INSTITUTO SUPERIOR TÉCNICO
Universidade Técnica de Lisboa

Stem Cells in Microfluidics

Controlling the Cellular Environment of Microspotted Stem Cells

David Soares da Conceição

Dissertação para a obtenção do grau de Mestre em Bioengenharia e Nanossistemas

Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Abstract: The main goal of this work was to develop a microfluidic device adequate for culturing mouse Embryonic Stem Cells (mESC) and to further evaluate the influence of microenvironmental factors, at microscale. Microfluidic technologies provide unprecedented savings in cost and time through the integration of complex chemical and biological assays on microfabricated devices. By means of soft lithography techniques together with a robotic spotting platform, the spotting of extracellular matrix (ECM) molecules was performed directly on polydimethylsiloxane (PDMS) sheets and PDMS microchannels, to develop mESC microarrays. HEK 293T cells were initially used to optimize important spotting parameters, such as the number of ECM droplets, the spot cellular capacity, spots diameter and also to select the best ECM molecule. The best result for this cell line was achieved with Laminin, and this molecule was immediately tested also for mESC culture. Laminin spots defined on a PDMS sheet and formed by 150 droplets provided the best mESC microarray, as it was possible to maintain these cells in culture within a 4-days time window. The microarray was then transferred into PDMS microchannels, where PDMS traps were placed to physically entrap mESC in order to allow their adhesion to the laminin spots, defined inside those PDMS traps. mESC were successfully immobilized in these structures, making the microdevice a good tool for immediate testing of cellular microenvironmental factors and a fair initial attempt to accomplish a final microfluidic device for long-term micro-scale mESC culture.

Keywords: Microfluidic device; mouse Embryonic Stem Cells; robotic spotting; Extracellular Matrix; Microarray

I. Introduction

I.1 Engineering the stem cell microenvironment

Stem cells (SC) are defined by the capacities for self-renewal and for differentiation into multiple cell types. Due to these properties they hold great promise for applications in regenerative medicine and as model systems for drug screening, toxicology and basic developmental biology studies[1]. Understanding how to preciously control the behavior of these cells is an important challenge for utilizing them in both therapeutic and scientific applications.

Therefore, it is clear the importance of having a sharp understanding of the cellular microenvironment and all of its variables (Figure 1) in order to control the cellular fate and to better develop cellular based therapies. While classical biological methodologies ranging from high-throughput gene expression analyses or fluorescence activated cell sorting (FACS) to *in vivo* experiments have significantly increased our understanding of the stem cell phenotype and the genetic mechanisms that control stem cell behavior, they are not ideally suited to elucidate the extrinsic mechanisms of stem cell regulation. *In vivo* experiments on stem cell niches are often hindered by low accessibility (e.g., niches in

the bone marrow, brain, and muscle) and by the difficulties to specifically manipulate

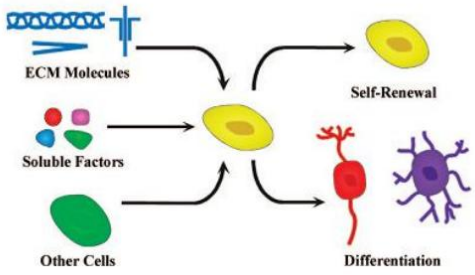


Figure 1. Components of the stem cell microenvironment. Stem cells are influenced by many components including extracellular matrix molecules, soluble factors, and other cells. The combination of all of these signals determines whether the cell undergoes self-renewal or differentiation. Adapted from [2].

niches genetically. In this framework, the field of biomaterials engineering together with the microfluidics technology is an important alternative in the development of stem cell research since both disciplines of engineering and stem cell biology are needed to implement an artificial stem cell environment. Microfluidic systems have the potential to be used for a wide range of applications such as in cell biology studies, neurobiology, pharmacology, and tissue engineering[3]. Miniaturization of devices to be used in these areas leads to many benefits, including decreased cost in manufacture with a use and disposal processing; decreased time of analysis; reduced consumption of reagents and analytes; reduced production of potentially harmful by-products; increased separation efficiency; and increased portability. In addition, some studies are difficult or impossible to perform in larger-scale devices. The protocol usually implied in the fabrication of such micro-devices includes several processes, including photolithography, etching, thin-film deposition, thermal oxidation, and wafer cleaning. However, techniques such conventional photolithography require clean room facilities, a large photolithographic equipment and besides that, it requires the use of several chemicals that are toxic to cells and that are not biocompatible. Taking that into account, for biological applications, techniques of soft lithography are usually used. These techniques use elastomeric stamps fabricated from patterned silicon wafers to mold materials and are used to create several unit operations components and structures such as micropumps and microchannels[4]. Such soft lithographic techniques include replica molding, microcontact printing, microtransfer molding and micromolding. The elastomer typically used is polydymethylsiloxane (PDMS) because it is biocompatible, optically transparent, permeable to gases, and durable[5]. It is also much less expensive when compared to other material used in conventional photolithography.

