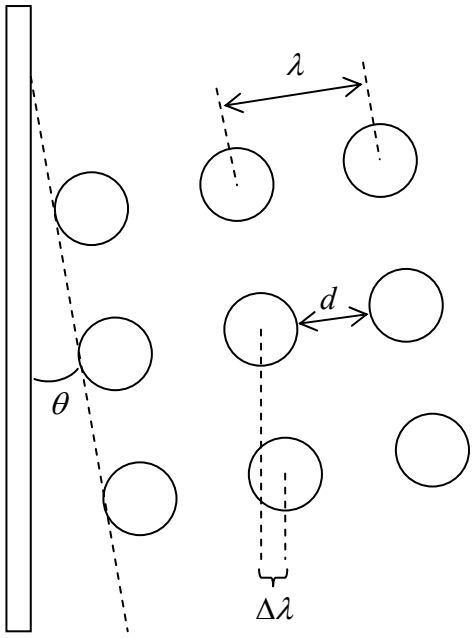

Figure 6. *Schematic picture of the geometry in the device.*

Details on the geometry and the design of the device can be found in appendix A.

2.4.3 Critical size

A bumper array has a property that is called the critical size. This size is usually expressed as the critical diameter, D_C, or as the critical radius, R_C. The critical size is the minimum size for which particles get bumped. That size depends on the geometry of the array. A particle get bumped when its center point falls into a neighboring streamline and that happens if $\Delta\lambda$ is smaller than the radius of the particle. This gives the following relationships.

$$R_C = \Delta\lambda$$
$$D_C = 2\Delta\lambda$$

Another way of expressing it is

$$R_C = \frac{d}{N}$$

Unfortunately, it is not this simple when dealing with micro systems. The flux, Φ, in every streamline between two posts is equal and depending on the velocity, v, and the cross sectional area, A [4].

$$\Phi = v \cdot A$$

As mentioned before, the flow profile in a micro channel is parabolic, so the velocities are not the same in every streamline between two posts. This indicates that a lower velocity in a streamline increases the cross sectional area of the streamline. The streamlines closest to the posts will therefore be wider than those in the middle between two posts. It is the width of the streamline closest to a post that determines the critical size, since that is where the particles get bumped. This means that the critical radius will be somewhat larger than d/N. To compensate for this a correction factor, α, could be calculated. The critical radius then becomes

$$R_C = \alpha \frac{d}{N}$$

An approximate value for α is calculated in a 2D model as follows [4]. At a point right between two posts the posts can be approximated with two infinite walls. Assume they are at a distance d from each other in a coordinate system where a y-axis is pointing along the bumper array walls and an x-axis is pointing perpendicularly to the bumper walls. The parabolic velocity profile is given by

$$v_y(x) = \frac{4v_{\max}}{d^2}\left(dx - x^2\right)$$

Now assume that the depth of the device is $w >> d$. The partial flow between a post and a plane a distance R_C from the post in the x-direction is then given by

$$\phi(R_C) = w\int_0^{R_C} \frac{4v_{\max}}{d^2}\left(du - u^2\right)\partial u$$

The relative flow of the partial flow is

$$\phi_{rel}(R_C) = \frac{\phi(R_C)}{\phi_{tot}} = \frac{w\int_0^{R_C} \frac{4v_{\max}}{d^2}\left(du - u^2\right)\partial u}{w\int_0^{d} \frac{4v_{\max}}{d^2}\left(du - u^2\right)\partial u} = 3\left(\frac{R_C}{d}\right)^2 - 2\left(\frac{R_C}{d}\right)^3$$

Assuming $\phi_{rel}(R_C) = \frac{1}{N}$ and substituting R_C with $\alpha\frac{d}{N}$ gives

$$\alpha \approx \sqrt{\frac{N}{3}}$$

For a period of $N = 20$ $\alpha \approx 2.6$.

It might be important to mention that cells are soft particles which may influence the critical radius. The cells might deform a little in the device due to the flow or when they are bumped. This might mean that the actual critical radius is larger than the theoretical one [6].

3 Fabrication of Bumper Arrays

There is a long line of steps to go through before a functional device is ready to be used. This work did not include the practical work of the first steps in the line since it would be too much to grasp within 10 weeks. But for a complete understanding the entire fabrication process will be presented anyway. Fabrication details can be found in appendix A.

3.1 Designing the bumper array

The first step is to design the device. Once the critical size is determined it was possible to choose a suiting distance, *d*, between the posts. If a larger *d* is wanted *N* can be increased to keep the desired critical radius.

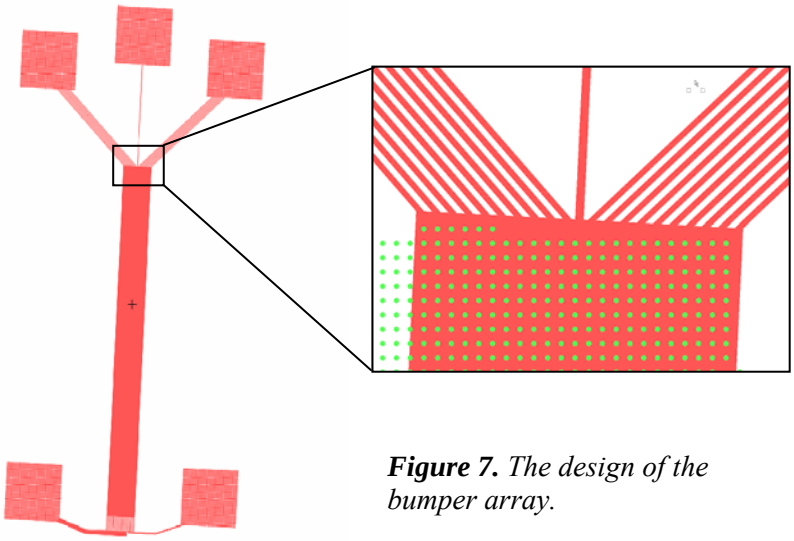

Figure 7. *The design of the bumper array.*

3.2 Creating the mask

Step two is to create a photo mask. The mask is created from a glass plate with a thin layer of chromium. On top of the chromium layer there is a thin layer of a positive resist, see figure 8. A resist is a polymer that is photo sensitive. It can be either positive or negative depending on whether it is weakened (+) or strengthened (-) by UV light [7].

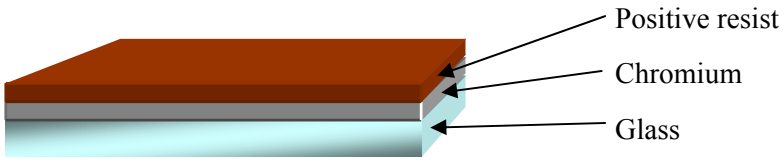

Figure 8. *The mask material.*

Then a laser beam marks tiny structures on the positive resist. The resist becomes weak (positive resist) where it has been exposed by the laser light. The mask is then dipped in a developer that dissolves the weak parts of the positive resist, see figure 9.

Figure 9. *The weak positive resist has dissolved.*

In the next step the master is dipped in chromium etch where all the exposed chromium parts are dissolved, see figure 10.

Figure 10. *The exposed chromium has dissolved.*

The mask is now finished and can be used to produce a master.

3.3 Creating the master

The master is made of a silicon wafer with a thin layer of SU-8, which is a negative resist. The mask is now used as a template when exposing the master with UV light, see figure 11.

