TÉCNICO LISBOA

Development and optimization of a microfluidic device for cell culture and personalized therapy of Cystic Fibrosis

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Abstract

Cystic fibrosis is the most common disease among the Caucasian race and leads to precipitated mortality. It is a genetic disease, which is transmitted by autosomal recessive inheritance affecting the long arm of chromosome 7. Many mutations that can cause this disease have been described, but the most common mutation is F508del, which affects the production of Cystic fibrosis transmembrane conductance regulatory protein (CFTR). This protein has the function of a channel of chlorine ions, so that, due to its malfunction or lack, the exchange with sodium ions can´t be carried out. This directly affects the hydration of the epithelium, producing thicker secretions and viscous and ultimately causes infections. We speak of a multisystem disease, because it affects all epithelia.
The microfluidic technology is currently under development and offers many new possibilities, among them the creation of the "Lab-on-a-chip", a device that integrates laboratory functions in its interior with the intention to simplify some analysis. This paper describes how bronchial epithelial cells from CF patients (CFBE) are cultured within a microfluidic device, and how certain CF treatments can be applied within this device in order to provide a more personalized medicine. The microfluidic device must be designed in the way to meet the needs of the experiment. Furthermore, it is imperative to know and control the parameters and characteristics (temperature, pH, cell density, contamination, etc.) so that the cell culture is viable and the experiment can be carried successfully.

Key words: Cystic Fibrosis, CFTR, CFBE, Lab-on-a-chip, microfluidic device.

INTRODUCTION

The aim of this project is to develop a microfluidic device, or more precisely a "Lab-on-a-chip", to carry out a cell culture inside through the necessary protocols. Benefiting from the cell´s ability to survive inside the device, the intention is to use these devices for the individual evaluation of different therapies based on the correction of the CFTR protein in the nasal epithelial cells of patients with CF.
For the realization of this work, the first step was the development of a microfluidic device and the optimization of the CFBE cell culture inside it, doing the perfusion assays for a few days. The other task previously to the traffic assays, was the control of CFTR protein expression with m-Cherry fluorescence protein inside the device.

Cystic Fibrosis

CF is an autosomal recessive disease, which affects the chromosome 7. More than 1800 mutations have been funded in the CF gen, but not all cause CF. Approximately 40 of them lead to a lack of production or defective production of the CFTR protein. It regulates de Cl ion crossing cellular membranes, working as a chlorine channel.[1][2]
The non-equilibrium between Na and Cl ions, affects to the lumen hydration because of their accumulation. It causes a leak of liquidity in the epithelial surface followed by the dysfunction of the cilium. The mucus produced becomes denser and consequently the bronchial tubes are obstructed as it is possible to see in figure 1.1. [3][4]

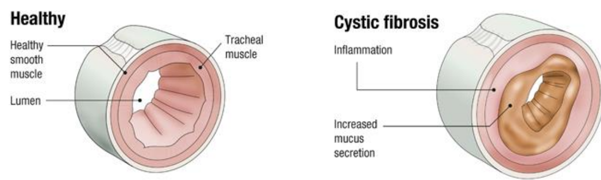

Figure 1.1 - Comparative between a healthy vas and with cystic fibrosis. [30]

There are six different types of CFTR mutation, depending on the level of protein production when the fail takes place.

Category I → Protein synthesis defect.
Category II → Maturation defect to cross to the endoplasmic reticulum.
Category III → Activation blocking.
Category IV → Conduction defect.
Category V → Incorrect connection with the membrane.
Category VI → Regulation defect.

