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Bachelor of Science in Materials Engineering

Development of an *in vitro* platform for a 3D microphysiological systems of human iPS-derived endothelial cells

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If your dreams do not scare you, they are not big enough.

Ellen Johnson Sirleaf

Abstract

In this work, we presented the original development of a chip for co-culture of cells to build up a 3D microvascular networks. The chip layout was designed in Autodesk AutoCAD2020 to obtain three interconnected tissue chambers where the cells will be seeding and growing. The cell compartments are interconnected and fed with culture medium through the hourglass shaped side pores of the chamber (two pores for each chamber). The cell chambers are connected with inlet/outlet pairs for medium, which is delivered by an automatic feeding system.

One of the initial goals for this work was to have the vessels grow towards both pores, so a pressure gradient was necessary. A medium delivery system was designed in, built with a 3D printer, and operated by a digital actuator connected to a microcontroller board. The oscillating system maintains a difference of level (static pressure) between the two culture medium containers, changing their positions automatically by previously defined time intervals, promoting uniform cell growth. The level height difference of culture medium and the time between changes can be adjusted by the programmable control system.

Furthermore, the design of the chip layout allows for the growth of cells towards mimicking a 3D network of blood vessels. After seeding and polymerization of the fibrin gel, the cells grew occupying all the chamber space. That can be observed by microscopy without stopping the culture, as the chip support is transparent. These indicate that a suitable platform for biological assays using different cell types in co-culture (e.g. fibroblast with human endothelial cells) can be carried on.

Keywords: Organ-on-a-Chip, Microfluidics, 3D vascular network, Fibrin, Fibroblasts, Tissue engineering

Resumo

Neste trabalho apresentamos o desenvolvimento original de um chip para co-cultura de células para a construção de redes microvasculares 3D. O layout do chip foi projetado no Autodesk AutoCAD2020 para obter 3 câmaras de tecido interconectadas onde as células serão semeadas e irão crescer. Os compartimentos das células são interligados e alimentados com o meio de cultura através dos poros laterais em forma de ampulheta (dois poros para cada câmara). As câmaras celulares estão conectadas à entrada/saída de meio, que é entregue por um sistema de alimentação automático.

Um dos objetivos iniciais deste trabalho era fazer com que os vasos crescessem em direção a ambos os poros, para isso um gradiente de pressão era necessário. Um sistema de entrega de meio foi projetado e construído com uma impressora 3D e operado por um atuador digital com uma placa microcontrolador. O sistema oscilante mantém uma diferença de nível (pressão estática) entre os dois recipientes do meio de cultura, mudando as suas posições automaticamente por intervalos de tempo previamente definidos, promovendo um crescimento celular uniforme. A diferença de altura do meio de cultura e o tempo entre as mudanças podem ser ajustados pelo sistema de controle programável.

Para além disso, o design do layout do chip permite o crescimento de células para simular uma rede 3D de vasos sanguíneos. Após a semeação e polimerização do gel de fibrina, as células cresceram ocupando todo o espaço da câmara. Isto pode ser observado por microscopia sem interrupção da cultura, pois o suporte do chip é transparente. Estes indicam que, uma plataforma adequada para ensaios biológicos usando diferentes tipos de células em co-cultura (por exemplo, fibroblasto com células endoteliais humanas) pode ser realizada.

Palavras-chave: Organ-on-a-Chip, Microfluídica, Rede vascular 3D, Fibrina, Fibroblastos, Engenharia de tecidos

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Acronyms

CBV	Capillary Burst Valve
DMEM	Dulbecco's Modified Eagle's Medium
DF	Dilution Factor
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGM-2	Endothelial Cell Growth Medium-2
FBS	Fetal Bovine Serum
HDFn	Human Dermal Fibroblasts, neonatal
HUVEC	Human Umbilical Vein Endothelial Cell
iPS-EC	Induced Pluripotent Stem cell-derived Endothelial Cell
OoC	Organ-on-a-Chip
PBS	Phosphate-Buffered Saline
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PGMEA	Propylene Glycol Methyl Ether Acetate
PS	Pen/Strep
UVO	Ultraviolet Ozone

1. Introduction

Cardiovascular diseases are the most common type of disease as well as one of the leading causes of death worldwide. The study of therapies for the treatment of the disease requires the testing of drugs and new molecules, which is usually made through animal experimentation in the laboratory. The need for an accurate *in vitro* model provided by an Organ-on-a-Chip (OoC) originates from the lack of suitable drugs for medical approval after testing phases, due to severe or undesirable side effects. After positive results in the animal assay, most of the drugs (90%) do not meet the requirements for human therapy and therefore fail the medical approval.

OoC allows for a better understanding and observation of these side effects by mimicking the necessary tissue, assisting in the development of new drugs and treatments on human models without the need for animal testing, and reducing failure in human trials [1].

1.1 Microfluidics

Microfluidic has been defined as a science that studies the behavior, control and manipulation of fluids through channels with dimensions between 10 and 100 µm and a volume capacity of 100 nL to 10 µL. Beyond that, this technology evolved quickly and became a powerful tool for bioengineering and biomedical engineering applications, since the amount of reagents used is small and contamination risks are reduced [2].

Later the idea of “Lab-on-a-chip” appears to miniaturize laboratory experiments, leading to new organs-on-a-chip concepts. Soon these devices started to be developed because they provided better mimicking of cellular environment and they were an important tool for biology and medicine applications.

Development of microfluidic bridges the gap between *in vitro* and *in vivo* models.

1.2 What is an Organ-on-a-Chip?

Clinical studies take a long time to be completed; they are expensive and expend many animal lives in the process. In addition, the results do not always translate the expected therapeutic effect to the human body, as the animal models do not mimic human pathophysiology entirely.

For the development of new drugs and new advances in medicine, there is a need to create more accurate models of human tissue and diseases *in vitro*. The OoC is described as a microfluidic cell culture device, where different types of cells are seeded in small structures with hollow chambers, usually casted with a flexible and synthetic polymer (Polydimethylsiloxane - PDMS) that can be patterned to mimic the microarchitecture and functions of human organs [2].

With OoC it is possible to study molecular and cellular activities associated to organ functions outside the human body and specific assays can be performed by adding bacteria, cells of the immunological system, drugs or diseased tissue cells to the chip. It becomes possible to apply these models for disease states, without dispensing animal

lives, providing a useful tool for the study of the physiology of human diseases and as a tool for the development of specific therapies [2].

It is thought that the genetic characteristics of each patient are the reason why the same disease can be more severe in some patients than in others and why the drugs work better in one patient but are ineffective in another [2].

The strategy of using stem cells in an OoC could be the solution. The stem cells can be collected from patients of diverse ages and genetic backgrounds, as these cells contain specific genetic characteristics (e.g. disease). The stem cells could be cultured inside the chip, differentiated to a specific kind of cell, and grown in co-culture until the production of an OoC, allowing for a large range of physiological studies and drug testing for therapies.

1.3 Types of organs-on-chip

As an answer to the need for accurate models of human organs in the medical field numerous OoCs have been developed [3]. With the improvement of the technology behind the OoCs development, they have become more complex and complete, enabling new possibilitie of tests for drug development and evaluation, as well as providing insights into how certain diseases develop. OoCs provide us a tool to better understand and visualize how e.g. cancer cells develop, multiply and interact with the different types of surrounding cells. Some examples of developed OoCs are Lungs-on-chip, Liver-on-Chip, Kidney-on-Chip, Skin-on-Chip and Heart-on-Chip.

Another type of OoC is Vasculature-on-a-Chip. The vascular network of the human body differentiates its composition and morphology in different and complex structures, namely arteries, veins, arterioles, venules and capillaries. Tasking one single system to mimic all these tissues becomes a rather hard task, so it is preferred to emulate specific tissues separately. Recently, multiple microfluidic-based platforms have been described in the literature for various vascular-based applications including tissue-engineered vascular networks and vascular disease models. The main goal of these developments is to better understand the vascular biology processes [1],[2].

1.4 Vasculature-on-a-Chip: fabrication methods

In recent years, hydrogel has been used in the setup of microfluidics chips to provide a 3D matrix for vascular cells. Kim et al demonstrated that is possible to make a microfluidic device with a flexible material that can hold the fibrin matrices [4].

1.4.1 Using collagen type I

Some of the first studies using soft lithography for the development of a vessel in a chip included the use of materials unfit for encapsulating cells during the fabrication process [5]. Attempting to reach a solution, Morgan et al. [1] proposed the micropatterning of type I collagen, as a natural hydrogel, to achieve enclosed and perfusable vessels and, as such, allowing for the creation of defined microvascular endothelialized structures.

1.4.2 Sodium Alginate

Although there are several methods capable of producing microfluidic structures from hydrogels, most share the common limitation of forming only a straight lumen with uniform size and morphology [5]–[8]. In his work, Wang et al. [9] use sodium alginate mixed with a divalent ion, such as Ca^{2+}. This mixture forms a hydrogel that is dissolved in an Ethylenediaminetetraacetic Acid (EDTA) solution. In PDMS, the channels are designed with sodium alginate and when EDTA dissolves, it creates a 3D PDMS structure for the culture of cells and tissues.

1.4.3 Soft lithography

Some groups have demonstrated [10]–[15] the possibility for analogous structures of *in vitro* perfusable vessels that can mimic Endothelial Cells (ECs) organization, vascular barrier function, inflammatory response, thrombosis and tumor cell intravasation under defined biomolecular and mechanical stimulations. However, none of these approaches follow the natural endothelial morphogenesis in forming the lumenized structure. Therefore, these approaches are limited to reconstitute responses of *in vivo* endothelia. Kim et al. proposed a multichannel device in order to study vasculogenesis and angiogenesis [16]. The novel microfluidic platform proposed creates a new way for the formation of perfusable and functional microvascular networks in 3D Extracellular Matrix (ECM) construction. This process occurs by searching the advantage of controlled heterotypic cell-cell interactions on a co-culture to emulate the physiological morphogenesis of ECs that lead to interconnected networks of microvessels.