I.2 Microfluidics in Cellular assays

Cellular fate changes due to high shear stresses are potential confounding factors in microfluidic cultures. Conversely, perfusion is crucial for the delivery of fresh nutrients and growth factors. Also, the isolation and immobilization of a determined cell type within a heterogeneous cellular mixture is a crucial issue in many biological fields. There are several biological microelectromechanical systems (bio-MEMS) approaches for cell isolation and immobilization. Typical mechanisms include a combination of gravity and hydrodynamic forces, dielectrophoretic (DEP), electrokinetic, magnetic, acoustic, laminar flow control, and microfiltering approaches[6,7].

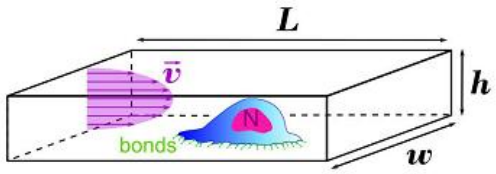


Figure 2. Cell trapping inside a microchannel. The velocity magnitude will influence the cellular fate.

As fluid flow mediates both mass transport of nutrients and shear stress, the perfusion flow rate needs to be balanced between supplying sufficient oxygen and nutrients for cell survival and minimizing shear stress damage. A viscous fluid (including air and water) moving along a solid boundary will incur a shear stress on that boundary. The no-slip condition dictates that the speed of the fluid at the boundary is zero, but at some height from the boundary the flow speed must be equal that of the fluid[8,9]. The shear

stress imparted onto the boundary is a result of this loss of velocity and can be expressed as

$$\tau(y) = \mu \frac{du}{dy} \qquad \text{(I.1)}$$

where μ is the dynamic viscosity of the fluid, u is the velocity of the fluid along the boundary, and y is the height of the boundary.

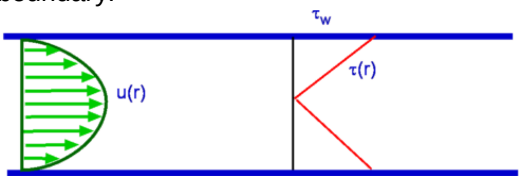


Figure 3. Velocity and shear stress distribution in a pipe flow.

From Equation I.1, if the shear stress of a fluid is directly proportional to the velocity gradient, the fluid is called Newtonian fluid. In the other fluids, however, the viscosity changes with the shear stress; these liquids are hence termed non-Newtonian. Other important dimensionless numbers can be referred, such as the Reynolds number and the Péclet number. Reynolds numbers larger than about 2300 correspond to turbulent flow and under this regime, inertial forces are dominant. The region in which Reynolds number is below about 2000 is referred to as the laminar flow regime. From equation I.2, it is obvious that low Reynolds numbers are attained at lower velocities, smaller dimensions, smaller densities, or higher viscosities. Therefore, in microchannels, laminar flow regime is dominant due to the small dimensions, where the velocities of flow would have to exceed the speed of sound before the onset of turbulence.

$$Re = \frac{\rho d v}{\eta} \qquad \text{(I.2)}$$

where d is the typical length scale (e.g. diameter or channel depth), v is the average velocity of the moving liquid, and p is the fluids density. The Péclet number, Pe, provides an indication of the relative importance of diffusion and convection, with diffusion being the random thermal motion of molecules within their surrounding environment and convection the transport as a result of bulk motion of a fluid. The Péclet number is defined as:

$$Pe = \frac{vL}{D} \qquad \text{(I.3)}$$

where v is the average velocity of the flow, L is the characteristic length of the system perpendicular to the direction of the flow, and D is the diffusion coefficient of the particle or molecule of interest.

Thus, microfluidic devices are ideal for mimicking stem cell niches in that they allow for the establishment of gradients of the soluble environment and control of mechanical forces that contribute to control the fate of stem cells.

II. Materials and Methods

II.1 Designing a cellular array on PDMS

In the first experiments, as an initial approach for microarray culture system optimization and characterization, Human Embryonic Kydney (HEK) 293T cells were used as the cellular model. Then, after all proper optimizations, mESC were used. The goal was to evaluate the possibility of culturing cells and their behavior on a simple PDMS sheet. For this purpose, using a non contact piezoelectric printer platform, a group of extracellular matrices and synthetic polymers were spotted on PDMS and afterwards these spots were evaluated concerning their capability to support cellular adhesion, as described in the following sections.

