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PRODUCTION OF DEVICE FOR THE CONTROLLED CULTURE OF MULTIPLE CELL TYPES

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Abstract

The central theme of this work is microfluidics applied on cancer cells studies. Microfluidics is a technology that permits the engineered manipulation of fluids at the micro-scale. The promising results obtained in recent years allowed fast development of this field in the global scientific community. Properties like rapid sample processing, increased control of the fluids, and decrease in costs, all make microfluidics an ideal candidate to support conventional experimental methods in biological science. Thus, the aim of this thesis is the design and development of an automated microfluidic platform for the controlled culture of multiple cell types within a single device to better recreate the tumor microenvironment. The control is guaranteed by the use of an on-chip microvalve system. This new microfluidic device should have the following features: simplicity and reproducibility, reliability and resistance, easiness of automation, and easy integration with different analysis and imaging techniques. The device was manufactured using multilayer soft lithographic techniques and replica molding. The geometry of the platform was designed to allow ease of production, use, and capability to recreate cancer metastatic sites. The mechanical compliance of the platform has been tested with positive results, and the platform and integrated microvalves were able to work properly for several weeks.The biological validation has shown promising results since the cells were able to properly attach and grow without contaminations issues and cell death, demonstrating that this platform is suitable for biological applications.The preliminary experiments involving the co-culture of different cell types inside the microfluidic device were performed using Neuroblastoma (NB) cancer cells, represent the main cancer site, and two other cell types representative of NB metastatic target sites: the endothelium and bone marrow, reproduced by umbilical cord derived (HUVECs) and Mesenchymal Stem Cells (hMSCs), respectively. Our device enabled analysing the NB cancer microenvironment focusing on the study of the interactions between the primary tumor and its target metastatic sites.

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Introduction

Microfluidics is an emerging technology that has seen its potential grow considerably in recent years. It allows the manipulation of fluids on the microliter scale within specific devices with a high degree of spatio and temporal control. The advantages of this technology derive from the fact that the motion of fluids on the micrometer scale exhibits unique behaviors that can be exploited for various applications in biological studies, and reduces the use of reagents and the loss of samples. These reasons have made microfluidic devices highly successful in various fields such as biotechnology, biomedical, and pharmaceutical research fields. The main technique used for the fabrication of the devices is soft lithography combined with the use of polymeric materials, in particular Polydimethylsiloxane (PDMS), for replica molding. The laboratory in which this thesis work was carried out is the BIAMET (Biomedical Applications of Multiscale Engineering Technologies) laboratory where biology, engineering, and biotechnology meet to study human tumor diseases, and Neuroblastoma in particular.

Neuroblastoma (NB) is a childhood solid tumor that originates from progenitor cells of the sympathetic nervous system. Metastases are present in about half of NB patients and are mainly located in the bones, bone marrow, lymph nodes, liver, and skin. The complex molecular mechanisms involved in tumor dissemination are not fully understood, due to the lack of appropriate means to study them.

The improvement of the soft lithography technique led to the creation of a multilayer microfluidic device. The advantage introduced by this technique consists in the possibility to create inside the device itself an automated system of microvalves that allow the control and a precise movement of the fluid, thus reducing the number of external devices needed. Moreover, the whole fluid handling inside the platform can be controlled via software, increasing the precision, and reducing the operations required by the human operator.

Thus, the aim of this thesis is the design and development of an automated microfluidic platform for the controlled culture of multiple cell types. The device is specifically designed to study Neuroblastoma tumor behavior and the interactions with its main metastatic target sites using a single device.

The structure of the thesis is the following:

The first Chapter contains a general introduction about microfluidics, the analysis of the physic phenomena occurring at microscale, the applications of microfluidic technologies on biology, and the description of the most common techniques used to produce the microfluidic platforms with all their advantages and disadvantages. It is also included a short description of Neuroblastoma.

The second Chapter describes all the protocols and the materials used during the experiments. In order, are reported: the development of the design, the fabrication of the platform through multilayer soft lithography and replica molding technique, the protocol to automate the device, and all the mechanical and biological validation protocols.
The third Chapter reports the results obtained from all the experiments performed: the final geometry and the shape of the platform, the automation system, the results of experiments for the mechanical validation and all the data derived from the biological validation experiments and the co-culture test.

Chapter 1

State of the art

This first chapter introduces the general characteristics of microfluidics and its role on biomedical research. It will present a general overview on microfluidic devices, their construction methods and the materials used. It will discuss their use and key advantages for biomedical applications. Then, some studies about co-culture in controlled multilayer platforms, a brief description of Neuroblastoma, and the aim of this thesis will be given.

