Master thesis

Design and optimization of a microfluidic chip for single cell analysis of circulating tumor cells

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Microfluidic device,
CTC analysis

ABSTRACT

Breast cancer remains one of the leading causes of death in women. The prognosis of cancer patients is predominantly influenced by the formation of metastases, with 10 to 15% of patients developing distant metastases within 3 years of primary tumour detection. Tumour cells with epithelial origin circulating in the peripheral blood are called circulating tumour cells (CTCs). Breast cancer therapeutic decisions are based on tumour markers such as ER, PR and HER2 which makes tumour cells characterization extremely important. Cancer progression is normally monitored by biopsy, an invasive procedure not well accepted in the clinic nor by the patient. An alternative approach is to analyse a blood sample, liquid biopsies are minimally invasive and they can be done repeatedly, allowing for real-time monitoring of the cancer evolution. The development of cheap, fast and reliable devices capable of isolating and characterizing CTCs is urgent. In this thesis, a microfluidic device to purify and analyze CTCs is reported. It was proved that this device can isolate MCF-7 and SKBR3 cells with an average trapping efficiency of 98% (N=9) with the cells remaining viable. It was also proved that it is possible to perform single cell analysis. Immunocytochemistry was done to characterize the phenotype of CTCs by targeting membrane receptors. The possibility to perform fast phenotypic characterization of CTCs with this microfluidic device has the potential to vastly improve the monitoring of cancer treatment and, in some cases, lead to therapeutic changes that can profoundly impact patient prognostic.

1. Introduction

Improved health care is a worldwide necessity. Lack of resources and technical expertise in developing nations restrict their ability to perform simple clinical diagnostic tests even for easily treatable conditions. On the other hand, developed countries are trying to shift from curative to predictive and preemptive medicine by bringing diagnostics into the physician’s room or even to patient’s home. Preventing both these realities to become true is the fact that the majority of clinical diagnostics is still being performed using conventional bench-top analysis methods. These techniques are effective but expensive, slow, labor intensive, need high-end equipment and consume large volumes of reagents. The increase necessity for novel diagnostic tools is evident. These new tools must be reliable, fast, cost-effective, easy to fabricate and to handle.

In an attempt to achieve these targets, researchers adapted photolithography and chemical-etching methodologies from the microelectronics industry to make microfluidic analysis devices. These devices have been in constant evolution over the past 20 years, continuously trying to demonstrate their substantial advantages over existing techniques. Despite some of these devices already being in the market, this new technology is facing an increasing number of barriers created not only by the medical community but also by the well-established clinical diagnostic laboratories which have multimillion dollar businesses to protect.

This thesis, which focus on the designing, fabrication and optimization of a microfluidic device to isolate and analyze circulating tumor cells, intends to contribute to the improvement of the state of the art of the diagnostic microfluidic devices. At the same time, it hopes to indirectly contribute to bring these new platforms to the patients, not only in rich and developed countries but also, and mainly, in developing nations where clinical diagnostic is, unfortunately, still just a dream.

1.1. Microfluidics and single cell analysis

Single cell analysis is the assessment of a cell’s functional properties as well as its transcription, translation, regulatory and signaling events. This methodology facilitates not only the understanding of how molecular events in a single cell relate to the properties of tissues, organs and the organism itself, but also the analysis of tissues composed by multiple cell types. Various studies have been revealing the heterogeneity of single cells relative to whole cell populations. This heterogeneity can derive from differences in cell development, cell cycle or cell aging as well as the inherent stochastic character of cellular processes. While regular cell analysis methodologies, such as flow cytometry, delivers averaged data on a cell population, cell heterogeneity and its importance in the population response is still concealed. Such findings have led to a transition from bulk to single cell analysis with low-throughput single cell analysis techniques being already extensively used. While this approach is useful to understand events in individual cells, to extract statistically relevant information from a cellular level onto a population level, a huge number
of cells must be analyzed. This challenge of developing high throughput single cell analysis systems has gradually found a solution in the advances of microfluidics and microfabrication techniques.10

The first microfluidic devices were made of silicon and glass using the microfabrication techniques from the microelectronics industry. These techniques require specialized facilities (clean room with costly and dedicated machinery), very expensive processes and usually a lot of time to produce a device, characteristics that are not compatible with the rapid prototyping necessary for the microfluidics industry.11 Polymers and their potential for mass replication technologies are easy to use materials, reducing the complexity, the cost and the time of fabrication.12 Polydimethylsiloxane (PDMS) is one of the most used materials for the fabrication of microfluidic devices having several properties that make it suitable to work with living cells. PDMS is nontoxic, flexible, biocompatible, porous which allows oxygen exchange and physically transparent allowing the optical monitoring of the processes. PDMS will be used to fabricate every microfluidic device in this thesis.13 At the micro level, the scale factor makes fluid behavior completely different from the macro level. Fluid properties are less influenced by inertial forces and more by viscous forces with Reynolds number (a dimensionless ratio of inertial forces to viscous forces within a fluid) being generally low. With a low Reynolds number the flow is fundamentally laminar with mixing of molecules between two parallel streams occurring only through diffusion.6,14-16 Microfluidic systems have been emerging as the leading technology when it comes to detect, isolate, count, mix, lyse and analyze cells. Their small dimensions enable single-cell manipulation, while reducing the quantity of reagents necessary and providing easy automation and parallelization capabilities. Furthermore, these devices also present a high level of integration and automation of complex processes such as electrical cell lysis and on-chip PCR.15,16