II.1.1 mESC Culture

In this work, the 46C mouse ES cell line (established at the laboratory of Professor Austin Smith, Wellcome Trust Centre for Stem Cell Research, University of Cambridge, England, UK,) was used. The mESC expansion was done in 60mm tissue culture plates, initially coated with gelatin (0.1% (v/v) gelatin in phosphate-buffered saline (PBS; Gibco)), in fresh culture medium Knockout DMEM (Gibco) supplemented with 15% (v/v) Knockout serum-replacement (SR; according to

manufacturer's instructions, Gibco), 2 mM glutamine, 1% (v/v) penicilin (50 U/ml)/streptomycin (50 ug/ml) (Pen/Strep; Gibco), 1% (v/v) non-essential amino acids and 1 mM β-mercaptoethanol (β-ME). The medium was supplemented with 0.1% (v/v) LIF and with CHIR99021 (Stemgent) at 0.3 μM. These specific conditions were used in order to maintain mESC pluripotency and avoid their differentiation[10]. Cells were dissociated every 2 days using accutase solution (Sigma) and re-plated with a concentration of $5x10^5$ cells/ml in 5 ml of culture medium.

II.1.2 HEK 293T Culture

HEK 293T cells (ATCC-LGC Nr: CRL-11268; Middlesex, UK) were grown adherently to T_{75} culture flasks in the presence of Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5 g/l), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (100 lg/ml) (Invitrogen) and were maintained at 37ºC in a 5% CO_2-humidified atmosphere. They were dissociated every 2 days using trypsin solution and re-plated at the same initial concentration ($3x10^6$ cells/ml in 10 ml of culture medium).

II.1.3 PDMS surface treatment

PDMS was firstly prepared by mixing a liquid silicone rubber base and a curing agent (Sylgard, Dow Corning) in a proportion of 10:1, measured by weight. The mixture was then taken to degas in a vaccum desiccator (Ted Pella, Inc.) until all the air bubbles were removed. Following that, the PDMS was spin coated onto a silicon wafer, to achieve a thickness of 500 μm, and taken to the oven to cure for 2 hours at 60ºC. To facilitate the adhesion of biological samples to the PDMS surface, the substrate was submitted to a chemical treatment performed in three stages: a first immersion for two hours in triethylamine, a second immersion in ethyl acetate also for two hours, and a third immersion in acetone to clean the remaining residues. After that, further surface modifications were done by means of a UVO Cleaner (144AX, Jelight Company Inc.) for 3 cycles of 15 minutes (10 minutes of UV lamp + 5 minutes of exhaustion). When dealing with microchannels, Corona Discharge (Electro-Technic Products) was employed during a time-window of approximately 30 seconds per cm^2. This procedure allowed increasing the efficiency of PDMS-PDMS bonding and also facilitated the fluids flow.

II.1.4. The HEK 293T / mESC cellular microarrays

To define both the HEK 293T and mESC cellular array, each extracellular matrix molecules/synthetic polymers tested were prepared and used in a concentration of 0.1mg/ml (dilution in PBS). By means of the GeSim NanoPlotter Np2.1 (NP), a microarray of ECM molecules, constituted by 150, 250 and 500 droplets was defined and plotted onto a PDMS flat sheet. A pulse of 50 V with a width of 10 μs was usually applied, and functionality tests including the stroboscope and the yellow paper test were always executed for droplet dispensing optimization. In this work, the NP Pico-Tip was used, enabling droplets of ~ 0.05 nl (50 to 60 pl).

The number of cells in each microarray spot was determined namely as a means of evaluating the capacity of the different conditions and settings to support cell culture. Cells were always visually examined on the microscope for evaluation of morphology, but cell counting was also performed by staining cells with the fluorescence dye 4',6-diamidino-2-phenylindole (DAPI, Sigma).

II.2 Microchannel fabrication

The structures designed and tested in this work were obtained through a microfabrication procedure including the construction of a hard mask, a SU-8 (Microchem) mold, and the PDMS replica molding, to accomplish the final microfluidic device. The first microchannel was used for mESC immobilization and static mESC culture (Figure 4-a). It was constituted by an inlet and an outlet, and by three microchambers designed to allocate and immobilize cells. The channel was constructed with 7,5 mm long and had 300

µm of width. The second microchannel was used to attempt a microscale perfusion culture of mESC, and it was made with two inlets and one outlet, and also with the three microchambers. It was designed with 12 mm long and with 300 µm width.

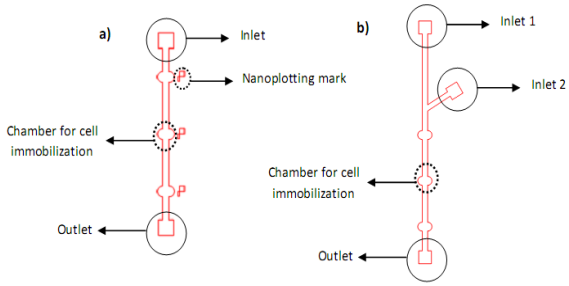


Figure 4. AutoCAD design of the final microchannels used during this thesis. a) mESC immobilization microchannel; b) microdevice for constant perfused culture medium.