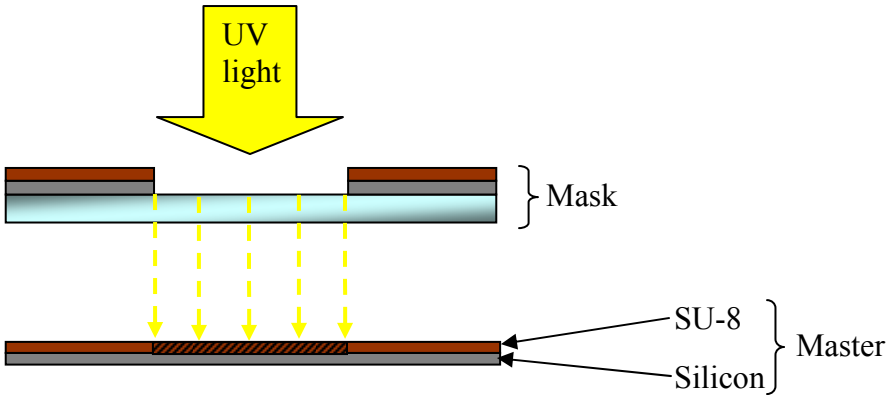

Figure 11. *The mask is used as a template when exposing the master with UV light.*

Where there is only glass left on the mask the light will shine through and where the light hits the master the SU-8 will polymerize and harden. The master is then put in a developer that dissolves all the SU-8 that has not been polymerized, see figure 12.

Figure 12. *The master after dissolving the unpolymerized SU-8.*

The master is then treated with an anti-sticking coating. After that the master is used as a template for making the final devices.

3.4 Creating the device

The devices are made of PDMS (polydimethylsiloxane), which is an elastomer. PDMS is a relatively cheep material and it is transparent for wavelengths between 230 nm and 1100 nm, which is suitable for microscopic biological studies. Other important properties of PDMS are that it is permeable to gases and impermeable to water. It is rather easy to use and it does not take so much time to create a new device once a master is made.

3.4.1 Baking the PDMS

The PDMS is mixed with a catalyst that cross-links the PDMS molecules. The cross-linking makes the PDMS harden. The mixture, that is still fluid since the hardening reaction is very slow in room temperature, is then put in a vacuum chamber to remove gas bubbles. When the gas is gone the PDMS is poured over the master and put in the oven. There the polymerizing reaction speeds up and the PDMS hardens. The PDMS is then carefully loosened from the master.

3.4.2 Checking whether the master is good enough

The first PDMS piece you make from a new made master can be sliced and put under a microscope to check whether the posts are straight or not. The master might have been over- or under exposed by the UV light in the creation process, which results in channels with tilted walls, see figure 13-14. If the walls are not straight, the flow profile will be different on different heights in the channel, which might be a problem since the critical size then is different on different heights too. It might lead to a lower resolution on the devices. If the walls are slanting too much a new master has to be made. Another problem that can occur if the master is too under exposed is that it will be difficult to remove the PDMS from the master during the fabrication.

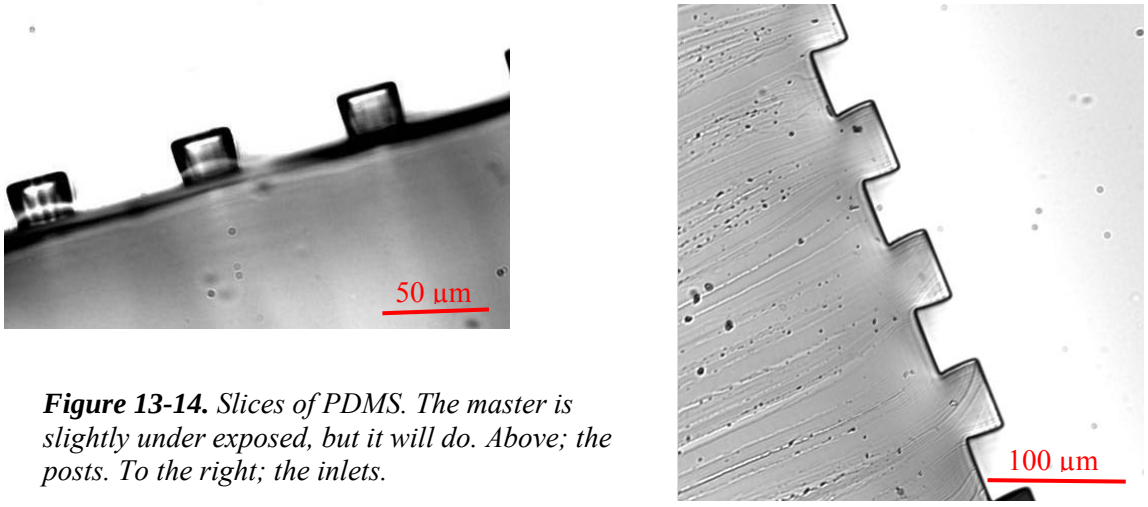

Figure 13-14. *Slices of PDMS. The master is slightly under exposed, but it will do. Above; the posts. To the right; the inlets.*

3.4.3 Making the holes for the inlets and the outlets

The channel in the PDMS is later on going to be sealed with a glass slide. Depending on whether the inlets and the outlets are to be on the PDMS side or on the glass side small holes are made either in the PDMS or in the glass. The holes in PDMS are easily made by pressing a thin metal pipe through the PDMS. If the holes are to be made in the glass

slide a sand blaster that is blowing pulverized aluminum oxide with high pressure is used to drill the small holes. On most of the devices used during this project the holes were made in the PDMS. The main reason for this was that it went a lot faster than drilling in the glass slides. The drilling is also more expensive, since it takes quite much pulverized aluminum oxide to drill holes for one device.

3.4.4 The oxygen plasma treatment and the bonding

Since PDMS is hydrophobic it can be hard to get the aqueous solutions into the channel once the PDMS and the glass slide is put together. So the next step is to treat the PDMS and the glass slide with oxygen plasma. This treatment makes the inside of the channel hydrophilic. The oxygen plasma treatment also fills other functions. It makes it possible to bond the PDMS to the glass. The bonds are O-Si-O (silanol) covalent bonds. The treatment also cleans the surfaces from organic materials and sterilizes the inside of the channel thanks to the UV light that is present during the treatment. The sterilization fills an important role when the purpose is to separate cells with the device. The glass slide and the PDMS is put together as quick as possible after the oxygen plasma treatment to prevent the oxidized hydrophilic surface from decaying. It decays in 15 minutes to one hour due to diffusion of polymers to the surface. It is not certain that the bonding will succeed, sometimes the bonding is not complete and sometimes it does not work at all. As long as the channel is sealed properly the device can be used without leaking. But if it is not, a new device has to be made. So the oxygen plasma treatment and the bonding is a critical stage in the fabrication process. The reason why the bonding sometimes does not work is not known. Speculations have been made that it might be due to the humidity. All the unsuccessful bondings that were made during the project happened over a couple of very warm days.

3.4.5 Gluing the pipes to the in- and outlets

After a successful bonding small and short rubber pipes are glued over the holes for the inlets and the outlets. This can also be a critical moment in the fabrication process. It takes some dexterity and patience to put the pipes in the right positions without getting glue in the holes. If an in- or outlet is clogged with glue a new device has to be made. The glue hardens in about 30 minutes. It is good to put some additional glue on once it has hardened to prevent leaking.

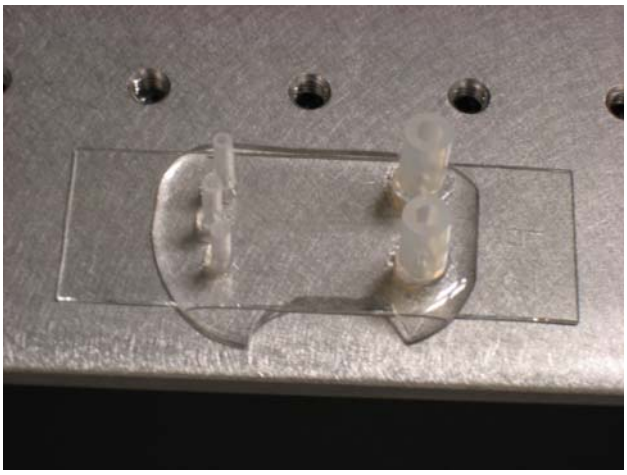

Figure 15. *A complete device.*

4 Preparation of Cells

The cells used during this project were human breast epithelia cells called MCF-10A (details can be found in appendix B). This type of cells were chosen because they are relatively robust and globular, which are good properties for basic studies in the device.

Before the devices were designed, size measurements were made on the cells to find out how wide the channels should be in order to separate the small cells from the large ones (results and details can be found in appendix B).