The first step of single cell analysis is to sort the cells from a generally complex and heterogeneous sample. A number of cell capturing methods, each with its own advantages and disadvantages, have been developed. These methods can be divided in active, which use external force fields to manipulate cells, and passive which use cell properties such as size, deformability or density. Electrophoresis, dielectrophoresis17, electro-osmotic flow18 and magnetic19, optical20 and acoustic21 based sorting are all examples of active methods while filtration22 and inertial22 based sorting are examples of passive methods. Active mechanisms are more specific and allow for a more precise control of the cells than passive mechanisms, but they normally require microfluidic devices with complicated features to apply, for example, electric or magnetic fields that increase the time of production and the cost of the device.5,24

The remaining step before being able to analyse a single cell is to isolate them one by one. To this end, several procedures have been developed. Isolation by optical tweezers25, dielectrophoretic trapping26, microwell trapping arrays27 and droplet compartmentalization28 are some examples of the more employed techniques. The more prominent method to isolate single cells and the one explored and optimized in this thesis is hydrodynamic cell trapping. This method is usually performed by creating side channels deriving from a main channel that such a fraction of the total flow. The cells are retained in the side channels due to the channels’ small dimensions. Examples of this are the dam structure reported by Yang. et al.29 where cells are immobilized against the dam structure when a fraction of the flow passes over the dam. The array of cell-pairs trapping channels by Lee. et al.30 where two cells are trapped on opposed trapping channels in order to study their interaction and the trap and release device presented by Tan and Takeuchi31 where the trapping side channels connect a meander-like main channel allowing for massive parallel trapping with a single inlet and outlet.

1.2. Breast Cancer and Circulating Tumour Cells

Breast cancer remains one of the leading causes of death in woman but a steady declined on the mortality rate has been reported over the past decades. This decline has been mainly influenced by the early detection of tumours which have a better prognosis, result of the increasing adoption of mammography screening. Advances in adjuvant therapies like radiotherapy, chemotherapy, immunotherapy or hormone therapy have also greatly contributed to this cancer’s mortality rate decline.32,33

Despite the advances on molecular characterization allowing for outcome prediction with gene-profiling models, therapeutic decisions are still based on readily available tumour markers as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).34,35 According to tumour grade and biomarker expression breast cancer can be categorized as luminal A, luminal B, HER2 enriched and triple negative, with each of the categories having specific treatment and possible prognosis.36 The prognosis of cancer patients is predominantly influenced by the formation of metastases, with 10 to 15% of patients with breast cancer developing distant metastases within 3 years of primary tumour detection.37 Metastases are caused by the dissemination of tumour cells from the primary tumour to distant sites through the lymphatic system of the peripheral blood. Tumour cells with epithelial origin circulating in the peripheral blood are called circulating tumour cells (CTCs) and their presence has been confirmed in several cancers including breast cancer.38 These cells have been demonstrated to be present not only in patients with metastatic disease but also in those with localized tumours.39

The monitoring of cancer progression is normally done by biopsy, an invasive procedure, very limited in location as there are anatomically inaccessible tumours and not well accepted in the clinic due to the high risk of post-biopsy complications. An alternative approach is to analyse blood samples for circulating tumour cells, a liquid biopsy. Liquid biopsies are a minimally invasive test that can be done repeatedly, allowing for real-time monitoring of the cancer evolution. CTC enumeration has in fact been correlated to chemotherapy response, having the possibility to provide regular insights into the effectiveness of the treatment.40-42 Several research groups have been reporting on the clinical relevance of detecting and enumerating CTC’s to predict progression-free survival and overall survival of cancer patients. The results show that the presence of five or more CTCs in 7.5ml of blood is associated with a shorter median progression-free survival and overall survival, regardless of the time of detection or the stage of breast cancer.43-45 The detection and analysis of CTCs in the peripheral blood of cancer patients holds great promise but, with the number of CTCs being as low as one cell per millilitre of blood, among billions of red blood cells and millions of white blood cells and platelets, CTCs unbiased and reliable isolation still represents a huge challenge.45,46

Among the technologies developed to sort CTCs, only CellSearchTM has been cleared by the U.S. Food and Drug Administration (FDA) for clinical use. CellSearchTM uses magnetic nanoparticles functionalized with antibodies against the epithelial cell adhesion molecule (EpCAM), overexpressed in epithelial cancer cells. Magnetically labelled CTCs are then sorted by applying a magnetic field. This system is currently
considered the gold standard in capturing and enumerating CTCs but there are limitations to it.