Soft lithography (Figure 1.1) and replica molding are used to fabricate the PDMS based chips, replicating from a silicon wafer. A thin film of negative photoresist, SU-8, is spin-coated and then patterned by UV exposure, causing the SU-8 dissolution and creating the master. For the replica molding, liquid pre-polymer of PDMS is casted against the master and peeled off, leaving a reversed imprint of the master [17].

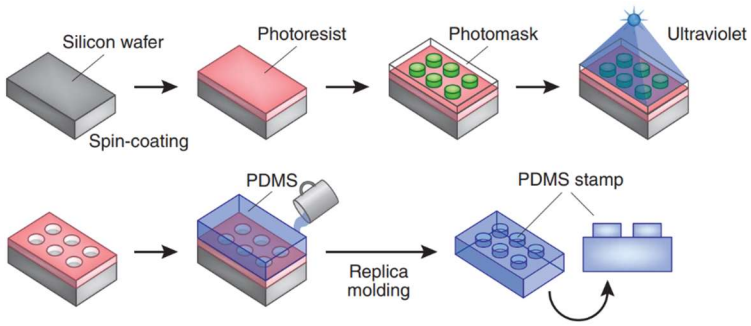

Figure 1.1: Soft lithography and replica molding. (adapted [17])

1.5 Our work

The present work describes the fabrication process of a microfluidic platform to develop an OoC, which ultimately recreates a vascular network. This fabrication process employed laser lithography to shape a chip-layer pattern onto SU-8, drawn with AutoCAD. PDMS was used as a substrate for the chip channels, as it is a low-cost polymer, flexible, breathable, biocompatible and transparent, allowing the monitoring of the cell development by microscopy [18]. The molded piece of PDMS with embedded channel structures was bonded by oxygen plasma to a glass coverslip and connected to a perfusion system for medium delivery.

Two chips layout were designed in AutoCAD. The similarities between the two are: the three tissue chambers (Figure 1.2 E) connected with each other and with the medium channels through hourglass shaped pores, serpentines to decrease the flow of the medium before it enters in the chambers, an input/output pair for the cells (Figure 1.2 A, B) and another pair for the medium (Figure 1.2 C, D). What distinguishes them from each other is the medium channels, while on chip I (Figure 1.2 I) the channels connect the medium inlet to the outlet, in the case of chip II (Figure 1.2 II) the channels do not connect them, leading the medium to pass only through the chambers .

The culture medium was delivered to the chip through two tubes that connect the inlet (Figure 1.2 C) and outlet (Figure 1.2 D) to two containers with medium. These two containers were mounted on an automatic feeding system that maintained a height level difference between them, generating a pressure gradient. This gradient allowed the medium to proliferate through the chip, promoting uniform cell growth and towards the two pores of the tissue chambers.

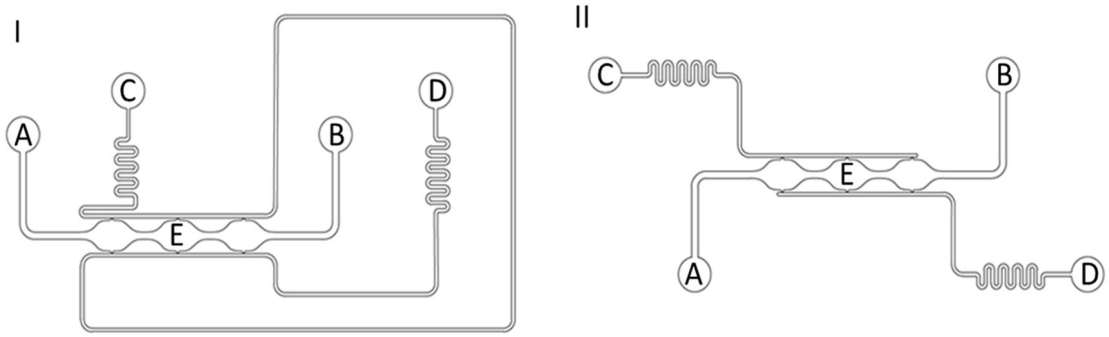

Figure 1.2: Design in AutoCAD of the complex (I) and simple (II) pattern. A- Cells inlet; B- Cells outlet; C- Medium inlet; D- Medium outlet; E- Tissue chambers.

Angiogenesis is a very important biological mechanism responsible for the ECs to undergo some changes, making them able to create new blood vessels through existing vasculatures. It has been proven that stromal cells, like fibroblasts, have a crucial role in this process since their main function is the synthesis and maintenance of the cell matrix, also, stromal cells secrete growth factors that help in the proliferation and adhesion of ECs [19]. Fibroblasts are large, flat and elongated cells that release a substance similar

to an amorphous gel (ground substance) that helps the flow of nutrients, the migration of ECs during angiogenesis, and works as an intercellular communication route [20].

To place the cells inside the chip, a hydrogel that contains thrombin mixed with fibrinogen (fibrin) was used. Fibrinogen is a soluble macromolecule responsible for healing wounds, inflammation and angiogenesis and in the presence of thrombin becomes an insoluble clot [21].

For this reason, in the first phase of the work, fibroblasts (Human Dermal Fibroblasts, neonatal - HDFn) [22] mixed with fibrin had been seeded and cultivated inside the chip, this allowed to oversee the cell viability and progression can be followed during development. This initial phase also enables the testing and optimization of the perfusion capability of the set up for cell differentiation and migration. The fibroblasts form a layer inside the microfluidic chambers and, thereafter, start to migrate as an effect of a lateral medium flux, as they normally do inside our body, allowing the recreation of the vascular network by recreating it inside the chip [23].

In a second phase, these cells will be switched for a mix of fibroblasts with ECs and later for Induced Pluripotent Stem cell-derived Endothelial Cells (iPS-ECs, provided by the CEDOC) to obtain a network of differentiated ECs. Endothelial cells form an internal monolayered lining in blood vessels, especially in blood capillaries, forming part of their walls. The structure and functional integrity of these cells are important for maintaining the wall and circulatory function, as they provide an anticoagulant barrier between the vessel wall and the blood and react with physical and chemical stimuli within the circulation [24]. These differentiated ECs have physiological functions capable of recreating a vascular disease network model, suitable for physiological studies and therapeutic tests of pharmacological compounds.

To determine if any or both of the layouts promoted the growth of cells mimicking a 3D network of blood vessels, some tests had to be carried out, such as:

- How to ensure the chip is free from contaminations for cell seeding;
- How to sterilize the chip;
- Analyzing how long it takes the fibrin to polymerize, since the mixture of fibrinogen + thrombin + cells and the placement of this mixture on the chip has to be done within that time interval;
- How to place fibrin + cells in the tissue chambers without displacing the hydrogel into the channels through the pores;
- How to put the culture medium on the chip without creating air bubbles;
- For the automatic feeding system, understanding what the best difference in level is for the containers;

2. Materials and Methods

2.1 Chip Design

The chambers were designed in a diamond shape (1 mm × 2 mm × 0.1 mm) so that the contractile force generated by the stromal cells is symmetrical [25].

Each chamber has 2 pores (30 µm) shaped like hourglasses to emulate the Capillary Burst Valve (CBV) and to stop the gel close to the medium channel [26]. In a CBV there is an increase in the size of the microchannel, where the gel is trapped in the form of a meniscus [27].

Despite inhibiting the leakage of fibrin into the microfluidic channel [18], the meniscus can burst if the driving force exceeds the resisting capillary force [27].

Next to the inlets/outlets, a serpentine was included to increase the resistance of the medium before it enters in the chambers, through the pores [26], making the liquid enters with controlled pressure and does not compromise fibrin and cells.

For the microfabrication of the chips, a molybdenum mask had been made using a high-resolution laser printing (Heidelberg µpg 101 Laser Writer). The patterns used for the chip layout were designed in AutoCAD.

2.2 Microfabrication

The first step to start the lithography process is to clean the silicon wafer. The wafer went to ultrasounds baths twice, the first one in acetone and the second one in isopropanol, each for 10 min at 60 °C, then the wafer was rinsed.

To ensure that the wafer was dry, compressed nitrogen and a hot plate at 180 °C were used. After the wafer cools down to room temperature, the SU-8 process can start.

This process includes several steps such as: spin coating, soft baking, UV exposure, post baking, development and hard baking (Figure 2.1).