1.1 Microfluidics

1.1.1 General principles and physics of microfluidics

Microfluidics is a research field in which small volumes of fluid in the order of nanometers and micrometers are treated. The major applications are for genetic analysis, amino acid analysis, protein crystallography, bioreactor, chemical synthesis, and single cell analysis. Microfluidic systems are developed following the guidelines of microelectronics and, more specifically, of MEMS (MicroElectroMechanical Systems), miniaturized system designed, assembled, and used to produced electronic modifications to the microscale. Besides practical difficulties in working at such small dimensional scales, the different physics of fluids on a microscopic scale brings several advantages:

1. Precise control of flow volumes of fluids reduces reagents usage and mitigates sample loss;
2. Surface and interfacial tension are more dominant at microscale than at the macroscale, these can be used for forming monodisperse droplets in multiphase fluid streams;
3. Effect of capillarity forces at the microscale are greatly increased compared to those at macroscale. Capillarity forces can be used for different applications such as passively pumping fluids in micro channels;
4. Laminar flows facilitate precise fluidic control, that is impossible to do with turbulent flows typical of macroscale system;
5. The size of microfluidic system increases the speed of common experiments techniques by orders of magnitude[1].

Overall, in microfluidics the physical phenomena that characterize the macroscale change significantly. At the macroscale, for example, mass transport is usually dominated by inertial forces and viscous dissipation is negligible, while in microfluidic devices the situation is the opposite. Despite the considerable advantages highlighted, microfluidic devices have not yet reached a widely success in the consumer market due to limitations in manufacturing capability and in the automation of the accurate control of flowsand volumes on a micro and nanometric scale. In fact, in micro channels the movement and mixing of fluids involve many physical parameters that escape the macroscopic rules.
To better understand how the motion of a fluid evolves within a micrometer scale channel, the most important properties of the fluid will be examined. The validity of the continuity approximation also on a microscopic scale, allows to exploit the principles of classical fluid mechanics for the study of microfluidics devices.
This approximation is very important from the computational point of view because it permits to simplify the calculations considering only the average values of the parameters that characterize the fluid motion , thus neglecting the most complex interactions between the molecules of the same fluid present in the Navier Stokes[2] equation (1.1):

$$\rho\, D\boldsymbol{v}/Dt = \rho\,(\partial \boldsymbol{v}/\partial t + \boldsymbol{v}\cdot\nabla\boldsymbol{v}) = -\nabla p + \mu\nabla 2\boldsymbol{v} + \rho \mathrm{g} \tag{1.1}$$

Osborne Reynolds in 1883 determined that the critical velocity v_c of a fluid was at the same time proportional to its viscosity η, and inversely proportional to its density ρ and to the diameter d of the pipe through which it flows, in particular v_c is the limit velocity, reported in equation 1.2, that define the change of regime of fluid motion:

$$v_c = \frac{k\eta}{pd} \tag{1.2}$$

The constant of proportionality k, has a value equal to 2000 for most fluids. When equation 1.2 is rewritten replacing v_c with the velocity of the fluid v, the factor k will no longer be a constant but a variable which takes the name of Reynolds Number (Re)[3]. Its value is calculated with the following equation:

$$Re = \frac{\rho v d}{\eta} \tag{1.3}$$

where v is the velocity of the fluid inside the channel in [m/s], ρ is the fluid density in [kg/m^3], d is the diameter of the channel in [m] and η is the shear viscosity of the fluid in [Pa·s].

When the value of Reynolds number is high it means that inertial forces dominate the system, while the viscous forces are more relevant with low value of *Re*. In microfluidic channel the Reynolds number is usually well below 100. So this value indicates that viscous forces are dominant in the system with respect to inertial ones, and since a flow is considered laminar for Reynolds number lower than 2000, it is possible to state that the velocity profile inside a microfluidic channel is laminar[3].

The microscale physics can be analyzed not only with the Reynolds number but also with other two important dimensionless numbers: Péclet and Capillary numbers. The Péclet number *Pe* is an indicator of the relevance of convection and diffusion, so it determines which mass transport mechanism is the most important in a specific system[3]. Its value is calculated using equation 1.4:

$$Pe = \frac{vd}{\mathcal{D}} \tag{1.4}$$

where v is the fluid velocity inside the channel in [m/s], d is the diameter of the channel in [m] and $\mathcal{D}$ is the diffusion coefficient of a generic species in [m2/s]. High values of Pe mean that convection dominates the system, while low value mean that diffusion is more relevant. In microfluidic channels the diffusion has the dominant role on mass transfer of species because Péclet number have a typical value of 500 far from the value on the order on 10^3 where the convective mass transport is dominant. Finally, the Capillarity number *Ca* represents the relative effect of viscous forces against surface tension in a specific system. This dimensionless number is calculated with the following equation:

$$Ca = \frac{vy}{\gamma} \tag{1.5}$$

where v is the velocity of fluid inside the channel in [m/s], η is the shear viscosity of the fluid in [Pa·s] and γ is the surface tension of the fluid (usually with air)[1] in [kg/s^2]. In this case, high values of Capillarity number mean that viscous forces dominate the system, while low values mean that interfacial forces are more relevant. A typical value for a microfluidic channel is about $1.4 \cdot 10^{-4}$ and indicates that interfacial forces are completely dominant in the system with respect to viscous forces.