CTCs that undergo epithelial-mesenchymal transition (EMT), where epithelial cells lose their characteristics and acquire the mesenchymal phenotype, have been proved to be involved in tumour dissemination and the metastatic process. This cells represent an important potential biomarker for metastases however, their capture by the CellSearchTM system is very low since they no longer overexpress the EpCAM marker.  

The system has also been reported to have low purity and to perform worse than the most recent developed technologies. Other platforms to sort and enumerate CTCs depend either on biological characteristics, with markers like N-Cadherin and VCAM-1, or physical properties as size, deformability and dielectric properties.  

The next generation of CTC oriented devices, like the one this thesis aims to develop, intends not only to isolate the cells but also to characterize their properties. Molecular and functional characterization of CTCs will enable researchers to better understand the metastatic process, with single cell whole-genome sequencing already available. It will also enable the monitoring of the evolution of the disease and its treatment, paving the way to therapy personalization.

The development of cheap, fast and reliable devices capable of characterizing CTCs is increasingly urgent. Recent studies indicate that patients with HER2 negative primary tumours can develop CTCs with HER2 phenotype. In this case, the monitoring and characterization of the HER-2 status can have profound impact in the treatment of cancer patients, with positive responses to HER2 chemotherapy, Trastuzumab, already reported by patients with HER2 negative primary tumour.

2. Materials and Methods

2.1. Microfluidic Device Design

The design of the microfluidic device employed in this work is based on a device reported by Van der Brink et al. The reported device design was modified in order to achieve a higher trapping efficiency and to simplify its fabrication and operation. The device was designed to trap single cells in 16 different locations where single cell analysis can be performed. The device has 2 inlets corresponding to the number 1 and number 2 in figure 2, which join in the main channel (50µm width and height) and 2 outlets, the number 3 (also called side outlet), where the 16 side channels (50µm height and width varying between 25µm and 50µm) associated with each trap end, and number 4 (also called main outlet), where the main channel ends. Each of the traps, depicted in figure 1 is composed of a pocket (10µm radius, 50µm height), where the cell will be trapped, and a constriction (2µm height, 10µm length and a width of 2µm or 4µm) that prevents cells from going to the side channel.

<table>
<thead>
<tr>
<th>Design Code</th>
<th>Side Channels Width (µm)</th>
<th>Constriction Width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[25µm, 2µm]</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>[25µm, 4µm]</td>
<td>25</td>
<td>4</td>
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<tr>
<td>[50µm, 2µm]</td>
<td>50</td>
<td>2</td>
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<tr>
<td>[50µm, 4µm]</td>
<td>50</td>
<td>4</td>
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Table 1 - Dimensions of the two variables of the device with the code used to refer to each of the designs.

To find the optimal dimensions for the device, four different designs were developed with two variables, the side channel width and the constriction width, varying between two values. The width of the trap constriction was 2 µm or 4 µm while the side channel width was 25µm or 50µm. The four design dimensions are indicated in table 1.

2.2. Microfluidic Device Fabrication

To have a functional microfluidic device ready for testing, standard and soft lithography techniques were applied. The design of the devices’ photomask was drawn in “CleWin” software (PhoniX software, Enschede, the Netherlands). According to this design, two chromium/glass photomasks were fabricated by the company Delta Mule BV. The need for two photomasks arises from the height differences between features of the chip (2µm height for the constrictions and 50µm height for the channels). Two photolithography steps were performed, the first with a 2µm layer of SU-8 2005, a more fluid version of SU-8, to form the constrictions and the second with a 50µm layer of SU-8 50, a dense version of SU-8 that allows for thicker layers, to form the other channels. The two layers were aligned against aluminium alignment marks deposited on the silicon wafer before the process. The final silicon wafer with the SU-8 mould was cleaned with 10% nitric acid (Sigma-Aldrich, St. Louis, USA) for 10 minutes and coated with perfluorodecyltrichlorosilane (FDTS, Sigma-Aldrich, St. Louis, USA) to ease the later release of the final device.

The PDMS microfluidic devices were fabricated by soft lithography using the previously prepared SU-8 mould. The PDMS mixture was stirred and degassed through vacuum for 1 hour before being dispensed at the centre of the SU-8 mould. Degassing was performed again for 5 minutes in order to take out the pouring generated air bubbles. The mould with the PDMS was transferred to an oven at 60°C and left for 16 hours to cure. The PDMS wafer was then detached from the mould and tape applied to this surface in order to protect it from dust before being used. Each chip was cut from the PDMS wafer with a surgical blade and connection holes from the surface to the channels were made with a 2mm puncher.