Living cells usually like to stick to surfaces. To be able to stick to the surface the cells produce proteins that work kind of like glue. When sticking to the surface they flatten out a little. This is one of the biggest problems when trying to separate cells in micro channels. Clogging has to be avoided. To get the cells to pass through the bumper array without sticking to the surface they had to be chemically prepared before they were pumped trough the device. This was done with trypsin, which is an enzyme that catalyses the cleavage of peptides. (Trypsin is by the way produced naturally by the pancreas in humans and animals.) Trypsin breaks down the proteins that the cells have produced, which makes the cells loosen from the surface. At the same time they regain their globular shape. A more detailed recipe can be found in appendix B.

There are also other ways to make the device less sticky such as adding BSA. BSA stands for bovine serum albumine and stops the cells from sticking to the surface of the device to a greater extent. Other alternative additives are milk and pluronics. One can also pretreat the surface of the channels with PEG-silane or PLL-*g*-PEG (polylysine-*graft*-polyethyleneglycole). A detailed recipe of the cell culture medium used during the experiments can be found in appendix B.

5 Experiments

A lot of different experiments that could be interesting were discussed. Unfortunately there was not enough time to do them all, so the focus was put on the most fundamental experiments.

5.1 The set up

Every time an experiment was to be made the set up had to be put together. The device was put in the microscope and connected to three syringes containing the solutions via thin tubes. The inner diameter of the tubes was 100 micrometers. A camera controlled by a computer was connected to the microscope. This made it possible to look at the microscope image on the screen and to take photos and films during the experiments.

Figure 16. *The experimental set up.*

5.2 Over-pressure- or vacuum driven flow

A usual procedure before pumping the solutions through the device is to put the solutions in vacuum for a couple of hours. The purpose is to get rid of gas bubbles that might disturb the separation process. But the culture medium used had dissolved carbon dioxide in it as a nutritive substance. Degassing the culture medium led to a change in the pH value when the carbon dioxide disappeared. This is not good for the cells so the experiments had to be made without degassing the culture medium first. It turned out that skipping the degassing was not a problem in this case, since the solution was pumped through the device with the help of over-pressure. The flow rate and hence the pressure could be increased so that all gas bubbles went in solution and disappeared in a couple of minutes. Vacuum driven flow was also tested since that is a simpler method. It was tested on pure culture medium to see the effect. There were a lot of air bubbles and they would not disappear even on the highest rate possible. With vacuum driven flow the maximum rate is much lower than with flow driven by over-pressure. So it seems that as long as a culture medium not suitable for degassing is used, positive-pressure driven flow is the best method.

5.3 The clogging experiment

The first experiment was to test if the cells would be able to pass the device at all without sticking to the surface or clogging in any way.

The tubes and the device were sterilized with 70% ethanol. The new tubes had run out, so the ones that were used had been used before in another experiment. The ethanol was then washed out by pumping in a culture medium (PBS with BSA, where 5 % of the

weight was BSA) from the sides. The cells were in another culture medium. They had already been trypsinised and were connected to the device after a while.

5.4 The rate experiment

The second experiment was to test different fluid rates, especially higher rates to see how fast the separation could be done without harming too many cells. If the device should be useful to biologists for separating cells and be a better alternative to conventional cell separating methods, the separation is not allowed to take too much time. The cells were to be collected after passing through the device at a certain speed and then dyed with trypan blue. Trypan blue is a stain that only dyes dead cells. Living cells have a functioning cell membrane that prevents the trypan blue molecules from being absorbed. Dead cells, on the other hand, have ruptured cell membranes or cell membranes that are not functioning. This means that nothing is stopping the trypan blue molecules from entering the cells. Details for the trypan blue staining can be found in appendix B. The point was to count the amount of dyed dead cells in a microscope after each tested rate to see how many percent of the cells that had passed that was dead. Hopefully this would lead to a diagram that showed some kind of maximum rate that was suitable for cell separation.

Two attempts were made on this experiment. Two syringes with cells were prepared instead of one both times. They were connected to the two inlets on the sides of the device. The reason for that was that the two inlets on the sides are wider, which means that the solutions could be pumped through much faster so the experiment would not take so much time. The middle inlet was left alone. In this experiment it did not matter that the cells were pumped in from the sides since the purpose of the experiment was not to separate the cells.

The first attempt started at a low speed, but because of air bubbles the rate was raised to 20 μL/min so the bubbles would disappear faster. When the rubber pipes that were glued to the outlets were filled the pump was turned off. The solution in the outlet pipes was sucked out with a pipette and dyed with trypan blue. A drop of the dyed solution was put on a microscope slide and inspected in the microscope. The same procedure was done with the rates 5 μL/min and 40 μL/min.

For the second attempt the cells had already been trypsinised and it took quite some time to find a hemocytometer to borrow, so the measurements were delayed. A colour camera had also been installed. This time higher rates than in the last attempt were to be tested. Since the cells do not stay in solution too many hours after trypsinisation the highest rate was tried first. The rates evaluated this time were 100, 80, 60 and 40 μL/min. The times for which the different rates were tested were noted since it might matter how long after the trypsinisation the different tests were made.

5.5 The separation experiment

The third experiment was to test the device itself to see if it managed to separate the cells correctly. The point was to pump cells through the device and collect the solutions with separated cells. The collected solutions were then to be evaluated in a flow cytometer,

which registers the amount of DNA in every cell once the cell DNA has been dyed with propidium iodide. That way it should be possible to see a difference in DNA amount in the cells from the two separated solutions. Hopefully all the cells in the solution from the outlet for the small cells only have one set of DNA. To store the cells until they could be looked at in the flow cytometer they had to be centrifuged to become a pellet, fixed with ethanol and put in the freezer. Details on the ethanol fixation can be found in appendix B.

For this experiment three attempts were made. The first attempt started on 5 μL/min, but since only a few cells seemed to pass the device, the rate was increased to 100 μL/min. The separated solutions were collected and pelleted.

In the second attempt the rates were also kept quite high. A new bottle of culture medium was used since the old one had been contaminated. Only a few cells were seen passing the device. Because of the low cell concentration there was no point in trying to make a pellet of the cells.

In the third attempt the rates were kept low. The highest cell rate was 0.5 μL/min and the highest culture medium rate was 1 μL/min. Unfortunately, the outlet for the small cells had collapsed during the bonding so practically nothing came out from that outlet. The flow was disrupted in the end of the device because of that so most of the cells came out through the other outlet. Therefore it was quite pointless to run them trough the flow cytomenter. The cells were, however, centrifuged to se if there were enough cells to make a pellet.

6 Results

6.1 The clogging experiment

Cells were seen passing the device. There were very few cells though. Some cells had stuck to the surface but a lot of them managed to pass. Another thing to be mentioned is that it could be seen in the microscope that the two different culture mediums used had different viscosity and refraction indices. There were sharp edges between the two fluids.