Mutations in categories 1 to 3 are linked with severe problems in the protein production. Otherwise, mutations in categories 4 to 6 are associated with insufficient quantity or bad operation of CFTR protein. The most common mutation is the F508deland, which produces the loss of phenylalanine in the 508 position of the protein, and belongs to the second category. [1][5]

CFBE cells

Cells used in this work are named Cystic Fibroses Bronchial Epithelium cells. It is a cell line originating from the bronchial epithelium of patients with CF, who are homozygous for F508-CFTR. The morphology of CFBE cells changes when they are adhered and making a monolayer (see figure 1.2). Size of CFBE cells is about 10µm of diameter. [6]

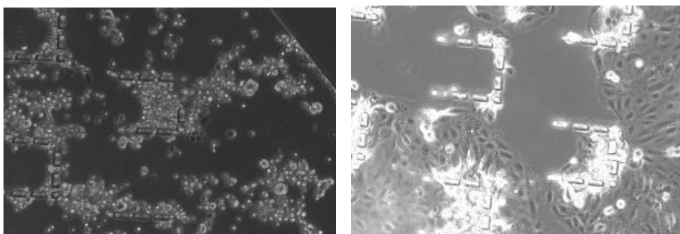

Figure 1.2 - Morphology of CFBE cells before and after adhesion.

For the experimental work performed in this project, two types of cells were used, that were derived from parental CFBE which express no detectable CFTR. The first, CFBE with constitutive wild type CFTR expression, express a normal, functional CFTR protein at the plasma membrane. The second, CFBE mCherry-wt-CFTR, express mCherry labeled wild type CFTR protein upon induction with doxycycline. In these cells, mCherry fluorescence can therefore be used as a marker of whole cell CFTR expression, whereas plasm mamebrane CFTR can be detected using an antibody against the extracellular flag tag (see figure 1.3). Another cell line (CFBE mCherry-F508del-CFTR) has been used to assess correction of the CFTR trafficking defect of the most common CF mutant, but was not used in this study. [7]

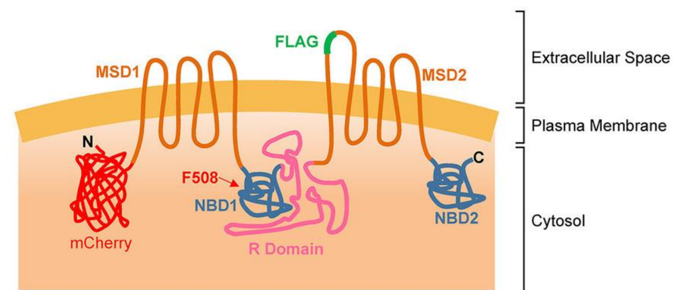

Figure 1.3 - Schematic representation of the CFTR molecule in CFBE mutated cells. [7]

Medical therapies. Modulators

CF is a disease without cure, for now. Being a multisystem disease, makes the treatment more complex. Therefore, there are different therapies that can help patients in their daily life. Depending on the patient different treatments are applied. But, actually, most promising solutions for CF were focused between: protein therapies, directed to correct or strengthen the CFTR protein and gene therapies with the future goal to correct the mutation causing CF.[8]

Modulators are molecules that increase the amount of F508del-CFTR delivered to the cell surface. One example of this is the VX-809 which is a CFTR corrector that can treat the CF type two.[9][10][11]

VX-809 works stabilising the N-terminal domain that contains MSD1 and, consequently, its resistant to the endoplasmic reticulum associated degradation. [12]

Microfluidics

Microfluidics technology appears in the beginning of the 1980's being a

multidisciplinary field. It consists in the studies, control, and manipulation of fluids in a sub-millimeter scale device.[27][29] Is a powerful instrument to realize analysis with high sensitive, high speed, high work rate and low cost characteristics. Furthermore, it is possible to establish a well-controlled microenvironment for manipulating fluids and particles in a microfluidic device.[13]

The behaviour of fluids in microscale differs from that in the macroscale, so, microfluidics studies how these changes can be useful and exploited for new uses. At small scales, some properties like surface tension, energy dissipation and fluidic resistance take more importance in the system control. Also, the Reynolds number, which determines the type of flux (laminar or turbulent) depending on their value. In channels with sizes smaller than 100μm becomes very low, what means that the flux is laminar, hence molecular transport between fluids is through diffusion. Another important effect is that the relation between the volume and the surface of contact is very high, and consequently, any chemical reaction is accelerated. [14]