These structures, accomodate three PDMS traps inside the three microchambers that were fabricated, accordingly to the layout shown in Figure 5, in order to spatially control the distribution of cells inside the channel.

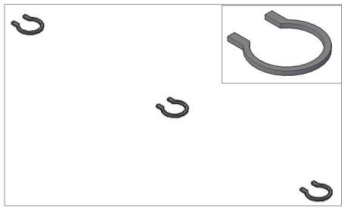


Figure 5. PDMS C-like structures for cellular trapping.

II.3 mESC culture in microfluidics

The immobilization of mESC in this microfluidic device consisted of several steps, as illustrated in Figure 6. The first step consisted on the spotting of 150 droplets of ECM solution inside the PDMS traps (Figure 6-a). This specific number of droplets was used because it was the best result obtained for the mESC microarray obtained on a PDMS surface (explained in a forward section). Since this surface was modified chemically and with UVO Cleaner, a well rounded shaped ECM spot was obtained. After that, the microchannel was bonded to this PDMS sheet (Figure 6-b), in order to make all the PDMS traps to coincide with the chambers of the microchannel. This alignment was made with the help of an optical microscope and by manually handling the two PDMS layers with tweezers. A small volume of cellular suspension (20 ul) was then pipetted at the inlet (Figure 6-c), and finally, flowed through the microchannel, by applying a negative pressure at the outlet, until all its volume was filled (Figure 6-d). The negative pressure was achieved by connecting a syringe pump to a capillary at the outlet. Since the final purpose was to promote mESC adhesion to the laminin spots, a flow rate of 2 ul/min was used while the channel was empty. Cells were then left to adhere for approximately one hour at 37ºC inside the CO_2 incubator.

After the mESC immobilization inside the microchannels, the goal was to submit the cells to a long term constant perfusion of culture medium. One of the objectives was to study the effect of different shear stress values in mESC microscale culture. This was done by flowing culture medium at different flow rate values. The microdevice used was the one depicted in section II.2 (Figure 4-b). The larger inlet was used to accomplish the standard mESC immobilization already explained in the previous section, and the smaller inlet was used to allow the culture medium entrance, by introducing a capillary in this inlet and connecting it to an external reservoir where the medium was stored.

III. Results and Discussion

III.1 HEK 293T cell culture on a PDMS flat surface

The HEK 293T cell culture was done on five different spots of natural ECM molecules or synthetic polymers, namely Laminin, Collagen, Fibronectin, Poly-Ornithin (PO) and Poly-D-lysin (PDL). It was also made a water spot, the negative control. When comparing the different ECM molecules, specially the natural molecules, some render a more structural function, such as collagen, whereas laminin and

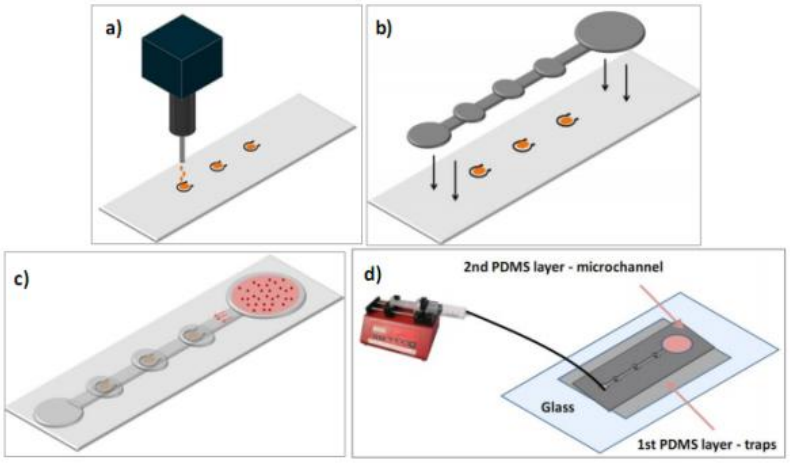


Figure 6. Schematical illustration of mESC immobilization and culture in microchannels. 150 droplets of laminin solution were spotted inside the PDMS traps (a). Following that, the microchannel was bonded to the PDMS sheet in order to make all the PDMS traps to coincide with the chambers of the microchannel (b). 20 ul was then pipetted to the inlet (c), and flowed through the microchannel, by applying negative pressure at the outlet (d).