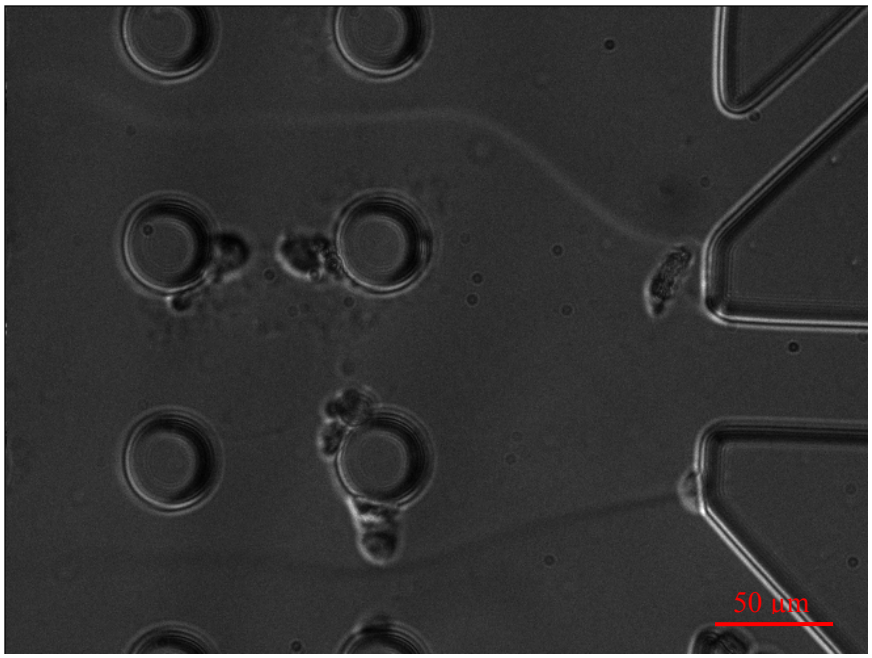

Figure 17. *The different culture mediums have different viscosities.*

6.2 The rate experiment

When looking at the dyed cells in the microscope after the first attempt both light and dark cells were seen. It turned out to be hard to distinguish between dark cells and other dead material, for example dust, since the microscope could not magnify the picture enough. This was not the only reason why it turned out to be impossible to count the cells. Another problem was the lack of a hemocytometer, which is a microscope slide with engraved lines that forms a net of rectangles with known areas. Hemocytometers are used to count cells and cell concentrations. Without a hemocytometer it is hard to keep track of which cells that have already been counted. A third problem was that the camera showed images in black and white only. With the camera the images were magnified more than in the microscope, so it might have been possible to distinguish dyed cells from dust and living cells from dead if the camera had been a colour camera. Despite not being able to count the percentage of dead cells the result was hopeful. After all there were an amount of cells that had not been dyed.

During the second attempt only a few cells had managed to pass the device, which means that the statistics behind the results were very bad. To get valid results at least 200 cells should be counted. Since the amount of cells was not that large (after running at 60

μL/min the total number of cells in one drop was 70) there was no point in counting the cells. However there were some cells that looked healthy after running at every rate. A couple of pictures were taken.

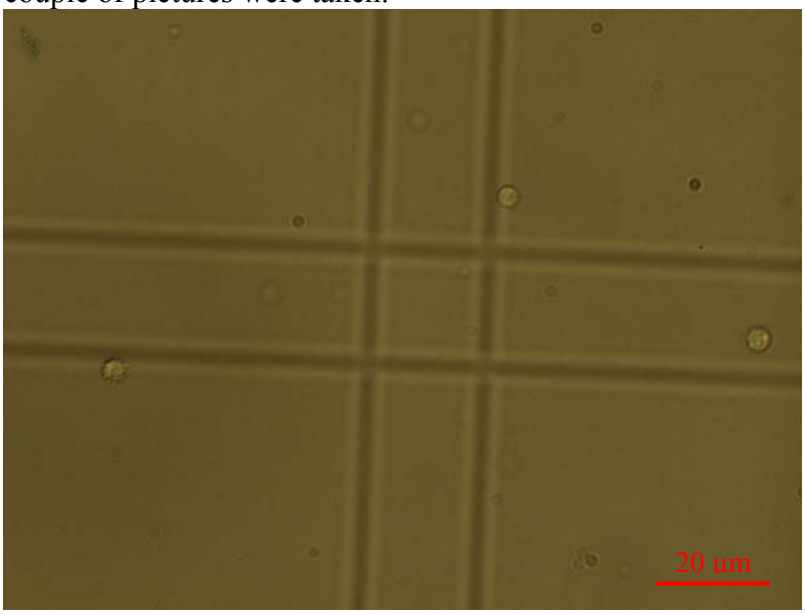

Figure 18. *Healthy looking cells after running at 80 μL/min.*

6.3 The separation experiment

The first experiment was not that successful. Not so many cells were seen passing the device. When the rate had been increased to 100 μL/min something strange was found in the device next to the inlets. There were thread-looking formations around the posts and among the threads there were cells that had been caught. The collected solutions were centrifuged to create a pellet, but no pellet could be seen.

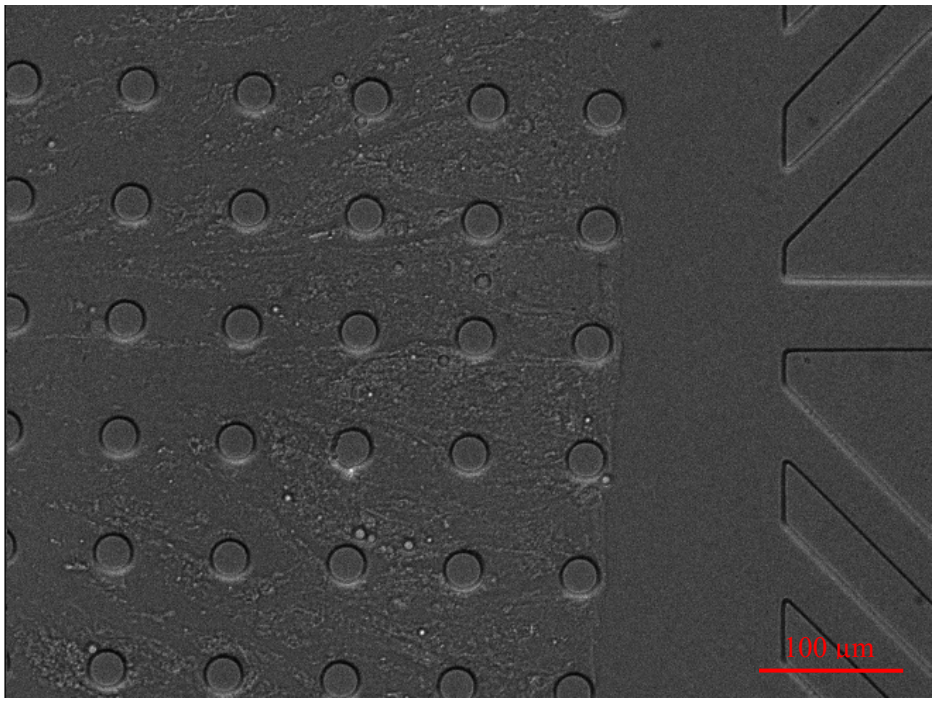

Figure 19. *Threads in the bumper. Some cells have got caught among the threads.*

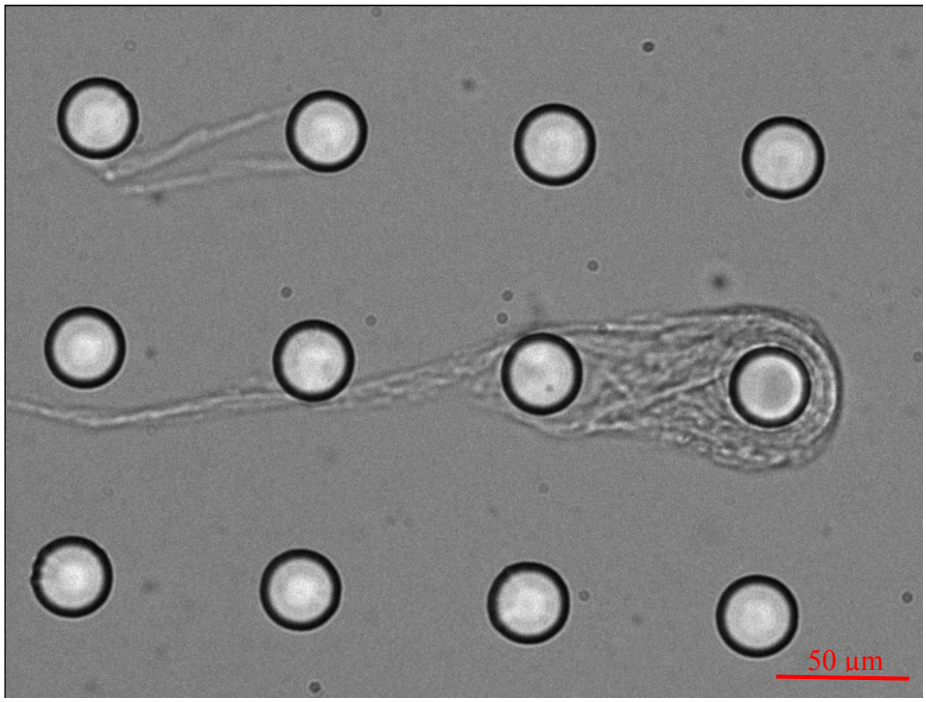

Figure 20. *Threads in the bumper.*

The second attempt turned out the same. The speeds were quite high this time as well. Threads were found around the posts by the inlets again and cells had got caught among the threads. This time no centrifugation was made since only a few cells were seen passing the device.