The materials used for microfluidics depend on the applications. There exist a lot of materials with different characteristics that can be used. The most typical ones are silicon, glass, elastomers, plastics, or combinations of them. In general, the evolution of microfluidic materials reflects the two major trends of microfluidic technology: microscale research and low-cost portable analyses. In this work, the material used is polydimethylsiloxane (PDMS), because of their biocompatibility, low cost and easy fabrication, well controllable microenvironment and especially, its gas permeability (which is crucial for long-term cell culture).[15]

Lab-on-a-chip

One of all the microfluidics applications is the called "Lab-on-a-chip". This term defines a device which integrates one or several laboratory functions in one single chip. It provides comparison to the traditional use of a petri dish or flask like the flexibility of design which can be adapted for each case, the miniaturization and high throughput experimentation, the real-time analysis and increased sensitivity, the minimization of fluid consumption and the capability to imitate a natural cell environment.

Moreover, LOC technology might someday be the key to powerful new diagnostic instruments that enable to identify patients who should receive the drugs and finally offer them a personalized medicine.[16]

Cell culturing inside microfluidic devices is a big challenge because the techniques to maintain alive the cells and make them grow in vitro, are still in development. There are a lot of items that can affect the experiments, like the device material, surface coating, cell density and cell type, properties as pH, CO2, temperature, and osmolality, such as shear stress, MRT, cell adhesion and medium composition.[17]

One of the most important things for a successful cell culture is to have a proper trapping mechanism. The method used in this work is the hydrodynamics, as it consists only in the variation of the surface topography designed with the objective to trap and immobilize the cells. It is the most useful because it is possible to adapt them to the cells dimensions, and their fabrication is inexpensive.

Another important point to control is an effective adhesion of the cells to avoid them flow out when the medium is introduced. The adhesion is promoted with proteins such as cadherins, integrins and proteoglycans, which help in the interaction between the cells. Also, and before the insertion of the cells, the culture device has to be coated with fibronectin (FN) or collagen type 4, to support the cell´s adhesion with the device.

MATERIALS AND METHODS

Microfluidic fabrication

There exist different methods for microfluidic fabrication, but the most commonly used and the one used in this work is soft-lithography. It involves the replication of a topographically defined structure in a soft elastomer. [18]

The first step in the process of microfluidic fabrication is comprised by designing a prototype of it, with the AutoCAD software. Afterwards, the hard mask was produced transferring the design to a glass with a deposition of Aluminium and spin-coated of

positive photoresist, with the Direct Write Laser machine (DWL). The next step was the fabrication of the mold (figure 2.1), starting with a spin-coated of a negative photoresist. Then the design in the mask was transferred with UV-light irradiation. The PDMS was mixed with a curing agent in a ratio of 1:10, and after 1h in the vacuum chamber, the mix was put in the mold, and baked for 1.30h. At the end, the PMDS structures were separated from the mold, the holes of inlets and outlets were drilled, and the structures were sealed in a glass slide with plasma oxidation treatment in a Harrick Plasma PDC-

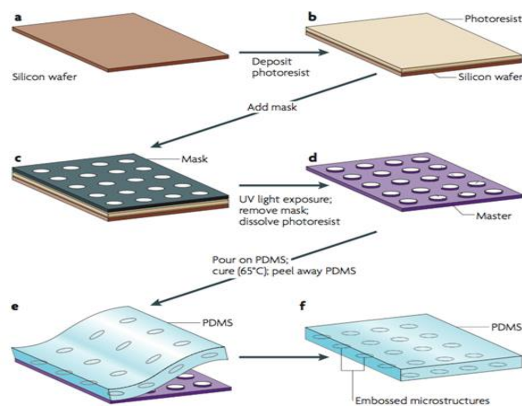

Figure 2.1 – Mold fabrication. [31]

Cell Preparation

CFBE cells were acquired from cell banks where they are frozen to be maintained. It is necessary to thaw the cells adding a cryoprotective agent, called Dimethyl sulfoxide (DMSO), to prevent the formation of ice crystals which can kill the cells during the thawing process. After that, cells were cultivated in the T75 cell culture flask using Eagle's minimal essential medium (EMEM) strengthen with 10% (v/v) of fetal calf serum (FBS), 1% of glutamine and 1% of antibiotic penicillin-streptomycin (pen-strep). The flask was introduced into an incubator until a confluence between 80% and 100% was achieved. Then, a treatment with Phosphate-buffered saline (PBS) to brush away the non-adherent cells, trypsin-EDTA to allow an enzymatic dissociation to separate the cells from the surface of the vessel was applied. After five minutes of incubation, EMEM with FBS was added, which does the opposite action of the trypsin, to provide the cells ready for usage.