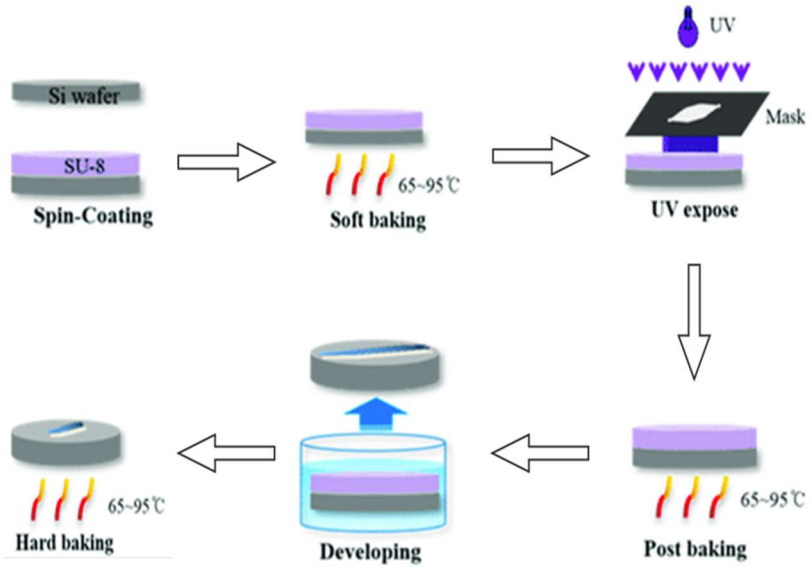

Figure 2.1 Representation of the SU-8 master fabrication. (adapted [30])

2.2.1 Spin Coating

A polymer (SU-8, Microchem) had to be deposited onto the surface of the wafer and the speed needed to spread it over the whole wafer is determined by the tables included in the SU-8 reference datasheet [28], taking into account the thickness of the chip channels and the room temperature.

SU-8 2050 was dispensed on a silicon wafer, pre-spun at 500 rpm for 7 s with an acceleration of 100 rpm/s, and then spun with an acceleration of 300 rpm/s, during 30 s and a speed of 1610 rpm, taking into consideration that the channels of the chips were 100 µm of thickness and the room were at 21.3 °C. Afterwards, the wafer rested for 10 min in a leveled place.

2.2.2 Soft Bake

To evaporate the solvent (cyclopentanone) contained within the SU-8 2050, the wafer was placed on a leveled hot plate at 65 °C for 5 min and to another one at 95 °C for 16 min.

2.2.3 Exposure

After the soft bake step and when the wafer cooled down, the sample was UV exposed (400 nm) in a mask aligner (MA6, Suss MicroTec, Germany) and the energy applied was chosen according to the SU-8 datasheet [28] specifications. The time was calculated by the equation:

$$Exposure\ time\ (s) = \frac{Dose\ (mJ/cm^2)}{Power\ Density\ (mW/cm^2)} \qquad \text{Equation (2.1)}$$

The sample was exposed on the mask aligner for 33.7 s with an exposure dose of 230 mJ/cm^2 and a power density of 6.83 mW/cm^2.

2.2.4 Post Bake

To complete the cross-linking process, the wafer was baked at 65 °C for 4 min and 95 °C for 9 min. While the process runs, the pattern should become visible.

2.2.5 Development

To dissolve the unexposed regions covered by the photoresist, the wafer was developed in a constant magnetic stirred agitation (50 rpm) bath of Propylene Glycol Methyl Ether Acetate (PGMEA) for 9 min. Afterwards the wafer was rinsed with a clean developer and isopropanol, subsequently washed with water, and finally dried with compressed nitrogen. If white traces are observed while the wafer is being washed with isopropanol, the wafer should return to the developer bath for one more minute and rinsed again until no white traces appear.

After the washing process, the sample was baked at 105 °C for 30 min and was optically analyzed by microscopy to verify if the wafer needed further development or another rinsing.

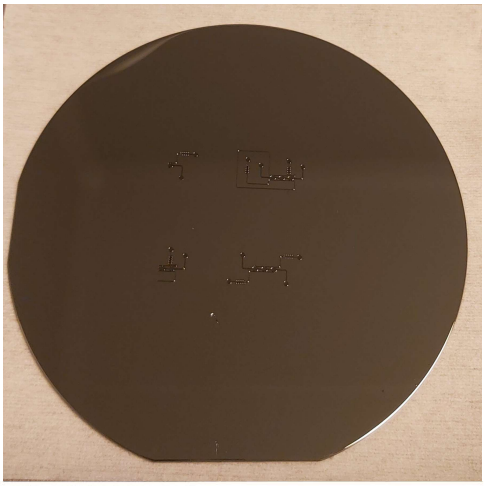

Figure 2.2: Silicon wafer with chip patterns.

2.2.6 PDMS

To obtain a master of PDMS, a curing agent was mixed to a base (Sylgard 184, Dow Corning) with a weight ratio of 10 : 1 without bubbles. The mix was previously de-gassed in a vacuum desiccator. After removing most bubbles, the mix was poured over the SU-8 mold and placed in a vacuum desiccator to completely eliminate any remaining bubbles (Figure 2.3 A).

Both PDMS and the wafer were inserted in the oven at 65 °C for 2 h for curing. After cooling, the PDMS was gently peeled off, removing the formed layer (Figure 2.3 B). Afterwards, the external insertions of the inlets and outlets were made with a 1.25 mm diameter puncher.

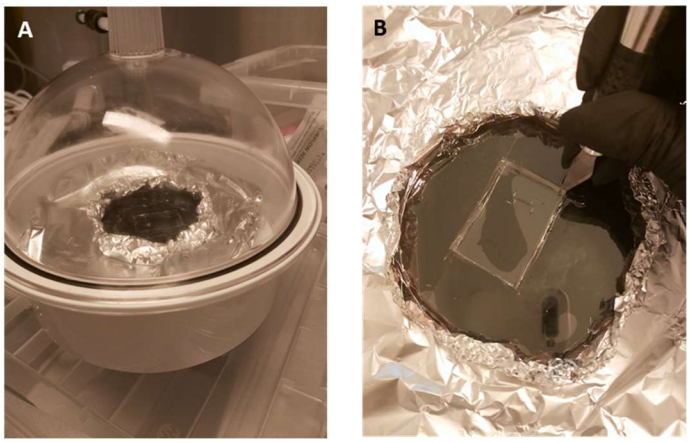

Figure 2.3: Photographs of the wafer with PDMS on a vacuum desiccator (A) and after the curing, the PDMS was cut and peeled off (B).

2.2.7 Sealing

Glasses slides and the casted chips were washed in ultrasound baths and bonded together by oxygen plasma.

After the equipment (Low-pressure plasma system: Zepto Plasma Cleaner) had been turned on (57 W of power), the cap was opened by pressing the “ventilation” button and the glass inside was removed. The glass slides and PDMS, with the channels facing up, were placed on it. The “ventilation” was turned off, the glass inserted in and closed by turning on the “pump” button. After the vacuum leveled 0.12 – 0.13 mbar, the O_2 valves were turned on. When the vacuum reached 0.5 mbar, the “Generator” button was turned on, waiting for a purple color to appear. After 1 min, the O_2 valves were turned off, followed by the “pump” button and the “ventilation” put in the “on” position. The glass pulled out and the PDMS blocks of the chips were put onto the glass slides, making some pressure on top. To guarantee the sealing condition, after the plasma process, the glass and the chip were baked at 150 °C for 5 min with some weight on them.

2.3 Chip Sterilization

All the components for the *in vitro* assay were treated for sterilization. That includes the oscillating system created to alter the direction of interstitial medium flow, the G23 needles with a previously rounded tip, the black paper and the tubing (Liquid Flows Tygon Tubing Coil 1/16 OD X 0.02" ID (10 m=33 ft)). All the components of the system were exposed to UV- C light for 15 min, after being sprayed with 70 % of ethanol.

Finally, the chip and tubing were cleaned inside with isopropanol and then repeatedly washed with Phosphate-Buffered Saline (PBS, Gibco). Then, the chip was emptied of PBS using a syringe and was ready to be seeded with the mix of cells and hydrogel.

2.4 Cells

2.4.1 Medium

The medium culture was made supplementing 89 % (v/v) Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma Life Science) with 10 % (v/v) Fetal Bovine Serum (FBS, Lonza) and 1 % (v/v) Pen/Strep (PS, Gibco), to be aliquot after.

2.4.2 Defrosting

Fibroblasts (Primary Human Dermal Fibroblasts, Cellntec) had been previously frozen at 1×10^5 cell/mL, 1 mL per vial, in liquid nitrogen.

To defrost the cells, the vial was placed in ice and then went to a bath at 37 °C. Medium and cells were inserted in a T-flask and placed into an incubator at 37 °C with a humidified atmosphere containing 5 % CO_2.

2.4.3 Passing

Cell passage is an important step to maintain the quality of the culture. When fibroblasts reach 100 % confluency, meaning, when cells cover the surface of the T-flask completely, the passage can be done. For this, the medium culture was removed and the surface of the flask with the cells washed with 10 mL of PBS to remove all DMEM.

After removing the PBS, the trypsinization to detach the cells from the flask was performed by adding 3 mL of trypsin-EDTA solution (Gibco) to cover the surface of the flask with the cells. Trypsin is an enzyme that, when added to cells, breaks down their proteins resulting in blocking the adherence to the surface of the flask, and is commonly used to detach cells from culture or seeding chambers - Trypsinization.

The flask was placed into an incubator 2 min, after that, the flask was taken out and the sides of it were gently tapped, to promote the release of the cells.

To interrupt the trypsinization, DMEM supplemented with FBS was added, in a volume at least twice the amount of trypsin. The medium was also used to wash the walls of the flask, cleaning all the cells.

The cells were transferred to a 50 mL falcon and centrifuged at 161 rcf (Biofuge 28 RS, Heraeus) for 5 min at room temperature. After the supernatant had been removed and the pellet had been resuspended in 300 µl of DMEM, 5 µl of the cell suspension were added to an eppendorf with 95 µl of 1:3 (v/v) trypan blue solution (two eppendorfs were prepared like that). From each eppendorf, 10 µl were pipetted and added to a hemocytometer to cell count.