fibronectin, serve less of a structural role and more of an adhesive or integral role within the ECM[11,12]. The results were classified in terms of two main parameters: spot uniformity and cell confluency. The spot uniformity gave an idea of how the ECM interacted with the PDMS material, and the cell confluency gave an understanding of how cells interacted with the ECM spot. The best results were achieved when inoculating cells (with a concentration of $2{,}5x10^5$ cells/ml) on laminin and collagen spots. Taking that into account, laminin and collagen were selected for further studies to evaluate if their affinity towards PDMS would change, as a function of time. It was observed that after 36 h, collagen lost its affinity towards PDMS, and no cellular spot was observed. This evidence showed that for this particular cell culture setting, at microscale, collagen would not work. On the opposite way, laminin spots remained perfectly defined during time, which gave the necessary motivation to further test this ECM molecule for further studies with HEK 293T cells and also for mESC culture.

After evaluating different natural and synthetic substrate molecules for microscale culture of HEK 293T cells, other important culture parameters were also tested and optimized, and cell and spot behavior characterized. This characterization was done by obtaining the cell saturation curve of the cell spots for three different droplet numbers (150, 250 and 500 droplets). For all the three conditions, the spot begun to saturate with a concentration of $2x10^5$ to $2{,}5x10^5$ cells/ml, probably due to the fact that cells did not adhere to the PDMS sheet like a monolayer, but filled the spot in a multilayer regime making it difficult to determine the exact number of cells in each spot. The bigger spot (500 droplets) had a capacity for approximately 420 cells, the intermediate spot (250 droplets) was filled with a maximum of approximately 350 cells and the smaller one (150 droplets) had a maximum number of approximately 230 cells. Another important aspect at the micro scale that was studied was the dimension of the spot. Figure 7 summarizes the spot diameter as well as the number of cells at the confluence obtained for particular number of laminin droplets, while staining cells with DAPI.

III.2 mESC culture on a PDMS flat surface

As performed with HEK 293T cells, micropatterning of mESC was evaluated using a PDMS flat sheet as the substrate. As the first step to the implementation of mESC patterning on PDMS sheets, it was evaluated the possibility of using laminin as the cellular adhesion substrate.

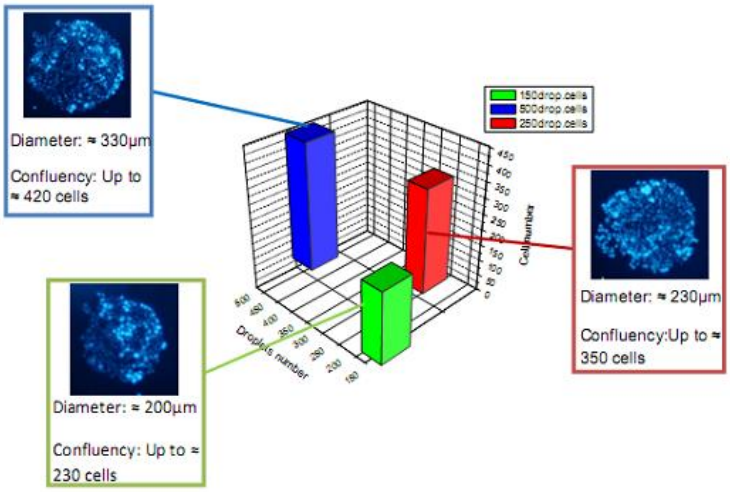


Figure 7. 3-D data plot, resuming the HEK 293T cellular spots features, regarding the diameter and confluency as a function of the number of ECM synthetic molecules droplets dispensed by the Nanoplotter.

A 150 droplet laminin spot was made with the help of the NP, and was submersed in a cellular suspension of mESC with an approximate a cellular concentration of $2x10^5$ cells/ml (Figure 8).

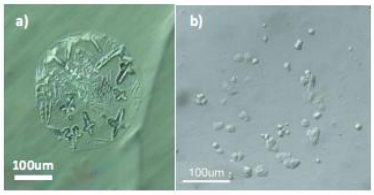


Figure 8. a) Laminin spot on PDMS flat sheet; b) mESC cellular spot after 12 hours of culture.

As can be observed in Figure 8-b), mESC adhered properly to the laminin spot. In addition, it was also observed that mESC prefer to be distributed in the periphery of the spot, rather than inside it.

In a second approach, and following the same experimental plan that was employed with HEK 293T cells, a more detailed characterization of mESC culture in the microarrays was performed. For that purpose, after executing the spot plan (spotting 150, 250 and 500 droplets), the PDMS substrates were submersed in a cellular suspension of mESC with a concentration of $2x10^5$ cells/ml. The substrates were observed every day to evaluate the adhesion of cells to laminin as well as mESC growth kinetics. Culture was performed during 4 days. Once again it was observed that mESC spot grow from the outside to the inside of the spot (Figure 9-a).