The PDMS chip was then bonded to a glass substrate using oxygen plasma. In order to do this the chip and the glass were exposed to a low intensity oxygen plasma for 40s at 2 mbar. The plasma reacts with the PDMS and the glass surface creating SI-OH bonds. After the exposure,
the chip was manually attached to the glass substrate and SI-OH bonds recombine and form an irreversible covalent bond SI-O-Si between the glass and the PDMS. Immediately after bonding, filtered PBS is inserted in the channels in order to preserve the hydrophilicity of the PDMS chip. The top part of the chip was covered with tape to protect against dust until the chip was used.

2.3. Cell capturing protocol

Three protocols were tested for cell capture; the positive pressure protocol and the passive pumping protocol were not successful. The cell trapping protocol used in this work was the negative pressure protocol. The hypothesis with this protocol is that, by applying controlled negative pressure in both outlets, the cells inserted in the inlet number 2 will be individually trapped in the pockets as a fraction of the solution containing them will be sucked into the side channels. This is facilitated by the flow focusing of the cell solution towards the channels’ wall containing the pockets. In the negative pressure protocol, a vacuum pump was connected to the MFCS-EZ FLUKENT system which was then connected, using two different channels, to two containers of a FLUIWELL 4C with tubing (OD:3mm Cole-Parmer, Illinois, USA). In these experiments one container was connected to the side outlet and another to the main outlet of the PDMS chip with 1.52 mm OD tubing (Cole-Parmer, Illinois, USA). The negative pressure applied to each channel was controlled individually through the MAESFLO software. A drop of PBS (7µL) was inserted on top of the inlet number 1 and a drop of the solution to be tested (7µL) on top of the inlet number 2. When this volume of liquid was sucked into the inlet, another drop was added right away in order to have a continuous flow of liquid. The PDMS chip was always positioned on an OLYMPUS microscope from where the experiments were monitored. The schematic of the chip during the negative pressure protocol can be seen in figure 2.

2.4. Cell culture

Two human breast adenocarcinoma cell lines were extensively used in this project, MCF-7 (diameter:19.1 ± 6.4 µm) and SKBR3 (diameter:18.1 ± 4.7 µm). Both these cell lines were gently provided by the department of Medical Cell BioPhysics of the University of Twente. Human astrocytes isolated from human cerebral cortex (ScienCell, California, USA) and HUVECs obtained from pooled donor (Cascade Biologics, Thermo Fisher Scientific, Waltham, USA) were used to show that the microfluidic device was capable of trapping and analysing cells with different origins and characteristics, its use not being limited to breast cancer cell lines. All cell lines were cultured in an incubator at 37°C with a humidified atmosphere containing 5% CO2 (v/v). The cells were passaged to a new flask with a total volume of 7µL of this dye solution was added to inlet number 2, 16 cells instead of a full well, 7µL at a time, while PBS was continuously added to inlet number 1 in order to take advantage of the flow focusing characteristics of the device.

2.6. On-chip live/dead staining experiments

To determine cell viability inside the chip two dyes were used, Calcein and Ethidium homodimer (EthD-1). Since the target of the dyes were only 16 cells instead of a full well, the stock solutions of Calcein AM (4 mM) and EthD-1 (2 mM) were diluted in PBS with a dilution factor of 20000. A total volume of 50µL of this dye solution was added to inlet number 2, 7µL at a time, while PBS was continuously added to inlet number 1 in order to prevent backflow from the device.

2.7. Immunocytochemistry on-chip

All the steps of the immunocytochemistry protocol were done with the chip. All the solutions were inserted in both inlets to achieve a constant flow. A volume of 7µL of cell solution was then introduced in inlet number 2 and the flow adjusted with the Fluigent system to the negative pressure values to be tested. Filtered PBS and culture medium were introduced when needed in the inlets number 1 and 2, respectively, in order to have a constant flow, until 16 cells entered the main channel (only one cell for each trap is introduced in the main channel in order to calculate the trapping efficiency of the chip). Afterwards the data on the number of cells trapped was collected and the process ended. When using the same chip for multiple experiments, the chip was cleaned inserting PBS on both inlets and varying the negative pressure from low to very high values (800mbar) until all the traps were clean. Only chips deemed completely clean (no cells or debris inside the channels) by microscopy observation were reused.
added to the second inlet to take advantage of the flow focusing characteristic of the device. A PBS solution was in every step being added in the same volume to the first inlet. The protocol for on-chip immunocytochemistry started by washing the chip with 5% BSA diluted in PBS in order to coat the microchannels and remove possible air bubbles. PBS was added to the inlet number 1 and SKBR3 cells in culture medium to the inlet number 2. The cells were captured and then fixed with 33µL of fixative solution (10 minutes incubation). A washing step was done with 66µL of PBS and then the cells were permeabilized by flushing 33 µL of permeabilization solution through the chip (5 minutes incubation). After another washing step, 66µL of blocking solution was introduced and remained in the chip for 25 minutes. The solution of Monoclonal mouse anti-HER2 (Her-81) (50µL) was added and remained in the chip for 50 minutes. This was succeeded by another washing step and the introduction of 50µL of Alexa Fluor® 594-conjugated donkey anti-mouse solution, as a secondary antibody, with an incubation time of 30 minutes. Finally, after another washing step, the cell nucleus was stained with 50µL of 1:100 DAPI solution (5 minutes incubation).