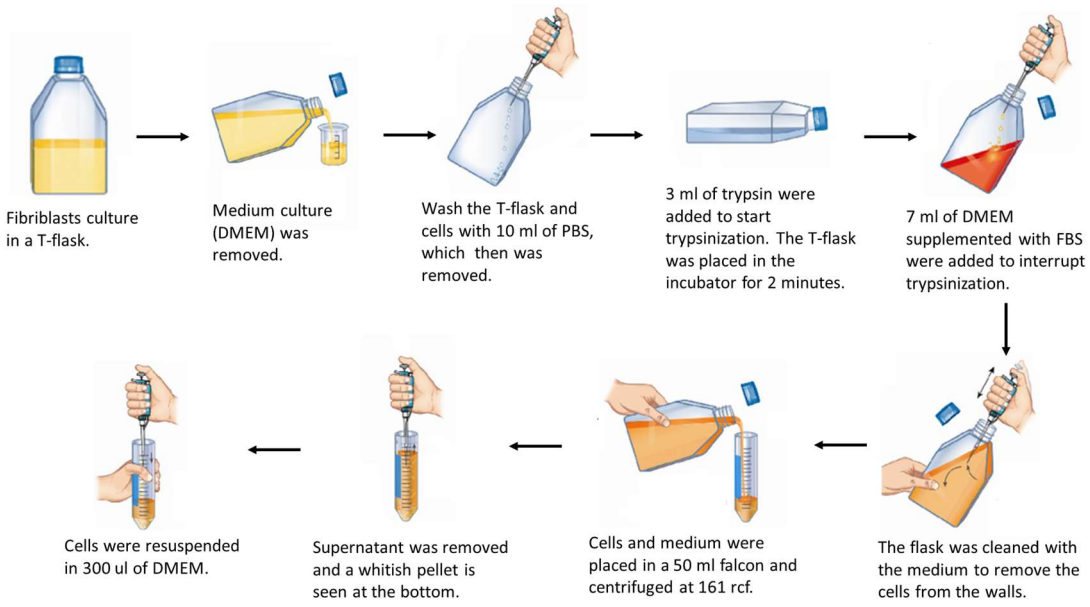

Figure 2.4: Method of passing and trypsinization. (adapted.[31])

The counting was made by microscopy, using a Nikon Eclipse TE 2000-S, bearing in mind that the cells attached to the top and left sides of each square were counted too, according to Figure 2.5.

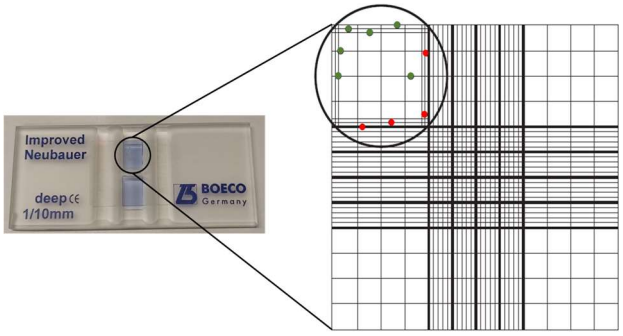

Figure 2.5: Hemocytometer used to determine cell concentration and the method to count the living cells, where the green spheres represent the cells to count at the top and left sides and the red ones the cells that were not counted.(adapted [32])

After counting the living cells, the estimated average was calculated with the ratio between the number of cells counted and the number of quadrants selected.

Knowing the average number of cells, the final concentration (C_f) and volume required for seeding in the chip (V_f), the initial concentration (C_i) and volume of cells for culturing (V_i) could be calculated by the following expressions:

$$Dilution\ Factor = \frac{V_{trypan\ blue} + V_{cel}}{V_{cells}} \qquad \text{Equation (2.2)}$$

$$C_i(cells/mL) = Average\ of\ Cells \times 10^4 \times Dilution\ Factor \qquad \text{Equation (2.3)}$$

$$C_i \times V_i = C_f \times V_f \Leftrightarrow V_i = \frac{C_f \times V_f}{C_i} \qquad \text{Equation (2.4)}$$

2.4.1 Counting

As an example, the counting procedure of one of the assays is described below, using the scheme of Figure 2.5. On Figure 2.6 it is shown the number of living cells counted on and then the average was calculated.

Knowing the average number of cells, the DF, the final concentration (7.5×10^6 cell/mL) and volume required (30 µL), the initial concentration and the initial volume was calculated with the Equation 2.2 – 2.4:

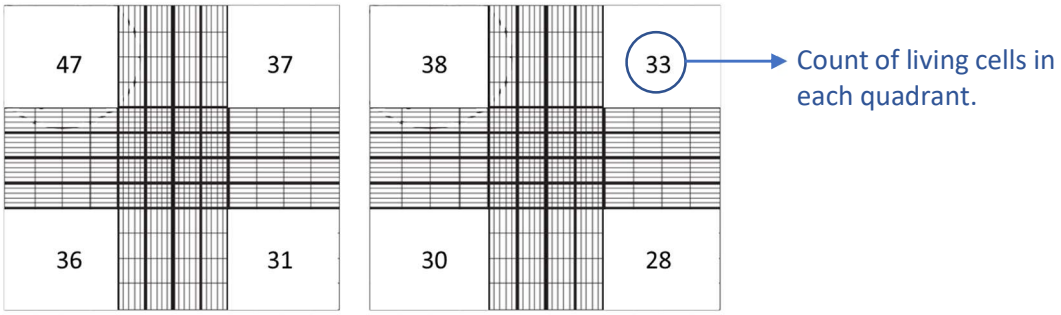

Figure 2.6: Scheme of the counting cells, after trypsinization.

$$Dilution\ Factor = \frac{V_{trypan\ blue} + V_{cells}}{V_{cells}} = \frac{95+5}{5} = 20 \qquad \text{Equation (2.5)}$$

$$C_i = \frac{47 + 37 + 36 + 31 + 38 + 33 + 30 + 28}{8} \times 10^4 \times 20 = \qquad \text{Equation (2.6)}$$

$$= 7 \times 10^6\ cells/mL$$

$$C_i \times V_i = C_f \times V_f \Leftrightarrow V_i = \frac{C_f \times V_f}{C_i} = \frac{7.5 \times 10^6 \times 30}{7 \times 10^6} = 32.14\ \mu L \qquad \text{Equation (2.7)}$$

2.5 Loading Device

2.5.1 Hydrogel Solution (Fibrin)

The hydrogel solution is prepared with fibrinogen (Fibrinogen from bovine plasma, Sigma) mixed with PBS, at room temperature, achieving a final concentration of 10 mg/1 mL that were dissolved in a 37 °C water bath for a minimum of 15 min. After that, the solution was filtered with a 0.22 µm syringe filter.

2.5.2 Seeding Cells

The initial volume of cells (v_i), calculated with equation 2.4, was centrifuged (Bio-fuge 13, Heraeus) at 744 rcf for 5 min and after removing the supernatant, was added to a 30 µl of fibrinogen solution which will be added at 1.8 µl of 50 U/mL thrombin. To delay the reaction between thrombin and fibrinogen the mixture of both reagents was kept in ice [29].

To fill the device with the cell suspension, the chip was placed on top of a black paper to better follow the liquid filling of the three chambers. The cells were introduced in the chip applying a positive pressure in the inlet and a negative one in the outlet, using two pipettes simultaneously.

After filling, the chip was placed in the incubator for 1 h, to allow full fibrin polymerization, and then the inlet/outlet of the cells were closed with transparent sealing film.

2.5.3 Introducing Medium

A pressure gradient between containers allowed the medium to flow through the channels and chambers, making a uniform pressure gradient and allowing the cells to distribute and grow inside the chambers.

The pressure gradient is obtained by the medium delivery system, design in AutoCAD 3D and built with a 3D printer (Ultimaker 2^{+}). The Arduino microcontroller board commands a servomotor, that manages the oscillating medium delivery system to achieve the medium level difference and therefore the pressure gradient.

With this system, a difference of level between two containers (each of 2 mL of medium) could be maintained. The container was automatically changing positions by a time interval defined previously to revert the flow.

2.5.4 Immunofluorescence

Immunofluorescence is a method that allows visualization and localization of various cellular components in cells. For this, it is necessary to use specifics immunolabels that attach to the cellular structures of interest.

Before immunofluorescence is performed, cells need to be fully attached to the chip, this is due to the fact that the cell membrane must be permeabilized to allow antibodies to access intracellular structures. Without fixation, the membrane structures would disintegrate and disperse the cellular content. .

To do the fixation, 2 mL of a 1 % Paraformaldehyde (PFA) solution was injected in the inlet and subsequently washed with PBS, twice. For permeabilization, 200 µl of 1 % Triton X-100 was placed and after 15 min was washed again with PBS, twice. The colorant used was phalloidin (0.5 µl + 200 µl PBS) and the chip stayed in a dark place between 30 min to 1 h, washed again with PBS and was observed on a microscope.

3. Results and Discussion

3.1 Chip

3.1.1 Design

Two different chip layout designs were made: a complex one (Figure 1.2 I) and a simple one (Figure 1.2 II). Both included three tissue chambers (1 mm x 2 mm) interconnected and linked to the medium channels by pores (30 µm). Furthermore, they had one inlet/outlet to cells and another one to medium.

Chip I had the medium channel straight from the inlet to the outlet enabling a permanent flow of medium culture independently from the flow through the chambers. The pressure difference between medium containers in the cell chambers is regulated by this channel "resistance" to the fluid.

Chip II was designed without a straight connection between inlet/outlet in order to force medium to go through the tissue chambers, promoting cell growth. The pressure gradient between containers acts directly on the cells inside the chambers.