It was also observed that, for large droplet number, the cellular spot never achieved confluence (results not shown). Indeed, although cells continued to divide, since the spot area was too big, the cell-cell communication was probably compromised and thus it was not possible to obtain a well rounded spot. This behavior was observed for 500 droplets of laminin and slightly for 250 droplets (results not shown). Hence, a droplet number around 150 enabled a better defined cellular spot, taking into account cell-cell interactions and also cell-laminin interactions. The results obtained for 150 droplets were quantified regarding the spots confluency along the four days of the experiment (Figure 9-b). As DAPI was not an adequate technique for quantification of these cluster-type cellular spots, a matrix of 495 squares was designed in AutoCAD and adjusted to the spots, enabling the calculation of squares filled by mESC (Figure 9-a), to give an overall understanding of the cellular growth within the laminin spots (Figure 9-b). It was found that 78 hours after the first inoculation, approximately 92% of the spot was filled with cells. Considering that 6 hours after submersing the PDMS sheets with cells the spot confluency was of 14%, a successful microscale expansion of mESC in laminin spots was accomplished with this system and with these droplet conditions.

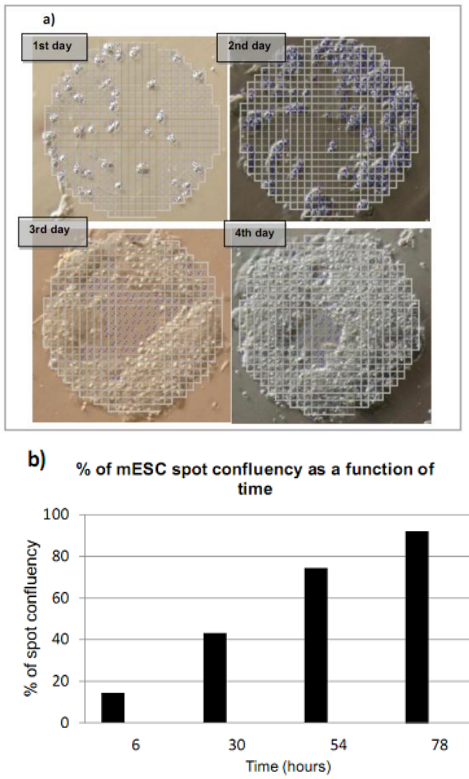


Figure 9. a) A matrix of 495 squares was made in AutoCAD and adjusted to the mESC spot. Using ImageJ, the squares filled by cells were counted and the proportion between squares filled and squares empty was calculated to obtain the percentage of the spots confluency, along the 4 days of experiment (b).

III.3 mESC culture in PDMS microchannels

After optimizing the design of the microchannel for mESC culture and analyzing the fluids behavior inside it with Comsol Multiphysics software, a final device constructed to physically entrap mESC in order to facilitate their adhesion to the laminin spot, by inserting auxiliary PDMS traps, with 20 μm of height. A 2D and 3D simulation showed that the velocity magnitude of a fluid, passing through such a structure, decreases at three specific sites and with great significance, since there are physical barriers which add another font of resistance to the fluid, in those specific locations. The 3-D simulation also confirmed that the liquid will still move along the channel, and will pass along the traps within the 20 μm of height that still remain to reach the top of the channel. This could theoretically enable cell seeding along all the three traps, making possible to obtain an array of three spots inside a single structure.

Figure 10 depicts the mESC immobilization inside a PDMS trap. Cells were able to "identify" the laminin spot, previously spotted inside the trap (Figure 10-a), and were distributed accordingly with the spots positioning. The mESC cellular concentration value was chosen according to the microdevice's volume, 90 nl. Indeed, in order to have an acceptable number of cells inside the microchannel, (i.e. a satisfactory concentration of cells per nanoliter) an initial concentration of $5x10^6$ cells/ml was initially tested. 450 cells were injected into the microdevice (Figure 10-b). Following that, a concentration of $3x10^6$ cells/ml was examined and it was observed that the number of cells immobilized inside the PDMS traps decreased (Figure 10-c), compared with the first initial concentration.

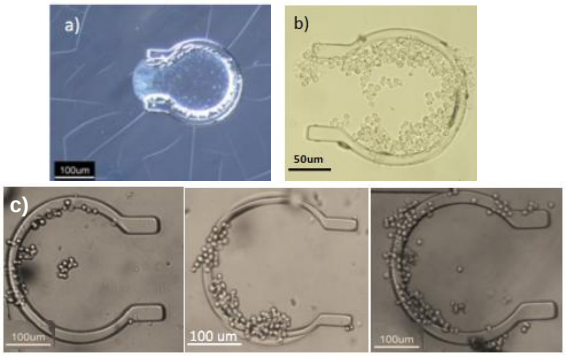
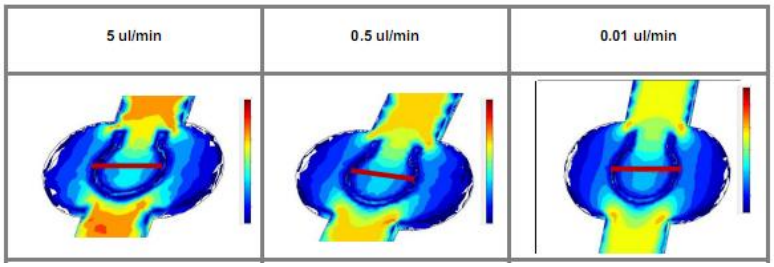