In the third attempt only a few threads were found and a lot more cells could be seen passing the device. It was also found that more cells came into the device if the syringe with the cells was tilted so that the opening was pointing downwards. Even more cells came when the syringe and the thin tube were shaken a little. Many cells were found in the little pipe that leads in to the device. Clearly, there is a problem of sedimentation. Since the cells are fairly large, quick sedimentation times should be expected. Disappointingly no pellet could be seen this time either. Although a successful separation could not be indicated by the flow cytometer the results were quite positive anyway. Films were taken on cells that were separating in the device. The small ones went straight ahead and the larger ones got bumped.

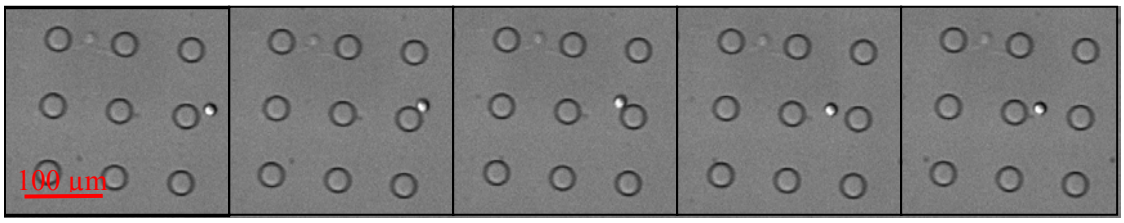

Figure 21. *A series of a cell getting bumped.*

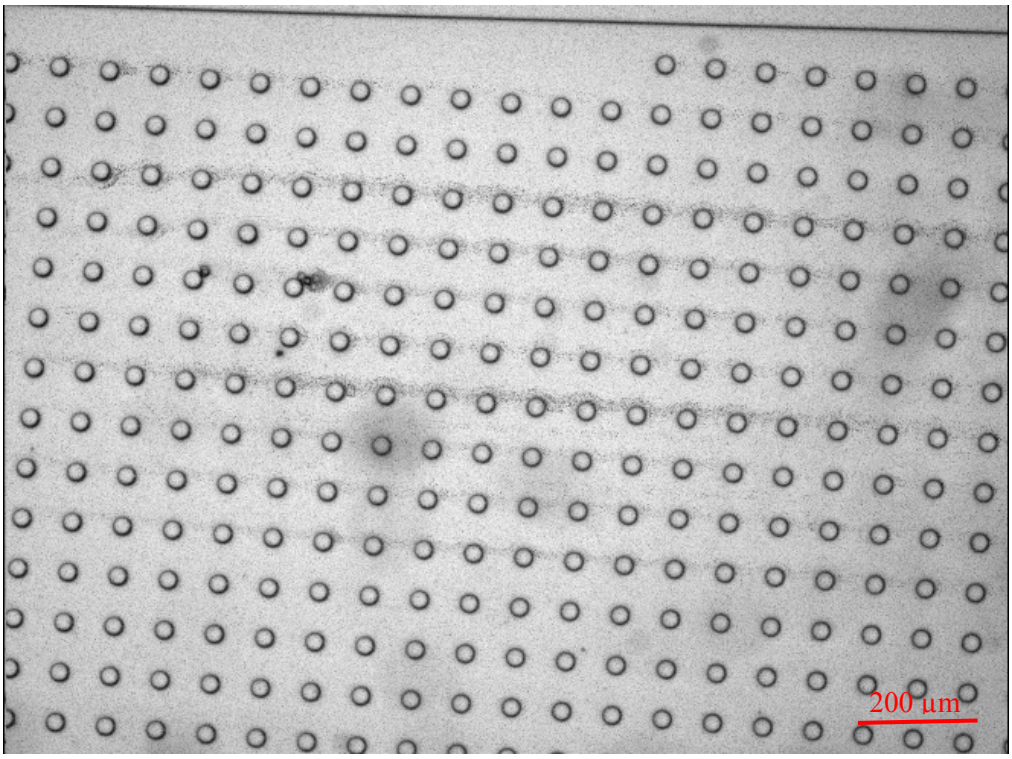

Figure 22. *A sum of many pictures over time. The picture shows the paths that the cells have taken. In this case most of them got bumped.*

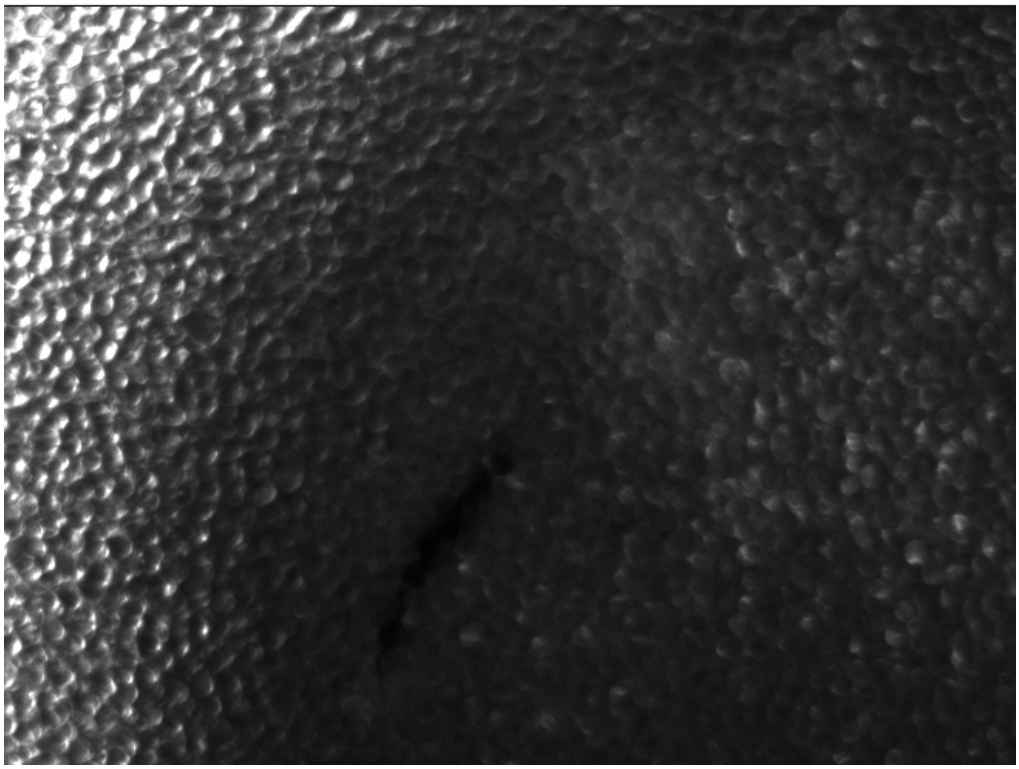

Figure 23. *The inside of the inlet pipe. A lot of cells have stuck to the surface.*

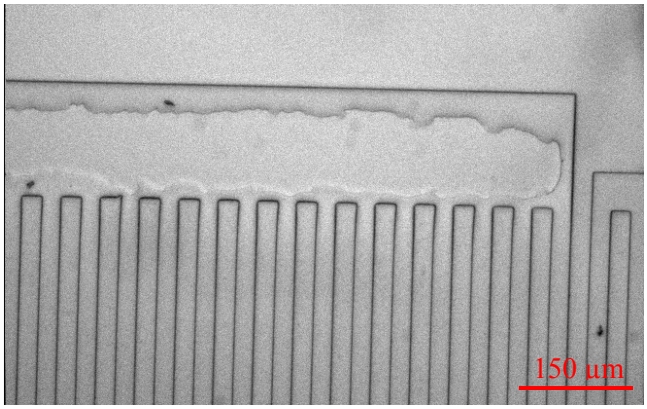

Figure 24. *One of the outlets had collapsed. Only a little fluid could pass.*

7 Discussion

7.1 Summary

One of the biggest problems in all experiments was the low cell concentration due to clogging. The cells got stuck in the tube that led into the device and could therefore not participate in the experiment.