3.1.2 Fluidic tests

The expected behavior in the chip I was: the liquid gets in the medium inlet, goes around the medium channels until it reaches the medium outlet and then goes inside the tissue chambers through the pores (Figure 3.1 blue arrows). Contrary to the expectations, after the liquid got in the medium inlet and acrrived at the first chamber pore, it went inside and spread out to the other chambers. When all the chambers were loaded, the liquid went to the inlet/outlet of cells chambers and passed the pore of the third chamber going to the medium outlet (Figure 3.1 rose arrows).

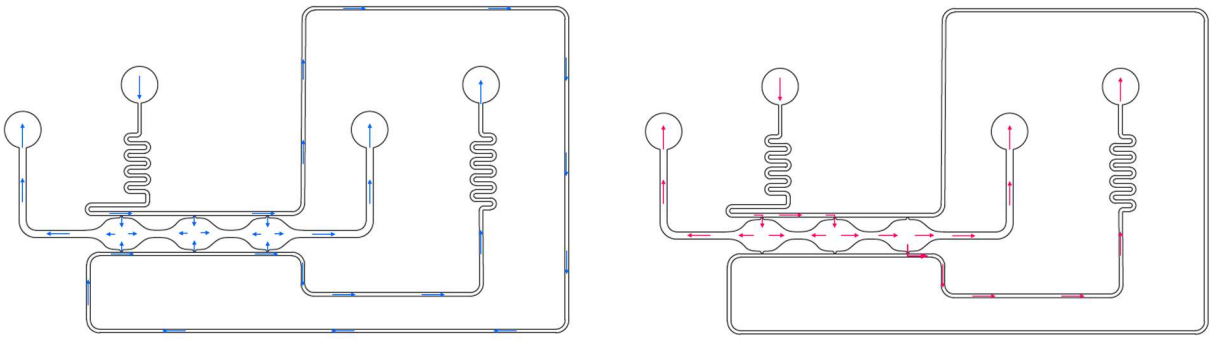

Figure 3.1: Fluidic behavior on the complex chip, what was expected (blue arrows) and what really happened (rose arrows).

In chip II the liquid inserted through the medium inlet goes to the end of the channel, getting inside of the chambers by the three pores, spreading itself to the cell inlet/outlet, and exiting through the pores on the other side to the medium outlet (Figure 3.2 blue arrows). In the experimental test, it had been observed that the liquid went to the medium inlet and flow all through the pore of the first chamber, proliferates until the cell inlet and through the other chambers, until the cell outlet. After that, the liquid

proceeded through the pore of the last chamber to the outlet medium (Figure 3.2 rose arrows).

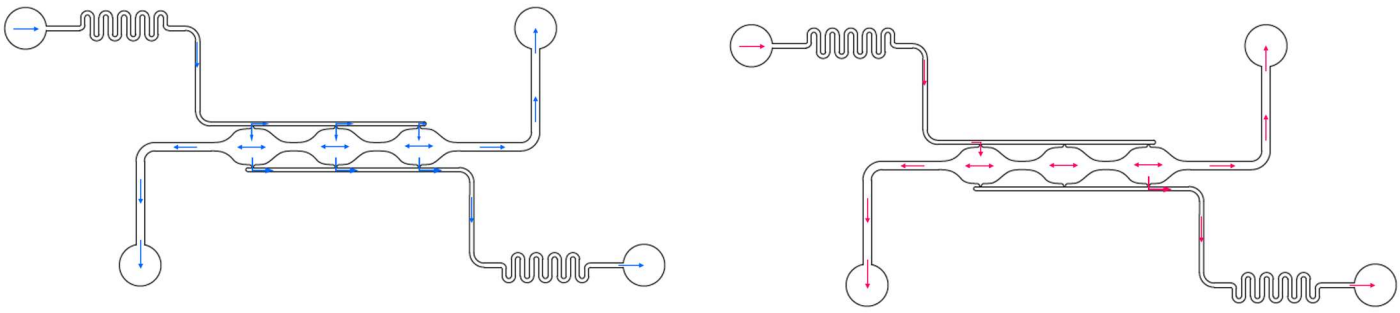

Figure 3.2: Fluidic behavior on the simple chip, what was expected (blue arrows) and what really happened (rose arrows).

3.1.3 PDMS particles in the channels

In the process of replicating the chips, when the inlet/outlet are made with the puncher, some care must be taken. The puncher must be very sharp so that the holes are well cut and not crushed. The holes also need to be straight vertical. If they are not well cut, small particles of PDMS will form that will enter the channels of the chip and clog them, as shown in Figure 3.3.

To reduce the particles, the chip was cleaned in ultrasound before doing the sealing, with the same procedure used to clean the wafers. Even so, in some cases, the particles remain in the inlets/outlets and after the tubes are connected and the liquid passes through them, the PDMS debris end up clogging the channels.

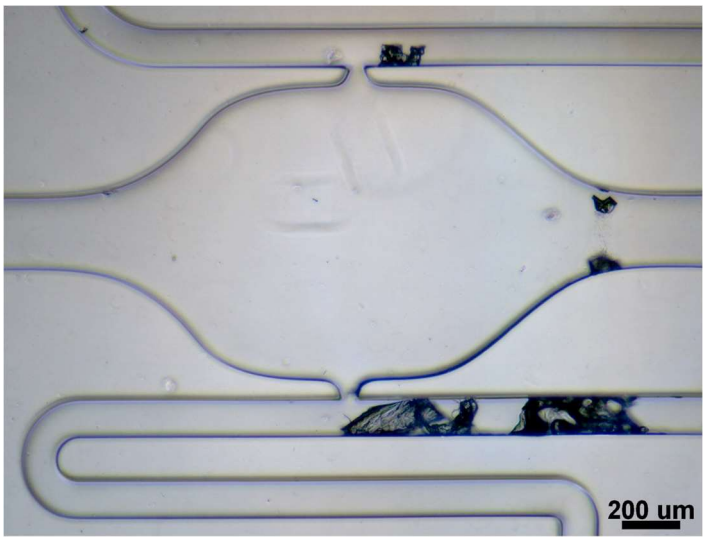

Figure 3.3: Chip clogged with PDMS particles.

3.1.4 Sealing problems

During the sealing procedure, it was observed that some chips did not adhere to the glass sheets. This can be related to the atmosphere of the plasma chamber.

When sealing with oxygen, the plasma has a bluish color. However, when sealed, some of the chips produced a fuchsia color, which indicates that instead of oxygen the device was using air to do the sealing.

The sealing with oxygen is better than with air because highly active oxygen species remove the methyl groups from the PDMS surface, forming polar silanol groups. After the surfaces are exposed to the plasma and in contact with each other, a covalent bond builds up through a condensation reaction, making the seal irreversible.

In addition to sealing with oxygen plasma, UV Ozone (Digital UV Ozone System, PSD Pro Series, Novascan) sealing was also carried out, but the results were even worse since the bond obtained between the PDMS and the glass is weaker.

3.1.5 Chip characterization

In this work, the thickness of the channels was measured by analyzing the cross-section by optical microscopy integrated with a measurement tool (Figure 3.4). The elasticity of the PDMS makes it easier to cut and measure the cross-section.

In order not to spend more resources, cutting and measuring were done on a chip in which the sealing did not result. Another measurement made on this chip was the pore width (Figure 3.5).

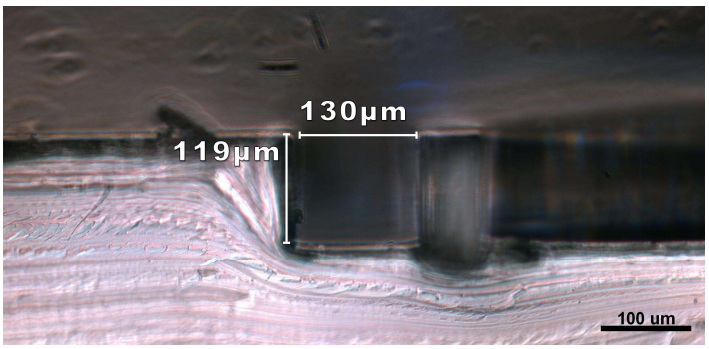

Figure 3.4: Measurement of the cross section by optical microscopy.

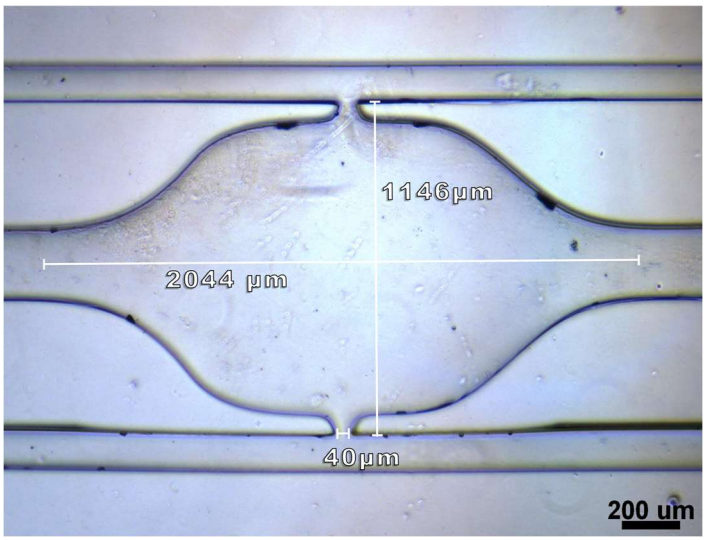

Figure 3.5: Measurement of the pore width.

3.2 Cellular confluency

The growth of the cells was monitored considering that the number is a crucial part of the assay. The goal confluency (Figure 3.6) was 100 % and after that mark was reached, the process described in 2.4.3 began.