Figure 10. Experimental results concerning the mESCs immobilization inside the microfluidic device.a) Laminin was spotted in the PDMS traps, in order to cover all the interior area comprised by them. b) PDMS trap aligned with the microchannel, enabling the immobilization of mESCs with a cell density of $5x10^6$ cells/ml. c) microarray of PDMS traps inside the microchannel. Using a cellular concentration of $3x10^6$ cells/ml, less cells were immobilized inside the PDMS traps, compared with (b). The fluid flowed from right to left.

As mESCs were successfully immobilized inside the microchannel, the next step was to study the long term response of cells within this microenvironment and to submit these cells to a constant perfusion of culture medium and analyze their response to specific values of culture medium residence time as well as flow rates and corresponding shear stress values. There are three orthogonal planes in each of which forces act in the three orthogonal directions,

Table 1. Comsol 3D simulation of the shear stress inside a PDMS trap in the microchannel for three different flow rates. The red lines depict the plot data that was further analyzed to calculate the shear stress variation.



giving nine stress components. Hence, remembering the definition of shear stress the total shear stress magnitude along the channel will be represented by the variations of the xx component of the velocity profile along yy and zz, and by the variations of the yy component along xx and zz. Therefore, six of the stress components were used to describe the total shear stress: $\epsilon_{xy}=\epsilon_{yx}$, $\epsilon_{yz}=\epsilon_{zy}$ and $\epsilon_{zx}=\epsilon_{xz}$. Using Comsol, a new 3D simulation was made in order to obtain the shear stress mapping inside the microdevice, for three different flow rates: 0.01 ul/min, 0.5 ul/min and 5 ul/min (Table 1). For this effect, it was adapted the viscosity of water, regarding the culture medium viscosity. Accordingly to the color scale that represents the shear stress magnitude, its value is higher on the top and bottom walls of the channel, compared with the lateral ones. On the three specific sites that contain the PDMS traps, the shear stress diminishes relatively to the straight parts, since the area increases. However, the most important factor to be analyzed is the shear stress distribution inside them, which could affect mESC fate and the reproducibility of the results. A 2D plot regarding the cross sectional view defined by the red line was simulated, in order to have the shear stress distribution across the x-axis perimeter that defines the center of the trap, where the mapping is fully specified, with a z-value equal to zero, which gave the shear stress right at the trap's surface. Within the PDMS traps, the shear stress values obtained with the Comsol simulation were as follow:

- 0.01 ul/min: From $4.5\text{x}10^{-3}$ to $5.6\text{x}10^{-3}$ dyn/cm^2;
- 0.5 ul/min: From $2.2\text{x}10^{-1}$ to $2.75\text{x}10^{-1}$ dyn/cm^2;
- 5 ul/min: From 2.2 to 2.75 dyn/cm^2;

Comparing this data with the results obtained by Kim et al[13], this microdevice can accomplish very similar shear stress values, although needing proper experimental analysis. A common characteristic that can easily be observed in the 3D plots corresponding to the three different flow rates is that the shear stress inside a trap is not constant and thus cells are subjected to different values of shear stress in different places of the trap. Thus, two different sets of cells can be considered, the ones that are closer to the traps walls, and the ones that fill the center of the trap. Taking that into account, the shear stress variation was calculated accordingly to the data depicted by the red circles and a percentage of variation of approximately 20% was determined.

The structure evaluated in this section is able to properly support the immobilization of mESC inside PDMS traps and can be employed simply by pippeting the culture medium at the inlet and by applying negative pressure at the outlet. Also, this microdevice enables the study of the long term effect of physical factors, like shear stress, in a time dependent manner, if the system is properly designed to maintain a viable mESC culture for several days. If under standard culture conditions a viable cell culture lasts for two days, in order to maintain the same residence time for culture medium, culture medium in micro-scale should be changed every 58 minutes, considering a cell density of $5\text{x}10^6$ cells/ml. However, this would be technically challenging and would turn difficult to maintain high cellular viabilities. In this