Experimental Setup

In the following part, the instruments needed to carry out the experiments were exposed. For cell insertion and perfusion assays, a syringe pump (New Era Pump Systems, Inc.) was used. Syringes of 10mL were applied in perfusion assays and 1mL ones to insert the cells, ethanol, and fibronectin. A filter was used to avoid contaminations and the syringes were connected to the device through a BTPE 90 nm tubing (INSTECH) and with a 20-ga plug insert in the holes. Also an incubator and a hot plate were used, and a microscope equipped with a camera.

Device Operation

The first step was the double sterilization of the device inside a laminar flow. Starting with 70% ethanol (v/v) at 5µl/min during 10 min, following by coating of the channels with a solution of 0,032mg/ml of collagen at 1,5µl/min for 10 min, to promote adhesion. Then the chip was put in the hood for 24h where it was irradiated with UV light. Then EMEM with antibiotics was introduced using a flow rate of 5µl/min for 5 minutes to remove waste and bubbles before the cell insertion. Cell insertion was performed with an initial flow rate of 2µl/min, which was gradually reduced to 0.4µl/min until enough cells where inside the chamber. Finally, all the holes were closed with closed plugs, and the device was introduced in the incubator for 24h. Next step was the perfusion assays, using a flow rate of 1µl/min.

Conditions

One of the most important aspects during the cell culture assays, was to reach the optimal conditions for the cell growth. The essential conditions to control were: the cell density, EMEM, pH, temperature, evaporation and O_2, contamination, MRT and shear stress. [17][24][25]

<u>Cell density</u>: If the density is high, it will be easier to insert cells because the chamber will fill with cells faster. On the other hand, too many cells occupying the chamber encourage a possible obstruction in the channels, what will be a problem during the perfusion assays, because the medium will not flow free. To do the cell insertion in one

device, 0,25mL of suspended CFBE cells were necessary. The density varied in each experiment but always was between 500.000 cells/mL and 1 million cells/mL.

EMEM: The cell culture medium is the most influential component, because as it is composed by salts, buffer substances and nutrients with supplements such as serum, hormones, cytokines, aminoacids, vitamins, antibiotics and antimycotics, which help to control of many parameters talked about, like pH, osmolality, glucose, and obviously, it has the capability to provide the nutrients needed to make the cells grow.

pH: The pH affects the cell viability directly, because can compromise the enzymatic activity of the cells. The pH value, has to be remained in a neutral range between 7.2 and 7.4, what reached by buffering the medium with sodium bicarbonate, ($NaCOH_3$). [26]

Temperature: The optimal temperature in this case was found around the 37ºC, which is the value that guarantees the major survival and grow rate. The control temperature can be acquired thanks to the incubation, or during the perfusion assays, with a hot-plate.

Evaporation and O_2: The temperature at 37ºC causes that the liquid inside the chamber quickly evaporates and, due to the permeability of PDMS, the gas passes through it. Oxygen is essential for the metabolic processes of cell. Hence, when the evaporation is processing, the osmolality of the cells increase and the cells can die. The typical method to control that factor is to introduce the microfluidic device inside an incubator after the insertion of cells, in the first 24h. Nevertheless, during the perfusion days it was impossible to put all the experimental setup inside an incubator, and therefore a continuous flow was used to culture the cells. In addition, the device was submerged in water, with the aim to mimic the conditions inside the incubator and to avoid the evaporation and consequently the bubble formation.