Another big problem was the threads (probably DNA from the cells) that accumulated in the device at high flow rates. The cells got caught among the threads.

To decrease the clogging BSA or dry milk could for example be added to the buffer. Pluronics could probably be added as long as the concentration is not too high. Diluting the buffer might help. The channel surfaces could be treated with PLL-*g*-PEG or PEG-silane.

To prevent the treads from accumulating the maximum rate for which the threads do not accumulate could be found. If this rate is too low to get the cells through the device in a reasonable time a higher flow rate could probably be used if nuclease is added to the buffer.

7.2 The clogging experiment

The results were quite positive. There were cells that stuck to the surface of the device but they were not so many that they prevented other cells from passing. The flow is of course a little disturbed from cells that stick to the surface. But the device is, on the other hand, relatively long compared to a cell so the cells should have time enough to get on the right track. The reason that there were so few cells might be because they were trypsinised a while before they were used. The cells stay in solution for a couple of hours only after they have been trypsinised, then they stick to the surface again. Another reason is of course the pure culture medium that is pumped in from the sides. The culture medium dilutes the cell solution. The fact that the cell solution is diluted might not be so good for the separation process, since it is desirable to collect as many cells as possible in a relatively short time. Another thing that probably was not so good for the cells is that the syringes, the tubes and the device were sterilized with ethanol before the experiment. If all the ethanol was not washed out cells might have died. But the washing with culture medium was going on for about an hour so hopefully there was not any ethanol left that could harm the cells.

7.3 The rate experiment

This experiment did not go as planned. Once the problems with the hemocytometer and the colour camera were solved the statistic material was still insufficient to produce a valid result. This time the low cell concentration was not due to pure culture medium that diluted the cell solution. Probably a lot of the cells had stuck to the surface in the syringe and in the tube. This time the trypsinisation was done right before the cells were used, so the low cell concentration was not due to an early trypsinisation. Obviously something has to be done about the clogging. BSA or dry milk could for example be added to the buffer. Pluronics could probably be added to the buffer if the concentration is not too high. The channel surfaces could be treated with PLL-*g*-PEG or PEG-silane. The better

the cells are feeling the easier they stick to the surface. So another thing that is rather easy to try is to dilute the culture medium. That way the cells might stay in solution longer. However, the results showed that there were an amount of cells that looked healthy after having passed the device. This might be enough to culture new colonies with cells that are synchronized. So when looking at the result from that angle it is a rather positive result.

7.4 The separation experiment

The clogging in the syringe and in the tube was obviously a problem in this experiment as well. Although the shaking on the syringe and the knocking on the tube and the inlet pipe helped a little, the clogging problem has to be solved if a higher cell concentration is wanted. Another problem was the threads that caught the cells so they could not pass. To be able to do something about this problem it is important to find out where the threads come from and what they are made of. After the first attempt speculations were made that the threads might be bacteria that had come into the device with the culture medium. It turned out that the culture medium was contamined so it was replaced with a new bottle in time for the second attempt. Despite this, threads were accumulating in the device the second attempt as well. New speculations were made that the threads might be DNA from cells that had burst. This is probably the case since there were not as many threads the third attempt when the rates were lower. But the threads have to be examined to know for sure. If the threads are bacteria the problem could easily be solved by working in a sterile environment. If the threads are DNA it will be a problem. That would mean that the cells cannot be separated on high rates, which means that the separation will take more time. This is not acceptable if the device should be used for cells. A solution to this problem would be to find the highest rate possible for which the cells do not burst and hope that this rate is fast enough so the separation time is not too long. Another solution could be to add something to the culture medium that breaks down DNA but cannot get through a healthy cell membrane, such as nuclease. That way the accumulated DNA could be removed from the device without harming the living cells.

8 Outlook

There is obviously a lot more to be done in this area of research. First of all some research on the clogging problem would be of interest. Is it possible to create a surface layer that cells would not want to sit on and that is not harmful to the cells? Or perhaps to find a suiting culture medium that keeps the cells in solution? It could also be of interest to do the rate experiment and the separation experiment again to see if they could succeed if the syringe and the tube are shaken during the experiments and if a device that has not collapsed is used. Another interesting question is whether it is possible to culture the cells after running them through the device. If the device is separating with a resolution high enough it should not matter that the cell concentration is low by the outlets. It should be possible to culture them anyway. If an experiment like that would succeed the device would probably be very attractive for biologists. Another thing that would be of interest is to do some research on the threads. What are they made of and what could be done about them?

Even though this research has given answers to some questions there are still a lot of question marks to be straightened out. Hopefully this could be done quite soon and who knows, perhaps a complete and functioning device can be found on the market in the future.

Appendix A

Fabrication details

The design, the mask and the master was done by Jason Beech. Most of the details on how the fabrication of these is done are taken from his Master thesis [4].

The design

The design of the device was done with the help of a CAD program (L-edit). The measurements were as follows.