### 3. Results and discussion

#### 3.1. Bead trapping experiments

To test the trapping efficiency of the devices, Dextran and polystyrene beads were used as a model of human cells. The trapping efficiency was defined, as the percentage of beads trapped of the total number of beads injected in the chip.

##### 3.1.1. Trapping efficiency of the different device designs

The trapping efficiency of the four designs of the chip was tested with polystyrene and Dextran beads with a constant negative pressure of 10mbar in the main outlet and 100mbar on the side outlet as a starting point. A flow focusing of 50% was maintained. The Dextran beads had 100% trapping efficiency on three of the chips tested, exceeding the efficiency of the polystyrene beads on these devices. The [25µm, 2µm] design had the worst results. It was found that all the repetitions of this design, which has the smallest features, were getting damaged in the SU-8 mold with each round of fabrication. Since the Dextran bead experiments were done after all the polystyrene experiments, it was expected that the chips would have a worse performance, with some of them not working at all. The lower trapping efficiency of the polystyrene beads was attributed to their size being 5 to 15µm smaller than the Dextran beads. Having a flow focusing of 50%, the bead solution was focused in half of the main channel, 25µm. The polystyrene beads, with 15µm, could be at a maximum distance of 10 µm of the trapping pockets while the Dextran beads, with a minimum dimension of 20µm, could be at a maximum distance of 5µm. With the increase in distance from the trapping pockets there was a decrease in the suction force which caused the volume near the frontier of the liquids to flow to the main outlet instead of flowing through the pockets where the beads would get trapped.

During the experiments, unwanted debris of large size were clogging the main channel of the chips, this was solved by using tape to clean the chip surface instead of dust free laboratory wipes. After implementing this change, it was found that small debris were still clogging the constriction part of the side channels, with more incidence on the 2µm constrictions. These debris were found to originate from the bead solution with the number of debris increasing if a bead solution was used for a high number of experiments. To reduce this problem each bead solution was used for only 6 experiments, after which a new solution was prepared.

##### 3.1.2. Trapping of multiple beads per trap

During the trapping efficiency experiments it was found that beads could get trapped in multiples of instead of a single bead per trap. It was noticed that one trap might contain as much as five beads. To understand to what extent the trapping of multiple beads per pocket could be a problem, more experiments were conducted. A constant negative pressure of 10mbar in the main outlet and 100mbar on the side outlet, as well as a flow focusing of 50% was employed in these experiments. It was found that polystyrene beads were more susceptible of being trapped in multiples than Dextran beads. This difference, seen in every chip design, is thought to be caused by the fact that Dextran beads are more deformable. When trapped, this type of beads will better occlude the trap and thus decrease the effect of the side negative pressure in the main channel. It should be noticed that the trap is not completely occluded, thus the high level of multiple bead trapping with both types of beads.

In order to try to stop the trapping of multiple beads per pocket, the experiments were repeated with 75% flow focusing of the bead solution. The hypothesis was that, by limiting the flow of the bead solution to 25% of the main channel, it was possible to reduce the multiple trapping since the limited space would prevent the beads from piling up, being transported with the flow to other pockets. The result was a reduction in the number of beads accumulated per trap, instead of one trap with three beads there were two traps with two beads each. Using a 75% flow focusing reduced the multiple bead trapping problem but did not solve it. For the convenience of not having to measure the punching spot to achieve a 75% flow focusing every time a test was performed, a 50% flow focusing was adopted in the following experiments. With the data from the previous experiments it was decided to exclude two of the designs. The [25µm, 2µm] design was excluded since most of the SU-8 mold repetitions of this design were damaged. The [50µm, 2µm] design was excluded since the 2µm constriction of this design made it extremely vulnerable to be clogged by unwanted particles. Even after implementing cleaner methods, like the aforementioned limited number of use for a bead solution, small particles would still clog some of the 2µm constrictions.

The polystyrene beads were also excluded, from the previous experiments it can be seen that this type of bead has a worse trapping efficiency and is more susceptible to be trapped in multiples than Dextran beads. The polystyrene beads are also less deformable than Dextran beads hence being a worse model for human cells.

##### 3.1.3. Trapping efficiency of dextran beads at different pressures

The previous experiments were all done with a constant negative pressure of 10mbar on the main outlet and 100mbar on the side outlet. In this experiments, the negative pressure value applied in the side outlet was varied and its influence on the trapping efficiency of Dextran beads was tested. A constant negative pressure of 10mbar on the main outlet and a flow focusing of 50% were maintained during this experiments. A trapping efficiency of 100% was achieved with both chip designs for all the side outlet negative pressure values tested. The chip was, at this point, considered optimized with all the beads injected in the chip, one bead for each pocket, being trapped. Multiple trapping still occurred, hence some of the channels were left empty in the end of the experiment. For
convenience, only the [25µm, 4µm] chip design was chosen to continue experiments.