Contamination: During the experiments in this work, a protocol for cell culture inside microfluidic devices was used to avoid contaminations. Starting to seal each PDMS structure in one different glass slide, with the objective to avoid cross contamination. Before the cell insertion, the double sterilization step was realized inside a laminar flow. The first one was made with ethanol and the second was an irradiation with UV light. Successively the cells were introduced in the device and all the holes were closed with plugs, before putting everything in the incubator. The perfusion started with a medium supplied with antibiotics.

MRT: The medium residence time (MRT) is defined as the time needed for the complete change of the medium in the chamber or channel. MRT parameter is used during the perfusion assays to know if the rate of medium renovation is sufficient for the nutrient delivery, oxygen transport and to control the accumulation of waste resulting from the metabolism of cells.[19][20]

MRT is calculated with the equation 3.1:

$$MRT = \frac{V}{Q} \qquad (3.1)$$

Q is the flow rate (in m^3/s) and (V) is, the useful volume obtained from the product of the width (w), length (l) and height (h) (in m). Considering the dimensions of the chamber established, and the flow rate used, 1 µl/min, the MRT obtained using the equations above, is 2,06s, an acceptable time.

Shear stress: The shear stress (τ) of a liquid is the parallel force that exerts on the surface of an object. In this case was calculated to determinate the characteristics of fluid that maintain viable cells because high levels can kill the cells.[20][21][22] It is determined by:

$$\tau = \frac{\mu Q}{w\, h^2} \qquad (3.2)$$

Where μ is the fluid viscosity (in kg/m·s), width (w) and height (h) (in m), Q is the flow rate (in m^3/s).
The shear stress was calculated, accepting the viscosity of EMEM similar to the viscosity of water at 37ºC which is 0,6913mPa·s and the maximum flow rate used in the experiments which was 1 µl/min. The value obtained for the shear stress is

0,286dyn/cm^2. For cells with a diameter of 10µm, the maximum value of shear stress to avoid a prejudicial effect is 0,3dyn/cm^2, consequently the flux used did not affect the cells.

RESULTS AND DISCUSSION

Device design

Two designs were used during this work (figures 3.2 and 3.4). Both have five inlets, one for each liquid introduced in the device, and one outlet (see table 3.1). The chamber has the same size, 1000x1720µm, and the channels are equal, too. They dispose of a width of 208µm and a height of 20µm and have five structures to divide the flux at the entrance of the chamber, with the objective to generate a homogeneous flux in the whole chamber (seen figure 3.1). The first notable differences between the two designs is the size of the traps inside the chamber. The traps in design B are bigger than in A, in order to trap the cells better and to waste less space inside the chamber.[19]

The other difference between both designs is that two channels were added above and under the chamber in design B. Their purpose is to remove the bubbles inside the chamber, pulling them with a syringe. This structures were designed because of the advantage of PDMS, and the property of its permeability to O_2, what enables air, but bot liquid, to cross the polymer. Therefore and to remove the air bubbles, the distance between the chamber and the channels must be really narrow. In this case, it is 50µm, which represents also the dimension that determines the size of this channels. The result obtained by using this channels was positive: if bubbles are in contact with the wall near these channels, they will disappear with an action of pulling and pushing repeatedly in the time. Finally, it has to be concluded that the best results were achieved by using design B.

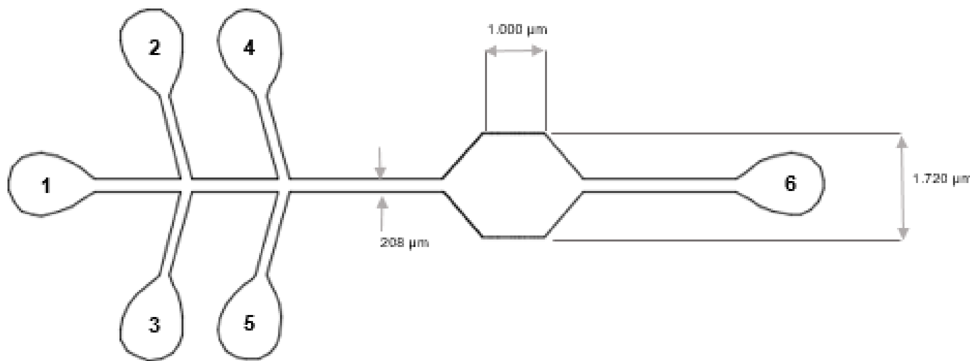

Figure 3.1 - General structure of the device.