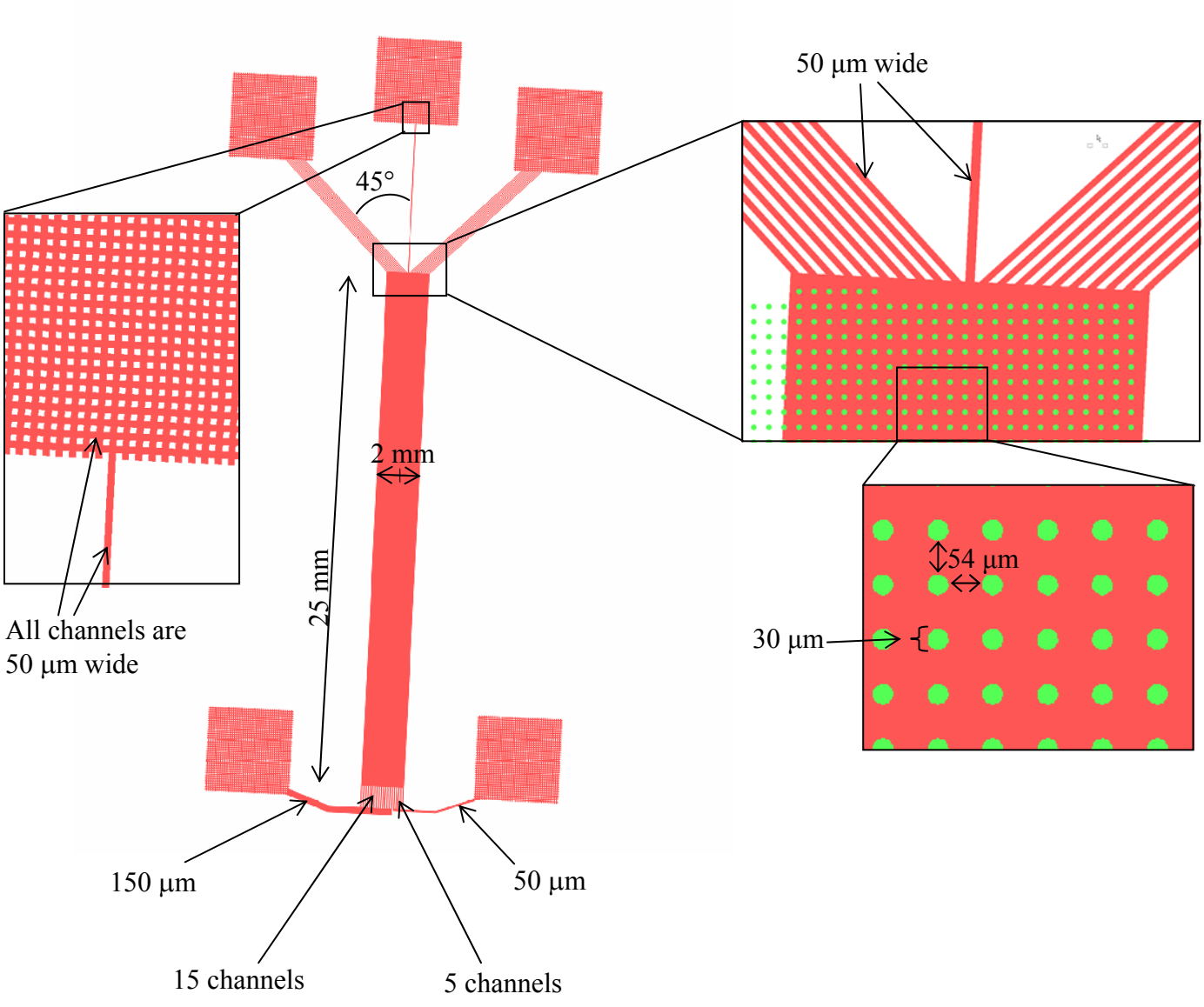

Figure 25. *The design and measurements of the micro channel.*

More explicitly
$d = 54$ μm
$N = 20$
α were estimated to be approximately 2.58

$$R_C = \alpha \frac{d}{N} = 6.966$$

$\lambda = 84$ μm
$\Delta\lambda = 4.2$ μm
$D_C = 2\,\Delta\lambda = 8.4$ μm

$$\theta = \arctan\left(\frac{1}{N}\right) \approx 2.86°$$

The posts were 30 μm in diameter.
The main channel was 25 mm long and 2 mm wide.
The small inlet channels were all 50 μm wide.
The angle between the middle inlet channel and the two outer ones were 45°.
The 20 small outlet channels were all 50 μm wide.
Five of the small outlet channels were led into one 50 μm wide outlet channel and the other 15 small outlet channels were led into a 150 μm wide outlet channel.

Mask fabrication

The transferring of the pattern from the blue-print to the mask with a laser beam was done by a Heidelberg mask generator.

Master fabrication in SU-8

The following recipe gives a 20 μm layer when used with SU-8 2010 and is based on recommendations from Microchem, the manufacturers of SU-8.

Substrate preparation

- Ensure that the silicon substrate (2" <100> wafer) is clean and planar.
- Bake in convection oven at 200°C for 30 minutes to remove surface water and promote the adhesion of SU-8 to the surface.

Spin coating of SU-8

- SU-8 should be applied to the wafer directly upon removal from the oven in order to minimize the amount of water that can adsorb to the surface
- Holding the wafer in one hand, and pouring from the SU-8 bottle with the other in as even a manner as possible, about one third of the wafer is covered.
- The wafer is then tilted so that the SU-8 flows over the entire surface.
- The wafer is centralized on the vacuum pad in the spinner and the following spin cycle is performed.
- 20 seconds at 500 rpm – to spread the coating evenly over the wafer.
- 120 seconds at 1000 rpm – to obtain 20 μm layer.
- These times are longer than those recommended by Microchem but gave more even coatings of SU-8 with smaller edge beads.

- Allowing the sample to relax on a level surface for 1-2 hours can minimize edge beads.

Note: One of the greatest problems encountered was that an excess of SU-8 was needed to achieve good coverage, and this excess, when thrown off the wafer during spinning had a tendency of finding its way to the surface. A piece of filter paper held close to the edge of the spinning wafer was used to catch this excess with good results.

Pre-baking
Pre- and post-exposure baking was performed on a hotplate with vacuum.

- 1 min at 65°C.
- Ramp to 95°C – takes 4-5 minutes.
- 2 minutes at 95°C.
- Relax for 10 minutes on a separate hotplate at 35°C.

Exposure
- 25 seconds at 12.5 $mJcm^{-2}s^{-1}$, which corresponds to a total dose of about 0.31 Jcm^{-2}. (The lamp intensity was 12.5 $mJcm^{-2}s^{-1}$ and the main wavelength was 365 nm, which is the wavelength for which SU8 absorbs most energy.)

Post exposure bake
- 1 minute at 65°C.
- Ramp to 95°C (takes 4-5 min).
- Ramp to 65°C (by setting the hotplate to 5°C – 8 min)
- Relax 10 minutes on second hotplate at 35°C.

Developing
- Sonicate at low amplitude (50 V) for two minutes in SU-8 developer.
- The developer is rinsed away with isopropanol and the wafer dried with nitrogen.
- A white film indicates the presence of undeveloped resist, in which case further developing is required. This should be done in 1-minute steps, with intermediate rinsing, until the white film is no longer seen.

Hard baking
- 200°C in a convection oven. This causes reflow reducing the size and number of cracks.

Anti-sticking treatment of master
The following steps about the anti-sticking treatment of the master are taken from an article by M. Beck (reference 8). The procedure described in his article was followed with the exception that 150 °C was found to give the best results, namely fewer excess deposits on the surface of the master, according to Jason Beech (reference 4).

- Hydroxylate the master in nitric acid, wash it thoroughly and dry it.

- Transfer it to an anhydrous glove box environment (nitrogen atmosphere, water content < 1 ppm).
- The master, situated in a Petri dish with a little hole near the edge of the cover, is placed on a hotplate.
- The hotplate is heated to 150 °C. Wait for about 10 min so that temperature equilibrium is reached.
- Inject a few microliters (how many depends on the size of the Petri dish) F_{13}-TCS (tridecafluoro-1,1,2,2-tetrahydrooctyl-trichlorosilane) with a microsyringe through the hole in the Petri dish. Cover the hole immediately with a glass slide. Wait for 2 h.
- Open the Petri dish chamber and wash off excess F_{13}-TCS with anhydrous hexane inside the glove box. The master can then be taken out of the glove box.

Baking the PDMS

The devices were fabricated in PDMS (RTV 615 from GE Bayer Silicones) and sealed with glass slides.

- The PDMS is mixed thoroughly with a hardener at a ratio of 9:1 by mass.
- The mixture is put in a vacuum chamber for at least 30 minutes to remove gas bubbles.
- The PDMS is poured onto the master.
- Bake at 80°C for one hour.
- The PDMS is removed carefully from the master.

Making the holes

The holes were made either in the PDMS or in the glass.

Holes in the PDMS

- Press straight down with a cannula (a sharp-edged thin metal pipe).

Holes in the glass

- Holes are made in the glass slides using 50 μm aluminum oxide in a micro-sand blaster (Microetcher™, Danville Materials).

Bonding the device

- The glass slide is wiped off with isopropanol to remove dust particles.
- The PDMS and the glass slide are then exposed to oxygen plasma at a pressure of 8 mbar for 30 seconds.
- The PDMS and the glass are carefully put together. Pressure is applied lightly with the fingertips.
- The device is filled with an aqueous solution immediately to conserve the hydrophilicity of the walls.

Gluing the tubes

Fluidic connections were made using silicone rubber tubing (Small by the inlets and larger by the outlets). The glue that was used was Wacker Elastosil A07.

- A small amount of glue was applied to the outside edge of the tubes that were then carefully aligned to the holes in either the PDMS or glass. The pipes were carefully put in the right place.
- The glue cures in about 40 minutes.
- Additional glue is applied around the pipes when the glue has hardened to ensure that the device will not leak.

Appendix B

Cell culture details

MCF-10A (human breast epithelial cells CRL-10317, American Type Culture Collection, Manassas, VA, USA) were seeded in 25 cm^2 tissue culture flasks (Nunc™, Roskilde, Denmark) in 5 mL of RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 1 % penicillin and streptomycin, 1 % non-essential amino acids, 10 μg/mL insulin, 20 mg/mL epidermal growth factor (Biochrome, Berline, Germany), 100 ng/mL cholera toxin and 500 ng/mL hydrocortisone (Sigma-Aldrich). The cells were incubated at 37 °C in a water-saturated atmosphere of 5 % CO_2 in air.

The cell size measurements were done by Kersti Alm. The following picture is a microscopic picture from the measurements of the cell sizes. Only the cells that were in focus were measured, since it is hard to determine the size of blurry cells.