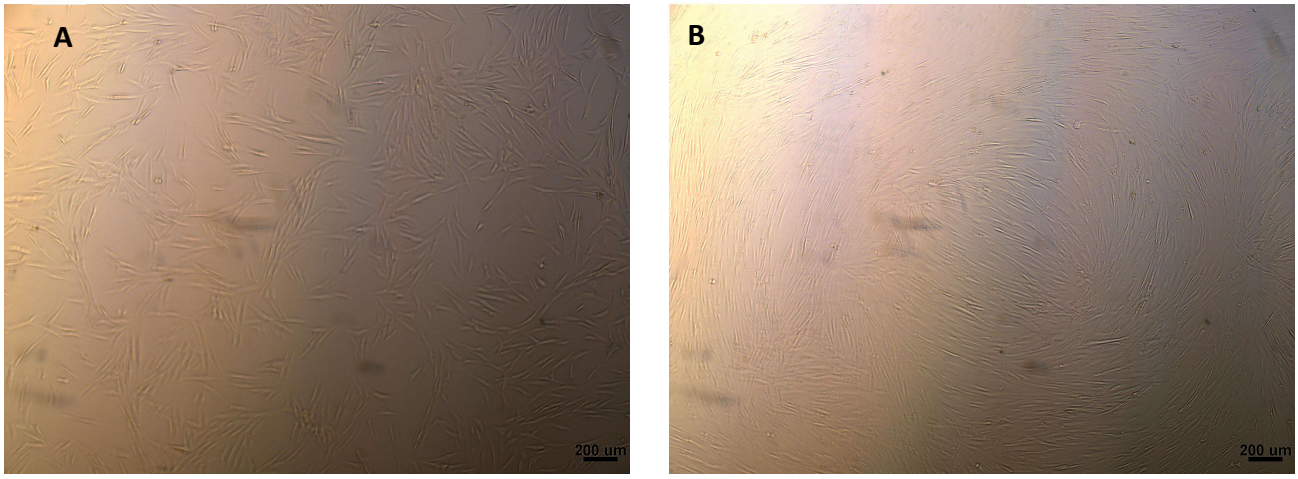

Figure 3.6: Photo A represents fibroblasts at 10 % confluence and photo B represents 100 % confluence.

3.3 Experimental Assays

Several assays with fibroblasts were carried out to optimize the experimental protocol. These made it possible to understand whether cells or medium should be placed first, how the fibrin polymerized, and what is the best method for placing the mixture of fibrin and cells in the tissue chambers without these going into the medium channels.

3.3.1 Testing the initial protocol and evaluating the polymerization interval for fibrin– Chip 1 (I)

In the first assay with chip l, to introduce the cells a syringe and tubes were used . It was observed that using a syringe is difficult to control the pressure and the progressing filling of the chambers leading to the creation of bubbles. The fibrin was placed inside the chambers with bubbles, and some cells escaped to the channels.

Without the centrifugation procedure described in 2.5.2, the supernatant of the cells was not removed, leading to fibrin dilution and resulting in an incomplete polymerization.

In this assay, the chip ended up with very few cells (2.5×10^6 cell/mL), almost empty. Besides to the small number of cells placed, it was also due to the fact that fibrin did not polymerize, resulting in a dispersion of cells through medium channels. This assay was useful to improve the filling process and reduce bubble formation.

3.3.2 Testing the use of pipettes to place the fibrin + cells mixture - Chip 2 (II)

In the second assay, the chip was previously filled with medium, before introducing the cells. Bearing in mind that if the channels were filled with medium, then the hydrogel would not tend to enter them and would only fill the three tissue chambers pushing the liquid to cells outlet.

Once again, the number of cells obtained was small and they had not been centrifuged with the procedure from 2.5.2. Although they were introduced with a pipette, the chambers remained with bubbles in them and some fibrin went to the channels.

In the previous assay, it had been observed that not removing the supernatant from the cells made polymerization incomplete. In this case and adding the fact of placing medium in the chambers, resulted that fibrin become even more diluted, making inviable the polymerization.

3.3.3 Testing and tuning the cell concentration to increase the number of cells seeded – Chip 3 (I)

To avoid the reduced number of cells that occurred on previous assays, in this third one the number of cells was increased to 7.5×10^6 cell/mL and a cells centrifugation process had been introduced to reduce the influence on polymerization. Besides that, since the process of fully filling the chip with medium before inserting the cells was used, the polymerization was still compromised. Believing that the use of a pipette was the better way to place the fibrin in the chambers. Unfortunately, some cells escaped again from the chambers and the formation of air bubbles was observed.

3.3.4 Chip reuse

With the number of tests increasing, the reutilization of chips was taken into account. There was a need for testing the best way to remove the remains of fibrin and cells debris from the channels. To achieve this, attempts were made to heat the chip on

a hot plate; to cool down placing it on ice; using acetic acid and a strong base solution (1 M NaOH), but without positive results.

Finally, trypsin was used to eliminate the cellular debris. The entire chip was filled with trypsin and placed inside the incubator for 20 to 30 min. Afterwards the chip was washed with PBS and observed under the microscope to check if all the cells/fibrin was removed, otherwise they may clog pores and channels. An efficient cleaning is very important to recover the chips and to allow further use, reducing costs and the need for longer times for new fabrication.

3.3.4.1 **Clogged Chips**

If a chip l becomes clogged (Figure 3.7), more intensive cleaning is required (using higher pressure). This is possible because the inlet and outlet of the culture medium are interconnected. Therefore, pushing at the inlet or outlet, the clog ends up leaving the channel. The serpentines are the most prone place to build clogs and the most difficult to clear.

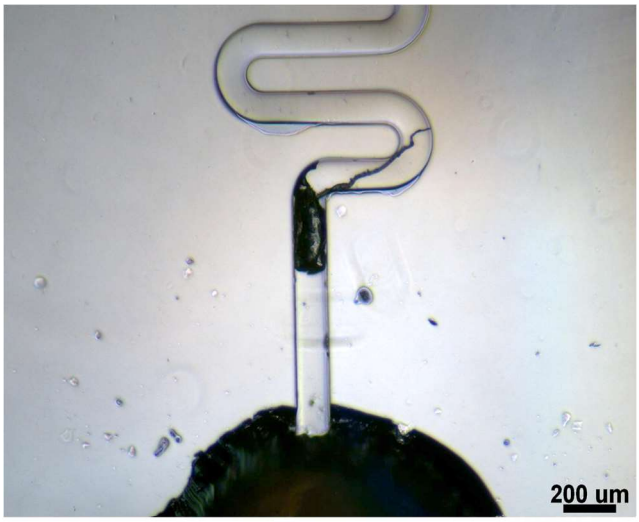

Figure 3.7: Chip l clogged with fibrin remains in the streamer.

On chip ll, even if a more thorough cleaning was performed, most of the clogging is retained in the channel (Figure 3.8). As this design does not have the inlet and outlet connected, often the clog blocks the pores, making its removal almost impossible.

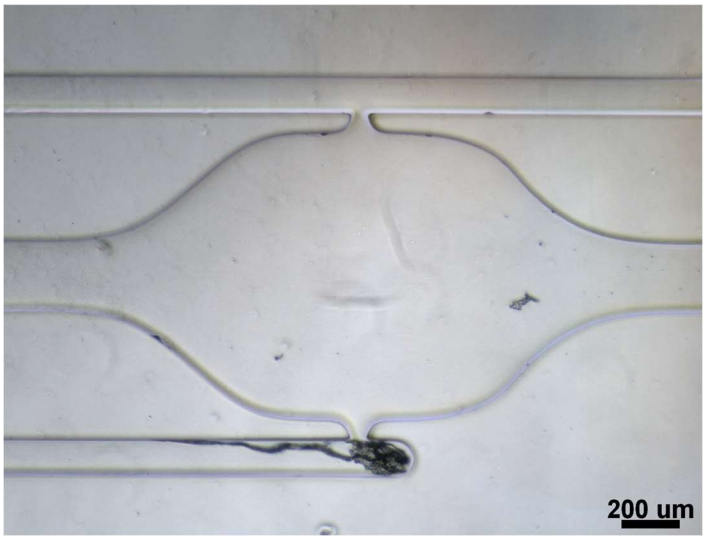

Figure 3.8: Chip l clogged with fibrin remains in the channels.

3.3.5 Polymerization

The polymerization time of the fibrinogen-thrombin-cells mixture had to be adjusted to 1 h, as it had been observed that sometimes thrombin did not fully polymerize (Figure 3.9 I) in 30 min inside the incubator, as described in the literature.

After performing several assays, it was noticed that when the thrombin was thawed and frozen repeatedly, it suffered degradation.

Therefore, it was necessary to optimize the process regarding the thrombin polymerization conditions. 30 µl of fibrinogen solution were prepared with 1.8 µl of thrombin and put in the incubator for 1 h. After 30 min it was possible to confirm whether the thrombin was polymerizing (fibrin turns whitish as shown in Figure 3.9 II) or not, nevertheless, it must stay 1 h inside the incubator to fully polymerize. This polymerization test was performed before each assay on the chip, to ensure that the fibrin mixture was behaving as intended.