Hole	Function
1	Cell inlet
2	Ethanol
3	VX – 809
4	FN
5	EMEM
6	Outlet

Table 3.1 - General structure of the device.

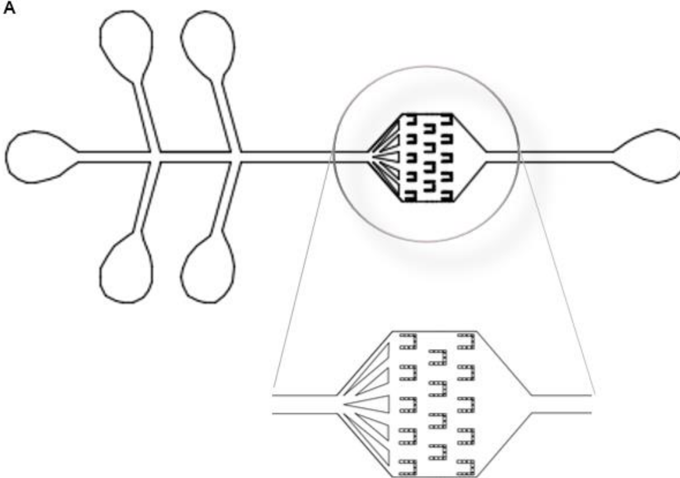

Figure 3.2 - General view of the design A and more detail of the chamber.

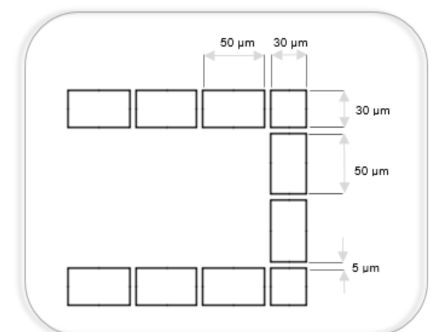

Figure 3.3 - Cell traps in design A.

Design B

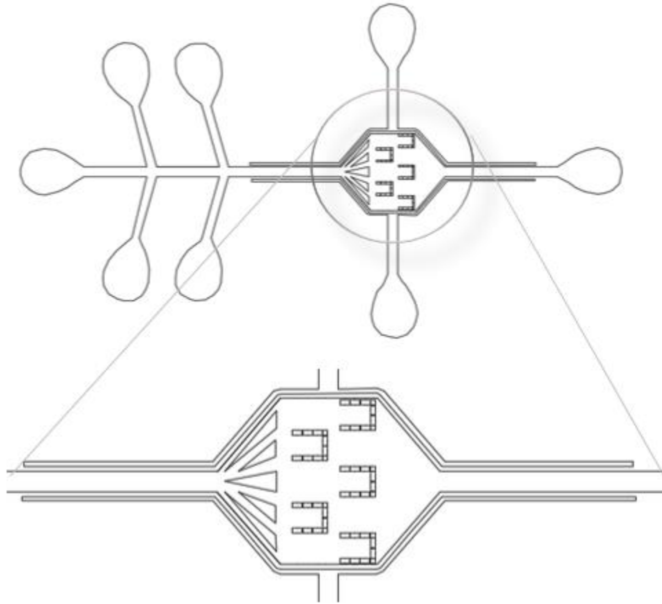

Figure 3.4 - General view of the design B and more detail of the chamber.

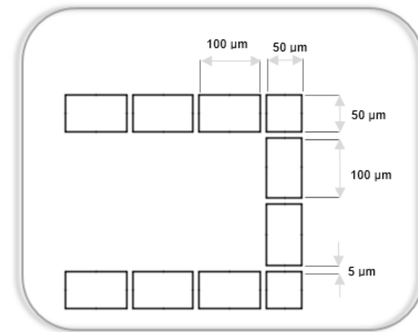

Figure 3.5 – Cell traps in design B.