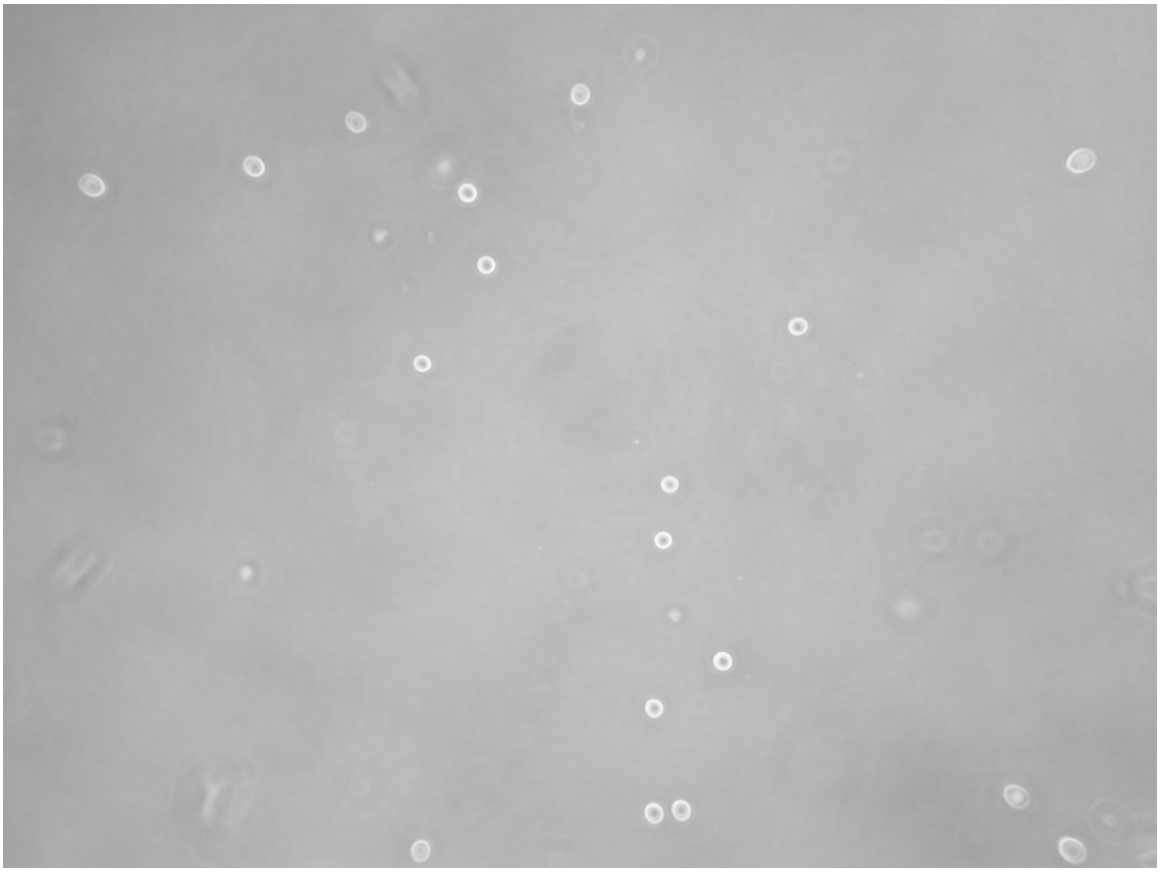

Figure 26. *A microscope picture from the size measurement of the cells.*

The diagram below shows the results of the size measurements. The x-axis shows the cell diameter in μm and the y-axis shows the number of cells. The total number of cells measured was 47. This means that the cell type MCF-10A has a size distribution that lies

between 10 and 22 µm. For the separation a threshold of 17 µm was chosen. Cells with a diameter less than 17 µm would be collected.

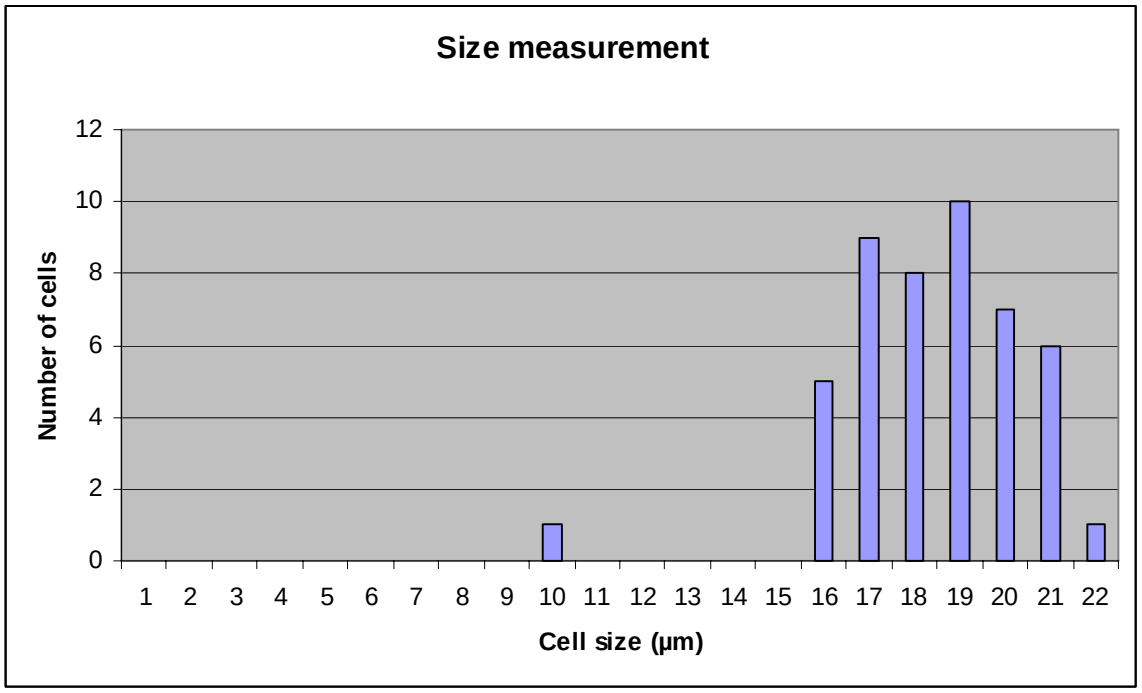

Figure 27. *The result of the size measurement.*

Trypsinisation of cells

The cells were cultivated in flasks with a liquid culture medium as described above. There were between half a million and five million cells in a bottle.

1. Pour out the culture medium or aspirate it with a pipette.
2. Add a couple of milliliters of trypsin to the flask. (4 ml were added to the 25 cm^2 flasks.) Tilt the flask carefully back and forth so that the last residue of cell culture medium is washed away without loosening the cells from the surface.
3. Aspirate the trypsin with a pipette.
4. Add new trypsin; 1 ml per 25 cm^2 flask.
5. Wait approximately 10 minutes for the cells to loosen. It goes faster if the flask is kept in a heat cabinet at 37 °C or if it is warmed in a person's hand (which was done during this project). If the temperature is cooler than 37 °C it can take more than ten minutes.
6. When the cells start to loosen, hit the bottle quite hard so that all the cells loosen from the surface and from each other.
7. Add culture medium to stop the trypsinisation. Add at least as much as the amount of trypsin. The amount of culture medium added determines the final cell concentration.
8. Use the cells as soon as possible. They will only be in solution for a couple of hours. If they can not be used right away keep them on ice (best) or in a refrigerator.

Trypan blue dying

Add 100 µL trypan blue to 400 µL cell solution.

Ethanol fixation

1. Put the cell solutions in small test tubes.
2. Centrifuge the cells at 600-700 *g*. (The first time the cells were centrifuged during this project they were centrifuged at 700 *g*, the second time they were centrifuged at 650 *g*. Both times the centrifugation temperature was 4°C.)
3. Aspirate the cell culture medium with a pipette (be careful not to disturb the pellet)
4. Add 2 ml 70% ethanol.
5. Store in the freezer at -14°C.

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