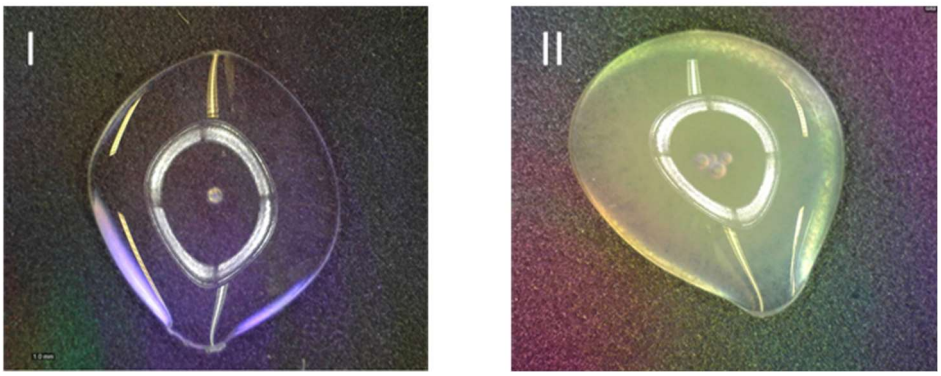

Figure 3.9: I- Fibrin not polymerized ; II- Fibrin polymerized.

3.3.6 Assay in which polymerization was still not carried out – Chip (I)

In this assay, the channels of the chip were previously filled with medium, such as those described in 3.3.1 to 3.3.3, following by injecting the cells. It had been observed that some cells went to the medium channels, confirming that when medium is added first, the fibrin gets diluted, provoking an incomplete polymerization and the occurrence of bubbles in the chambers and channels.

3.3.7 Testing the use of a black surface under the chip and using two pipettes to fill the tissue chambers – Chip 3 reused (I)

This assay was performed to confirm the suitability of the cleaning procedure to reuse the chips. It was also used to slightly change the filling protocol, applying two pipettes to transport the cell suspension to the chip chambers: one to inject the cells from the inlet and the other, empty, to make suction from the outlet. The filling process was followed by placing the device over a black surface to increase the contrast of filled/not filled channels and chambers (Figure 3.10).

Despite that, the pressure made with the pipette was high and lead the fibrin to the channels. Nonetheless, the mix polymerized well and without bubbles.

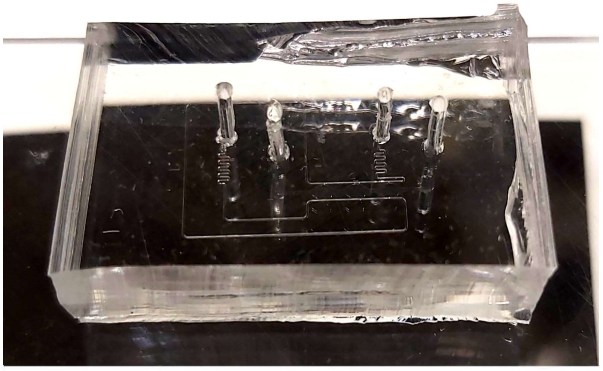

Figure 3.10: Chip on top of a black surface where the difference between empty channels and filled tissue chambers is visible. The chambers turn invisible when filled while the channels are still visible and empty.

3.3.8 First assay in which there are total polymerization and fibrin + cells did not go into the channels – Chip (I)

This time, the black surface was used again during the filling of the chip, to better see when the chambers were filled, due to the better contrast between the filled and unfilled tubes. To make sure that the fibrin was not diluted, in addition to the polymerization tests described in 3.3.5, a syringe was used to suck out any PBS that remained in the channels. As a result, the fibrin was completely polymerized, and the meniscus referred in chapter 2.1 was quite noticeable as required (Figure 3.11).

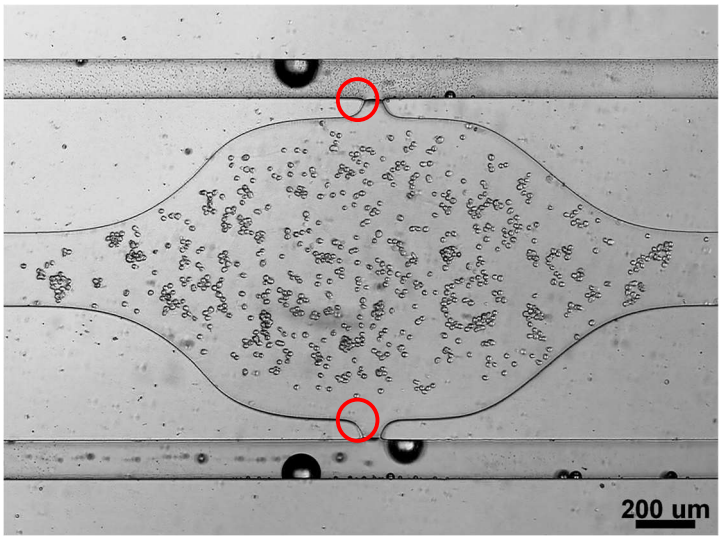

Figure 3.11:Chamber fill with cells and a noticeable meniscus.

When the medium was introduced, some bubbles appeared in the chambers but vanished in the next hours.

3.3.9 Repetition of all protocols of the previous assays to ensure their viability – Chip (II)

In this test, the previous protocol was repeated but it was noticed that the 3rd chamber contained a low number of cells, which could be related to a faulty seeding process. The immunofluorescence staining was performed with success (Figure 3.12), as the assay was done with extreme care, controlling the pressure by the level gradient that limited the rea-gents flow.

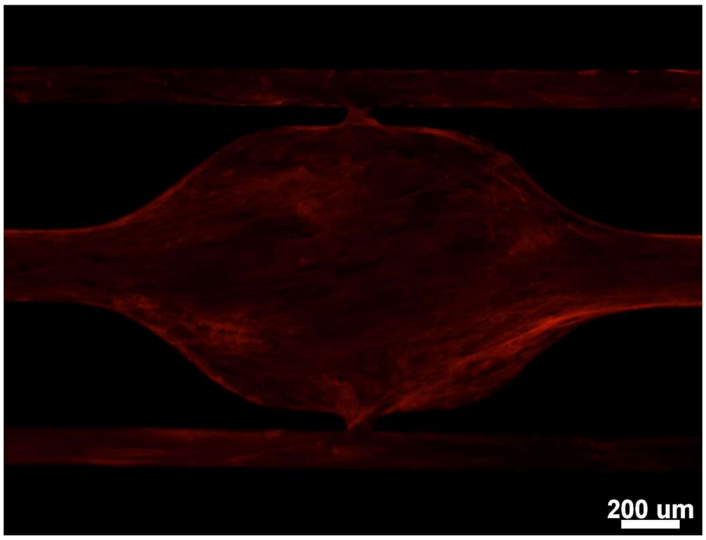

Figure 3.12: Immunofluorescence of fibroblast.

3.3.9.1 Reused chips after immunofluorescence

All chips were trypsinized in order to reuse them. The same procedure was applied to this chip, but trypsinization did not work as expected, and debris remained inside as shown in Figure 3.13. This may be due to the fact that a fixation of the cells was necessary for the immunofluorescence staining, which reduced the trypsin effect.

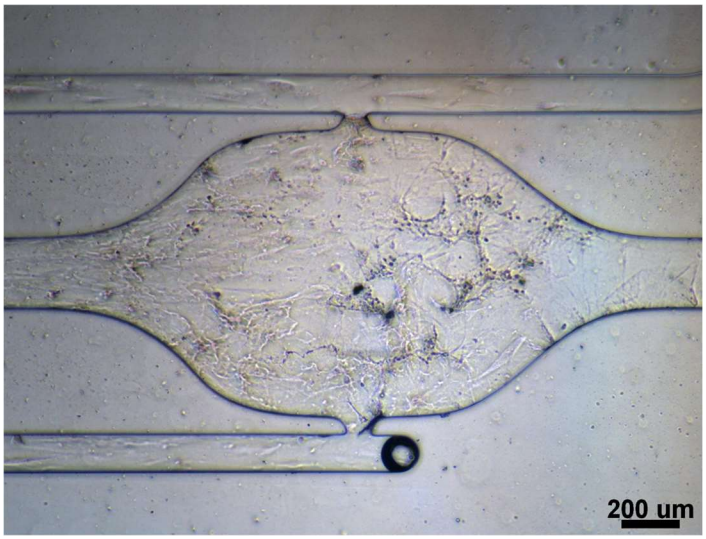

Figure 3.13: Chip on which after immunofluorescence was made trypsinization. Cell debris remains inside the chambers, making it impossible to reuse.

3.3.10 First assay with Endothelial Cells - Chip (II)

After seeding the cells using the optimized protocol and observing how the chips' microfluidics works, the first assay with fibroblasts and Human Umbilical Vein Endothelial Cells (HUVECs, Lonza) was made (5×10^6 cell/mL and 2.5×10^6 cell/mL respectively).

The trypsinization and counting process used with the HUVECs were similar to the used for fibroblasts. Only the time of trypsinization was changed to 4 min, the centrifugation was done at 308 rcf (section 2.4.3) and 268 rcf (section 2.5.2), the DF was 5 and the medium used was Endothelial Cell Growth Medium-2 (EGM-2, Lonza).

After trypsinization and the cell counting, the proportion to achieve was 1 :3 (v/v) mixture of ECs and fibroblasts. The cells were mixed with 30 µl of fibrinogen and 1.8 µl of thrombin and placed on the chip.

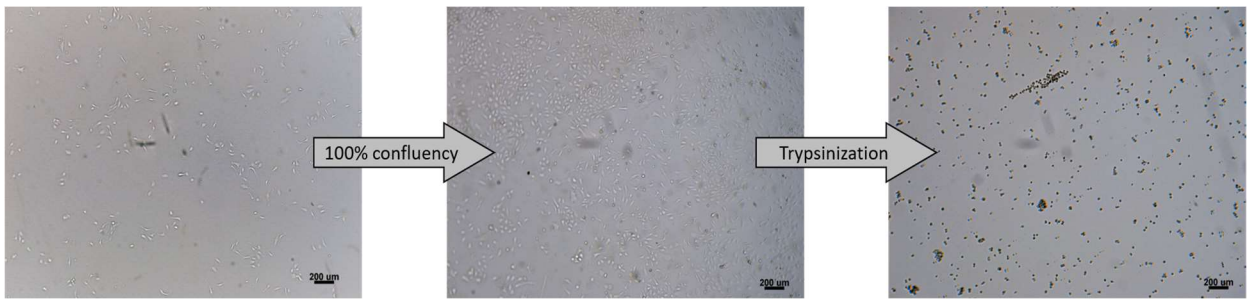

Figure 3.14: Confluency of HUVECs, from the first day until trypsinization.