Perfusion Assays

The perfusion assay was the most challenging step in this work due to the difficulties in the maintenance of the required conditions. After the insertion of cells and after having remained 24h inside the incubator in order to promote the cell adhesion, the perfusion assays start with a daily change of EMEM to guarantee the fresh medium. This process was performed with a 10mL syringe and a flow rate of 1µl/min.[23]

The biggest problem during the perfusion assays was the bubble formation. It is nearly impossible to prevent the appearance of bubbles since they can arise from residual air or spontaneous formation at defect sites, also when the plugs were connected or disconnected. With the control of humidity in the incubation step, bubbles disappear. As it is impossible to introduce all the experimental setup inside the incubator during the perfusion, different solutions were tested. Was very important to make sure that the liquid did not expire during the perfusion and have a good control of temperature. Also, the device was submerged totally in water. The evaporation through the PDMS was avoided with this process, because in both sides of the PDMS wall was water (figure 3.6). It was proved that due to the submerging in water, the bubbles disappeared and the formation of new bubbles was eluded as it is possible to see in figure 3.7. [28]

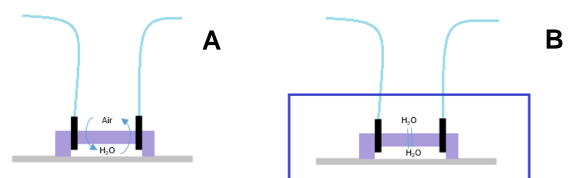

Figure 3.6 - Schematic representation of microfluidic device non-submerged in water (A), and submerged in water (B).

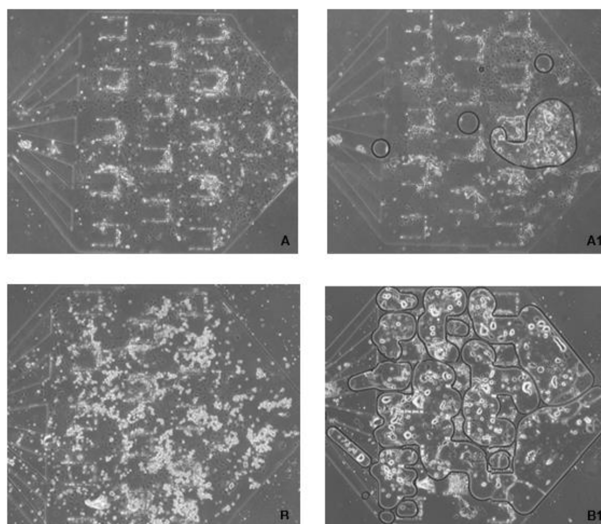

Figure 3.7 - Comparison between the first day of perfusion submerged in water (A) before the perfusion, (A1) after perfusion and the perfusion without submerging the device (B) before perfusion, (B1) after perfusion.

The other proposed solution, was the addition of two additional channels, with the intention to try to pull the bubbles and remove them from the chamber. Two mechanisms were tested: Pulling the bubbles out manually and doing so with a syringe pump. Sometimes they moved a little bit, but they did not disappear. The reason for this result is that it was impossible to pull more than 2mL of air, and as the channel became full of air, negative pressure inside the latter impedes to continue pulling. Similarly, it was tested to pull and push repeatedly during some seconds. It was possible to observe how the bubbles were vanished by the contact with the wall near these channels. The bubbles in the middle of the chamber, did not show these reaction. In their case, the channels also become full of air due to pulling but, as the air is pushed immediately, the exchange causes that the bubbles have some space to cross the PDMS and be eliminated. The image 3.8 are snapshots of the bubble absorption captured in a video.

Figure 3.8 - Bubbles disappearing through PDMS when pull and push the channel (10 seconds).

This method was tested with cells inside the chamber, too. The result was the same as before. To achieve this results, it is necessary to bear in mind that the cells must be totally adhered.