context, it is important to enable a constant perfusion of culture medium within the microdevice, lower enough to prevent the removal of important autocrine factors and to minimize the effect of shear stress, and sufficiently high to have a proper waste removal and a good nutrient concentration. In order to perform these experiments, it was decided to use the microdevice with two inlets depicted in Figure 4-b) and a system which could take advantage of the gravity effect to move the fluid across the channel, considering the height difference between the fluid at the inlet and at the outlet. Although the theoretical assumptions for this system seemed to be adequate in order to expose cells to different flow rate values, to the corresponding shear stresses, and then to evaluate their effects towards stem cell fate it was experimentally verified that the microdevice suffers intense perturbations when making the transition from the mESCs immobilization to the perfused microculture. Thus, it seems that such a system does not render the best solution and the best microenvironment for a long term mESCs micro-culture.

IV. Conclusions and future trends

The primary purpose of this thesis was to construct a microfluidic device capable of performing mESC immobilization and also perfusion of culture medium to enhance a viable micro-scale culture system. This would serve as an alternative to the macroscale standard protocols, reducing their cost and time of analysis, and would also give a solid initial attempt to a high throughput biochip for stem cell culture. By means of a NanoPlotter non contact dispensing platform, a microarray of ECM spots was made and submitted to cell culture both on PDMS sheets and inside PDMS microchannels. A complete spot characterization was done, in terms of spot diameter, spot cell density and the best ECM molecule was selected. Laminin was then used together with some PDMS traps to immobilize mESC inside the microchannels. Thus, this microdevice is surely a good tool for short time cellular analysis, approximately with a 1-hour time window, which was the calculated value for a viable mESC residence time. Regarding a long time perfused micro-culture, a theoretical distribution of the shear stress inside the PDMS traps was determined through a Comsol simulation, for high (5 ul/min), intermediate (0.5 ul/min) and low (0.01 ul/min) flow rates. The obtained results depicted a significant similarity with the experimental results presented in literature, and more importantly, exhibited a 20% variation of the shear stress inside a PDMS trap. After some experimental attempts, the microdevice did not render the desired long time perfused micro-culture, essentially because of the interference of some components of the microdevice, such as capillaries and external reservoirs. To avoid such significant problems and to optimize the accomplishment of small shear stress values, a good and valuable future solution would be to implement microvalves[14] or pressurized external valves into a microdevice such as the one studied in this work, to better control the entrance of the fluid into the microdevice. Also, the hypothesis of an autonomous capillary micropumping[15] to prevent the use of syringe pumps and external components to the microchannel would be good future attempt.

V. References

1. Mooney, D. J.; Vandenburgh, H. *Cell stem cell* **2008**, *2*, 205-213.
2. Little, L.; Healy, K. E.; Schaffer, D. *Chemical Reviews* **2008**, *108*, 1787-1796.
3. *Lab on a Chip* **2006**, *6*, 1118-1121.
4. Haeberle, S.; Zengerle, R. *Lab on a Chip* **2007**, *7*, 1094-1110.
5. Wheeler, A. R.; Throndset, W. R.; Whelan, R. J.; Leach, A. M.; Zare, R. N.; Liao, Y. H.; Farrell, K.; Manger, I. D.; Daridon, A. *Analytical Chemistry* **2003**, *75*, 3581-3586.
6. Wong, I.; Ho, C.-M. *Microfluidics and Nanofluidics* **2009**, *7*, 291-306.
7. McDonald, J. C., Duffy D. C., Anderson J. R., Chiu, D. T., Wu, H., Schueller, Olivier J., A., Whitesides G. M. *Electrophoresis* **2000**, *21*, 27-40.
8. Ong, S. E.; Zhang, S.; Du, H.; Fu, Y. *Front Biosci* **2008**, *13*, 2757-73.
9. Ho, C.-M. *Most* **2001**, 375-384.
10. Martin, G. R.; Timpl, R. *Annual Review of Cell Biology* **1987**, *3*, 57-85.
11. Lin, S., P., Chuang, T., L., Chen, J., J., Tzeng, S., F., *Journal of Medical and Biological Engineering* **2004**, *24*.
12. Alovskaya, A., Alekseeva, T., Phillips, J. B., King, V., Brown, R., *Topics in Tissue Engineering* **2007**, *3*.
13. Kim, L.; Vahey, M. D.; Lee, H.-Y.; Voldman, J. *Lab on a Chip* **2006**, *6*, 394-406.
14. Khademhosseini, A.; Yeh, J.; Eng, G.; Karp, J.; Kaji, H.; Borenstein, J.; Farokhzad, O. C.; Langer, R. *Lab on a Chip* **2005**, *5*, 1380-1386.
15. Zimmermann, M.; Schmid, H.; Hunziker, P.; Delamarche, E. *Lab on a Chip* **2007**, *7*, 119-125