Before starting the assay, the thrombin test described in 3.3.5 was performed to obtain a complete thrombin polymerization.

After the chip had been sterilized and dried, the cells were introduced before the culture medium with two pipettes, using the dark background to follow the process. After careful preparation, the chip went to the incubator for fibrin polymerization, with all the cells inside the chambers.

Although the thrombin test went well, it did not properly polymerize inside the chip. For this reason, some cells went into the channels when the culture medium was added. Despite the chip having fewer cells in its chambers, it was left growing in the incubator and after 2 weeks cells were observed (Figure 3.15).

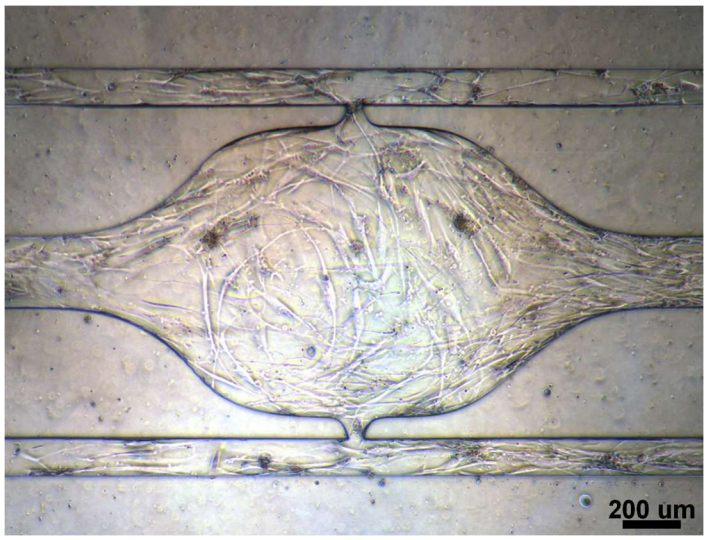

Figure 3.15: Growth of endothelial cells in the tissue chambers.

3.4 Fully automatic feeding system

The control of the servomotor is made by an Arduino board. With the Tinkercad program, a sketch of the system can be set up, and code adjusted to define time and speed of movement. The servomotor was chosen because it can rotate by code pre-defined angles, making it possible to adjust the movement easily and therefore the level difference between reservoirs, as well as to control the speed with which this movement is made.

All the pieces to build the feeding system were drawn 3D in AutoCAD and converted for the open source software Ultimaker Cura 4.6.1, to adapt the printing parameters (e.g. walls thickness, infill, speed, material, travel) to the design and function of the system. The parts were then printed on an Ultimaker 2^+ 3D printer that uses fused filament fabrication standards to fuse polypropylene in pieces.

The system consists of two bars where the containers with medium are fixed to two parallelepipeds, the bottom bar is linked to a wheel that is attached to the servo-motor and has a stake attached to the transparent acrylic where both bars are attached (Figure 3.16).

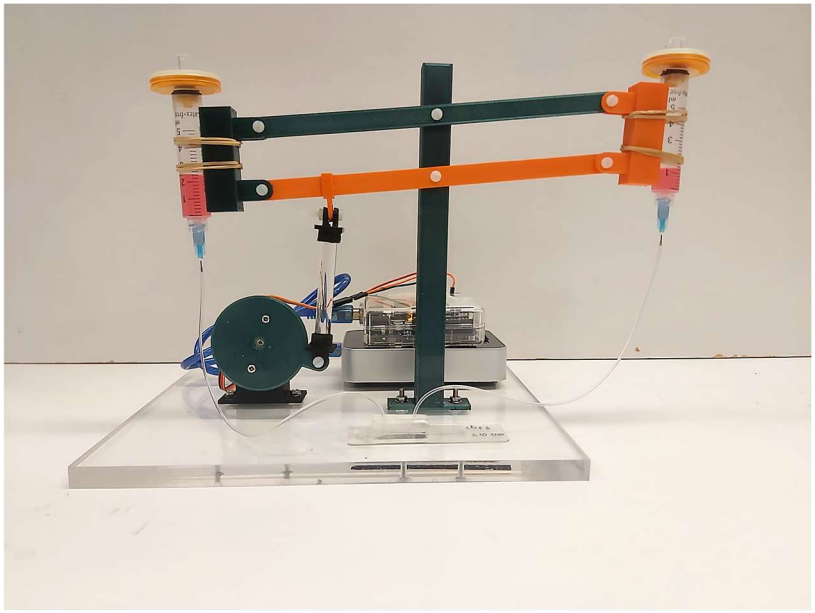

Figure 3.16: Automatic feeding system.

The code used to control the Arduino is depicted below (Figure 3.17). This code controls the system to change positions each 24 h. For the two containers to achieve a medium level difference of 2 cm, the angles used were 61.5 ° and 106.5 ° (position up and dawn).

The cycles are designed to control the speed with which the system changes positions, in this case the speed has been reduced, making the transition less abrupt.

```
#include <Servo.h>

int i = 0;

int m = 0;

Servo servo_2;

void setup()
{
  servo_2.attach(2);

}

void loop()
{
  servo_2.write(0);
  while (!(1 != 1)) {
    for (i = 61.5; i <= 106.5; i += 1) {
      servo_2.write(i);
      delay(100); // Wait for 100 millisecond(s)
    }
    delay(86400000); // Wait for 86400000 millisecond(s)
    for (m = 106.5; m >= 61.5; m -= 1) {
      servo_2.write(m);
      delay(100); // Wait for 100 millisecond(s)
    }
    delay(86400000); // Wait for 86400000 millisecond(s)
  }
}
```

86400000 milliseconds equals 24 h

Figure 3.17: Code used to control the automatic culture system.

4. Conclusions and Future Perspectives

The development of this project features two microfluidic platforms where cell growth is achieved, allowing the development of co-cultured human cells, and eventually building a 3D human vascular network on a PDMS chip.

Two different chip layout designs were conceived (I and II) and although both have permitted the growth of cells, the chip I is easier to trypsinize and to reuse if necessary.

The microfabrication of the chips in PDMS was successful; the only process that caused some problems was the sealing of the chips with oxygen plasma. Although attempts were made to seal the PDMS to the glass with UVO, the number of sealed chips was lower than expected. Therefore, the sealing procedure was kept with oxygen plasma, while changing the sealing parameters and making sure that the color of the plasma was bluish/purple, indicating that the sealing was done with oxygen and not with air.

The protocols for the cell matrix polymerization, the way to introduce the cells plus the fibrin with two pipettes and the contrast technique for chip filling have been optimized, which proved to be essential techniques for this work.

The entire cell growth process, from defrosting, seeding until achieving full growth inside the chip, using the automatic feeding system, was achieved. The cell culture was fed by the medium reservoirs and granted a continuous culturing process for two weeks. The communication from the pores to the cell-chambers proved to be adequate for ensuring the medium delivery, at the same time that the pores avoided the invasion of the channels. However, optimization of the assays protocol for specific kinds of cells and polymer had to be performed.

The automatic feeding system changed the position of the containers every 24 h and kept the medium level difference by 2 cm, allowing for the constant presence of medium in both containers and a constant flow. These two parameters avoided failures or fluctuation in the delivery of the medium to the cells that could compromise cell growth or lead to cell lysis.

This system was built economically, using only an Arduino control board, a servomotor, polypropylene connecting parts, an acrylic plate and fixtures. Furthermore, it allowed the culture to be maintained in culture for long periods of time without the need of human intervention.

Another advantage of this system is the possibility of being used with other cell cultures, requiring only to changing the protocols and the parameters of the Arduino code to the desired ones.

Although most of the tests were done with fibroblasts and only one was done with ECs and fibroblasts, the initial ones were crucial to adjust the sterilization protocol, placement of cells and the medium and to know the microfluidics of the chips and the behavior of the cells.

It was not possible to do more tests with ECs and fibroblasts, since the time to do the thesis was reduced and the pandemic situation limited the access to the laboratory

during this year. Even so, it was possible to demonstrate that the two developed chip designs are viable for long-term cell culture.

In terms of optimization of the chip design, the chip ll layout could be improved by ending the medium channels just after the pores, so that fibrin does not get stuck when the trypsinization of the chip takes place. Thus, the remains of fibrin end up passing through the pore to one of the outlets, without leaving any pores clogged and allowing the reuse of most of the used chips.

To help the adhesion of ECs to the glass and PDMS, and before introducing the medium in the chip, a pre-coating of the microfluidic channels with laminin could be done [29]. This is because the tissues and cells without a certain proportion of laminin become dysfunctional. Besides, laminin helps in migration through the pores facilitating the linkage between the channels and the vascular network.

In summary, the results of the project confirmed that the chips are viable to perform long-term cell culture and be useful to explore co-culture of cells and building vascular tissue in extended culture periods.

It is expected for further testing to be conducted with HUVECs and fibroblasts cell co-culture as well as experiments with iPS-derived endothelial cells, as originally planned. The iPS-derived cells assays will allow the study of disease related physiology and the testing of drugs for cardiovascular therapies.

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