The beads provided a cheap and fast way of testing the microfluidic devices, allowing the selection of the best experimental protocol and the understanding of some fundamental characteristics of the device design as the difference in hydrodynamic resistance of the side channels and the main channel. Despite this advantages, the use of beads was not a reliable model of human cells, as will be seen in the next set of results. Optimizing the devices with beads led to the assumption that the negative pressure values were optimized to always capture 100% of the cells injected but the problem with the trapping of multiple cells in a single trap remained. Both of these assumptions turned out not to be true as will be seen ahead. Beads should be used to perform initial tests on microfluidic devices but no optimization step should be done if the use of cells is the final goal of the device.

3.2. MCF-7 Cell experiments

3.2.1. Trapping efficiency of MCF-7 cells at different pressures

Having optimized the microfluidic device for 100% bead trapping efficiency, the next step was to test if the trapping efficiency of the same protocol remained the same when using a solution containing CTCs. A constant negative pressure of 10mbar on the main outlet and a flow focusing of 50% were maintained during this experiments. The [25µm, 4µm] chip design was employed. The results of the trapping efficiency experiments using different negative pressure values on the side outlet are depicted in figure 3. Both -100 and -80mbar side pressure values had 100% trapping efficiency, all 16 cells injected in the device were trapped. However, it was found that at this negative pressure, the cells would remain on the pocket just a few seconds before being sucked through the constriction towards the side outlet. Applying -50mbar on the side outlet resulted in an 94% average capture efficiency, very similar for -40 and -30mbar that had an average trapping efficiency of 98%. Having -20mbar applied on the side outlet resulted in only 48% of injected cells being trapped. This low trapping efficiency is thought to be due to this negative pressure value not being enough to counter the effect of the higher hydrodynamic resistance in the side channels compared to the main channel. This higher resistance favors the flow of the solution to the main outlet instead of the side outlet, inhibiting cell trapping.

With a negative pressure of -30mbar on the side outlet, an average single cell trapping efficiency of 98% was reported, with the goal of 100% efficiency achieved in some of the experiments. This results represent a big improvement compared to other devices that intend to capture and analyze single cells, such as the average single cell trapping efficiency of 40.1% reported by Oechetta et al.13 or the 83% reported by Riahi et al.52. The previous results using a similar device, reported by Van der Brink et al.39 in 2011, were also improved, with the highest single cell trapping efficiency achieved exceeding the previously reported 85% to the value of 100% cells trapped.

Regarding the multiple cell trapping problem, Van der Brink et al.39 reported an average of 29% of cells trapped in multiples while Oechetta et al.13 reported this value to be 4.4%. The microfluidic device designed in this thesis was able to completely eliminate the multiple trapping of cells. This is thought to be caused by the total occlusion of the trap’s constriction by the cells which removes the effect of the side negative pressure in the main channel.

From the cell behavior experiments it was found that, with a side negative pressure of 100mbar the cells are sucked into the outlet after approximately three minutes by squeezing through the constriction into the side channel. The same happens with a side negative pressure of 50mbar where the cells take approximately four minutes to be sucked into the outlet. This behavior makes it impossible to perform any following analysis on the cell since the time frame to do so would be extremely limited, in addition to the decreasing percentage of cytoplasm present in the pockets. With the last pressure value tested, -30mbar, the cells were captured and remained in the pocket even when the pressure applied on the outlets was inverted. This pressure value was selected to continue experiments even though there was a small portion of the cell’s cytoplasm inside the constriction that raised questions about the viability of the trapped cells. This questions will be addressed in the next set of experiments. This results are in agreement with the literature, a similar behavior of the cells deforming into a trap’s constriction and becoming strongly attached to it was already reported by Van der Brink et al.39, while Adams et al.45 reported that low negative pressure values must be kept, in order to preserve cells’ morphological structure when trapped in a pore.

3.2.2. Trapping behavior of MCF-7 cells at different pressures

On the previous experiments, it was found that the cells had different behaviours when trapped at different pressures. The cell behaviour of MCF-7 cells was studied at 3 different side outlet negative pressure values: 100mbar, 50mbar and 30mbar. The experiments were performed with the [25µm, 4µm] design, using a constant negative pressure of 10mbar on the main outlet and a flow focusing of 50%.