Fluorescence Assays

The aim of the fluorescence assays is to do a step for the future traffic protein assay, showing how it is possible to use a microfluidic device to localize the CFTR protein inside. With the aim, in the future, to be able to see how the proteins change their location when the drug is applied. To do so, the CFTR protein was marked with a fluorescent protein, called m-Cherry, which enables to indicate the exactly position of the CFTR protein. This fluorescence protein has its maximum excitation at 588 nm and the maximum of emission at 611 nm. [24]

CFBE mCherry wtCFTR flag were inserted in the microfluidic device. To notice the expression of mCherry-Flag-CFTR doxycycline (Dox) was introduced to the cells. For the perfusion, the devices were submerged in water. To avoid bubbles.

In 3.10 it is possible to see the fluorescence localized around the nucleus, which suggests that the imaging is not really optimised, because with CFBE-mCherry-WT the signal should be spread around the cytoplasm and visible at the plasma membrane. It is possible to see some differences between both images, in spite of the same cells are used, in 3.9 cells are in the right shape and good adhered, whereas in the other case that they

had not changed their spherical shape. The fluorescence of CFTR labeled with mCherry located in the membrane, it would be possible to see if cells were forming a monolayer and adhered. When this experiments where performed, the cell density was lower than necessary and as a consequence, cells can not form the monolayer, impeding the properly localization of the CFTR. [3][7]

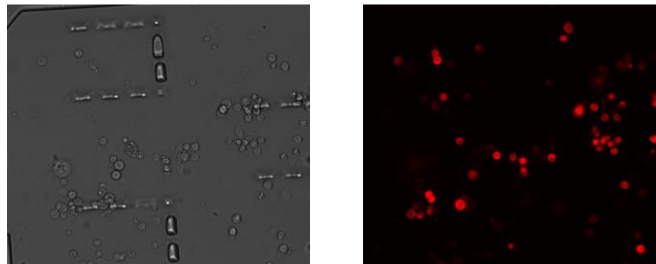

Figure 3.9 - CFBE – mCherry-WT-CFTR-Flag inside the microfluidic device

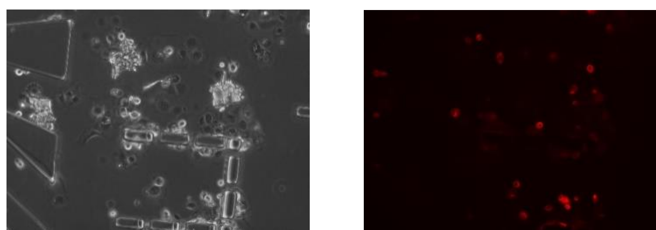

Figure 3.10 - CFBE – mCherry-WT-CFTR-Flag inside the microfluidic device

The next step must be the traffic protein assays but, the execution of VX-809 correction would have required multiple experiments and a statistical approach, that due to time problems was impossible to achieve.

CONCLUSIONS

Nanotechnology, and particularly microfluidic devices, have a really promising future as they can help to improve medical researches. The development of "Lab-on-a-chip" microfluidic devices, open the door to different applications, simulating all the characteristics of an entire laboratory, inside a chip. They offer the possibility to mimic situations of the human body with more accuracy and by reproducing the same characteristics, what enables to reach more realistic results.

In this work, a microfluidic chip was designed and tested with the aim to improve the cell culture inside it. Furthermore the objective was to stablish the protocols in order to maintain the cells alive during a few days by controlling the variables such as pH, temperature, the amount of O_2. The microfluidic device was designed in a way make it possible to culture the cells in its interior. It has to be mentioned that the cell density has a high importance. Once the perfusion assays were improved, contaminations were avoided, and the bubble formation was controlled by using the design B of the chip and submerging the devices in water, the assays with fluorescence cells were performed. It was possible to see and compare the location of the CFTR protein in mutated and non-mutated cells inside the microfluidic devices.

This whole process, has the final objective to offer a more personalized medicine to cystic fibrosis patients, by realizing faster tests to know the best therapy is for each patient. Thus, this could improve the daily life of this patients.

To conclude, it remains to explain that this work constituted a big challenge due to the lack of information and because the technology used is still in development. Even though, it was really interesting to work in this project and to help to advance the process of getting a better knowledge about the use and application of this technology in medical research with some little steps.

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