With the previous experiments, cell viability questions were raised by the deformation undergone by the cells when sucked into the 4µm constriction to the side channels. To address these questions, live/dead staining experiments were performed using calcein and ethidium homodimer (EthD-1). The experiments were performed with the [25µm, 4µm] design, a constant negative pressure of 10mbar on the main outlet and 30mbar on the side outlet and a flow focusing of 50%. The negative pressure used to trap the cells made them deform when entering a 4µm

![Trapping efficiency of single MCF-7 cells with different negative pressure values applied in the side outlet. N – number of experiments.](image)
constriction leading to the side channels. Despite this deformation, all the cells trapped with the negative pressure tested, 10mbar on the main outlet and 30mbar on the side outlet, had the intensely green fluorescent calcine inside their cytoplasm and no EthD-1. Since only viable cells with an intact membrane have active esterases that turn the non-fluorescent calcine AM into its fluorescent counterpart and keep out the membrane-impermeant EthD-1, we can conclude that all the cells trapped with this optimized protocol are viable.

3.3. SKBR3 Cell experiments

3.3.1. SKBR3 cells trapping efficiency and behaviour experiments

Having optimized the chip and the pressure applied in order to have a protocol capable of trapping single viable cells. The next step was to prove that the microfluidic device designed can perform single cell analysis on CTC’s. For this purpose, the phenotype of cancer cells was characterized through immunostaining of specific protein biomarkers. The membrane receptors ER, PR and HER2 were considered as possible targets since their presence in CTCs is of fundamental importance for therapeutic decisions. Having tested the microfluidic devices with the MCF-7 cell line, both ER and PR could be the target since this cell line is known for their overexpression but the protein HER2 was the chosen biomarker due to its therapeutic significance, with recent studies discovering that patients having negative HER2 primary tumours can develop HER2 positive CTCs.14–18

This circumstances led to the use of a new cell line known for overexpressing the HER2 receptor, the SKBR3 cell line.44 Before performing any immunostaining experiments the trapping efficiency and behavior of this cell line on the microfluidic devices had to be tested. The previous optimized conditions were used, the experiments were performed with the [25µm, 4µm] design, a constant negative pressure of 10mbar on the main outlet and 30mbar on the side outlet and a flow focusing of 50%. The results obtained were the same as the MCF-7 cell line. The trapping efficiency after six experiments with the previous optimized conditions was on average 98%. The SKBR3 cells also had the exact same behaviour as the MCF-7. With the previously optimized pressure, the cells were captured and remained in the pockets even though there was a small portion of the cell’s cytoplasm inside the constriction that led to the side channel. The use of another cell line for the immunostaining experiments was not expected in the beginning of the project but ended up supporting it by showing that the results were not dependent on the cell line. The already optimized conditions for the MCF-7 cell line were also found to be optimal for the SKBR3. The trapping efficiency was observed to be the same in both cell lines as well as the behaviour upon trapping of the cells. This can be explained by both cell lines having similar characteristics, both deriving from a human breast adenocarcinoma and having similar size.

3.3.2. Immunocytochemistry of HER2 Receptor in SKBR3 cell line

With the protocol optimized to maximize the single cell trapping efficiency of SKBR3 cells, the phenotype of this cell line as a model of CTCs was characterized through immunostaining of the membrane receptor HER2. A monoclonal mouse anti-HER2 (Her-81) antibody was used as a primary antibody followed by the detection of the fluorophore-conjugated secondary antibody Alexa Fluor® 594-conjugated donkey anti-mouse. The immunocytochemistry protocol was optimized off-chip with the controls for both the primary and secondary antibody and only after the procedure was executed on-chip. The cell nucleus was stained with DAPI to facilitate the localization of the cells. The results were observed in the EvosTM FL Imaging System fluorescence microscope. After accessing the primary and secondary antibody response and performing the nonspecific binding controls for both antibodies, the immunocytochemistry protocol was performed on-chip.

SKBR3 cells were trapped using the previous optimized conditions: the [25µm, 4µm] device design, a constant negative pressure of 10mbar on the main outlet and 30mbar on the side outlet with a constant flow focusing of 50%. Figure 4 shows four SKBR3 cells single trapped and immunostained for HER2 during three different experiments. The red fluorescence reveals the position of the primary antibodies connected to the targeted receptor and is emitted from the fluorophore-conjugated secondary antibody.

![Fig. 4 - Three images of single trapped SKBR3 immunostained for HER2. Red fluorescence from the Alexa Fluor® 594-conjugated secondary antibody. Cell nucleus is stained blue with DAPI.](image)

With this experiments it was proved that it was possible to analyze single cells trapped on the designed microfluidic device. More specifically to characterize the phenotype of SKBR3 cells as a model of CTCs through immunocytochemistry targeted at a membrane receptor. Regarding the one-chip immunocytochemistry, the adapted protocol worked well from the first experiment. When solutions needed to stay in the chip for several minutes the negative pressure was switched to zero on both outlets and the cells remained in the pockets. The full procedure should be done under the microscope where the final results will be observed, an attempt to move the chip with the immunostained trapped cells to another microscope was done without success. The negative pressure derived from removing the tubing of the outlets was too high, displacing the cells from the pockets.

The molecular and functional characterization of CTCs is a hot topic in cancer research.56 The phenotype characterization performed is an example of the potential of this type of microfluidic devices. The HER2 phenotype of CTCs is one of the most important characteristics to be monitored. Recent studies indicate that with the progression of the disease, patients who have negative HER2 primary tumours can develop HER2 positive CTCs. This status shift between the primary tumour and its CTCs can also occur in the opposite direction, meaning that patients with HER2 primary tumours can develop HER2 negative CTCs. If monitored and recognized on time, this status shift can provide clinicians the
information necessary to adjust patient treatments with positive results already reported in the literature.54–58

The continuous research in this area will represent a paradigm shift, where cancer treatment response can be regularly monitored through real-time, minimally invasive CTC isolation and characterization, which will lead to therapeutic personalization and prognosis improvement.

3.4. Other cell line experiments

To illustrate the potential of the microfluidic device designed in trapping and analysing cells with different origin and characteristics, behaviour and viability tests with HUVECs and human Astrocytes were performed. The trapping efficiency of this cell lines was not tested due to time constraints. The experiments were performed with the optimized conditions, using the [25µm, 4µm] design, a constant negative pressure of 10mbar on the main outlet and 30mbar on the side outlet and a flow focusing of 50%. This experiments show that the microfluidic device designed can be used for applications with different cell lines. Both HUVECs and human astrocytes were easily trapped with the optimized protocol but optimization of the pressure and flow focusing parameters might be needed to improve the trapping efficiency. The cells were in the pockets for periods of time longer than one hour. Experiments which last for more than one hour and need the cells to be viable were not performed but should be possible if a pumping system with cell medium is connected to the chip.

4. Next steps

The microfluidic device designed in this thesis should be tested with actual CTCs. The CellSearchTM system or similar cell isolation platform should be previously used to separate CTC’s from a patient’s blood. This CTC’s should then be inserted in the device for single cell analysis.

This chip can also be used to isolate CTCs directly from blood by using size based sorting. Size based sorting is considered to be a method of high sensitivity, being very efficient at recovering CTC from the blood but of low specificity since they also capture other blood cells. Erythrocytes have a diameter of 5-9µm and have been reported to 100% transverse pores of 3.3µm diameter, not being a problem for our current chip. The problem with using size sorting methods is the overlapping size of CTCs with white blood cells.22 This problem can be addressed by inserting electrodes on the bottom of each trap to measure cells’ electrical impedance. It is known that there is a difference between WBC and CTCs membrane impedance which can be used to differentiate this two group of cells.25,26 If each trap is connected to their own controllable source of negative pressure, it is possible to automate the device to suck every trapped WBC to the side outlet or remain constant if a CTC is trapped.

Special attention should be taken to the concentration of blood cells since the system must be able to go through the process of measuring the electrical impedance and decide to suck or keep the cell, fast enough for every cell with the size of a CTC to be analysed. This problem can be addressed by increasing the quantity of traps on the device. Studies report that, for similar devices, the trapping efficiency and the experimental reproducibility is consistent either using a solution only with CTCs or human blood spiked with cells.22 This represents a good reason to believe that the developed device can be used to directly isolate circulating tumour cells. The microfluidic device has also been proved to work with cell lines other than cancer cells. This makes this device potentially useful for an endless array of applications in all areas of research that perform single cell analysis.

5. Conclusion

In this thesis, a microfluidic device for single cell analysis was designed that presents an improvement on the average trapping efficiency regarding the state of the art devices. During the work, an optimization in terms of protocol and specific parameters was done. The use of beads provided a cheap and fast way of testing the microfluidic devices, allowing the selection of the best experimental protocol and the understanding of some not expected characteristics of the design such as the difference in hydrodynamic resistance of the side channels and the main channel. Despite the advantages, the use of beads was not a reliable model of human cells and should only be used to perform the initial tests of microfluidic devices, no optimization step should be done with beads if the use of cells is the final goal of the device.

It was proved that with this microfluidic device it is possible to isolate cells with an average single cell trapping efficiency of 98%, with the cells remaining viable. The cell lines possible to trap and analyze are not limited to cancer cells, experiments with HUVECs and human astrocytes were made with positive results. It was also proved that it is possible to perform single cell analysis in this device. Immunocytochemistry was done to characterize the phenotype of CTCs by targeting the membrane receptor HER2. The characterization of this phenotype of CTCs has tremendous implications, with recent studies indicating that during the progression of the disease, patients who have negative HER2 primary tumours can develop HER2 positive CTCs and vice versa. Using the developed microfluidic device, with which cancer treatment response can be regularly monitored through real-time, minimally invasive CTC isolation and characterization, this status shift between the primary tumour and its CTCs can be monitored and recognized on time, providing clinicians the information necessary to make therapeutic changes that can profoundly impact patient prognosis.

The continuous research in this area will represent a paradigm shift, with cancer treatment response being regularly monitored and therapeutic personalization achieved. Furthermore, the microfluidic device designed has the potential to become the only lab-on-a-chip device needed to monitor cancer treatment if the research on CTCs sorting and full automation of the device is pursued and accomplished.

REFERENCES