1.1.2 Application of microfluidics in biology

Cell cultures, in the field of contemporary biology, are certainly one of the most important experimental methods for the discovery of new drugs and therapies. The typical research method used to carry out these tests involves the use of Petri dishes or multi-well culture plates. Despite increases in throughput introduced by the latter, we can still identify gaps and imperfections in their use. The most significant advantages brought by microfluidic cell culture methods with respect to the traditional ones are reported in Figure 1.1[4].

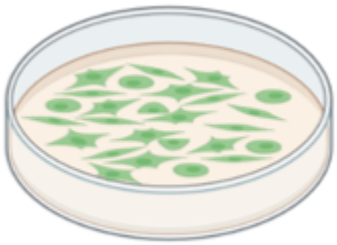
	Conventional cell culture 	Microfluidics cell culture 
Flexibility of device design	Not possible	Possible
Experimental flexibility and control	Low flexibility and low control	High control and flexibility of the experiments
Control of temperature and gases	Large fluid volumes prevent fast change	Small volumes allow dynamic control
Addition of nutrients and removal of metabolites	Infrequent, manual exchange of large volumes	Precisely measured, continuous or transient exchange of media
Stimulation with drugs/proteins and simultaneous imaging	Mostly not feasible	Feasible
Parallelization of cellular assay	Not feasible	High capability of parallelization
Single-cell manipulation and analysis	Manually involved, inaccurate, low throughput	Accurate and high throughput
Automation of cell culture task	Bulky, expensive fluid-handling robot must be use	High capability of automation in compact, inexpensive format

Figure 1.1 *Main advantages using microfluidic cell culture respect of traditional cell culture methods; adapted from[4].*

As said before, microfluidics increases the precision on the control of the fluid flow, also impacting cell culture; moreover, microfluidics permits simultaneous manipulation and analysis from a single cell level to larger cell populations and up to tissues culture. In a microfluidic device it is possible to simultaneously operate on high numbers of individual cell culture chamber, obtaining high parallelization of experiments, high throughput, and thus improved reproducibility and reduction in reagent costs[5]. Nevertheless, microfluidic devices used for biological application have some disadvantages[4]:

1. Using microfluidic device means small volumes, in turn resulting in faster consumption of nutrients and increase of the concentration of metabolites or secreted molecules, especially compared to the conventional cell culture techniques, as shown in Figure 1.2[5];

2. The culture protocols of conventional methods are well known, but they need to be revised and modified before using them in microfluidic platforms;

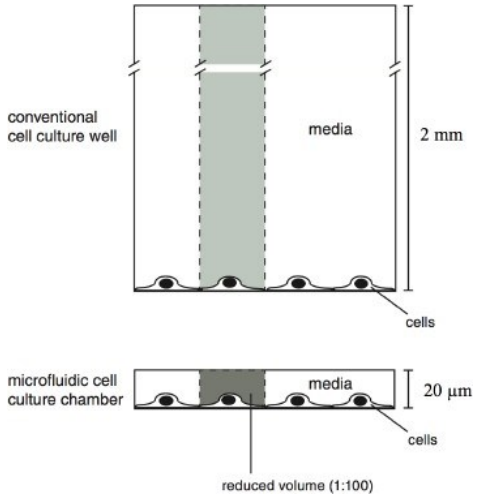


Figure 1.2 *Comparison between conventional cell culture well and microfluidic cell chamber; adapted from*[5].

In the last years there is an increasing demand for automated and quantitative cell culture technology, driven both by the intense activity in stem cell biology and by the emergence of systems biology. In this perspective, the use of microfluidic devices has become crucial. Microfluidic systems can be automated more easily than the standard cell culture platforms. Automation allows culturing cells for several days or weeks under defined conditions without manual intervention. In conventional cell culture application, robotics can be useful to eliminate time-consuming manual pipetting steps resulting in increased throughput and accuracy. The main problems are their bulky and complicated nature, and the substantial cost associated in their use. Microfluidic platforms have high capability for automation in compact and inexpensive form. For example, the possibility to integrate microvalves allows to control fluid flow to have precise chemical and physical control of the microenvironment. This enables precise adherence to the timing of protocols, which is of key importance when dynamic processes in short time intervals are involved[5].

1.2 Materials and technologies for programmable microfluidics

In the last ten years microfluidics has seen a considerable development. Now it is possible to create devices with hundreds of different components that can constitute a real *"Lab-on-Chip"* (LoC) as reported in Figure 1.3.

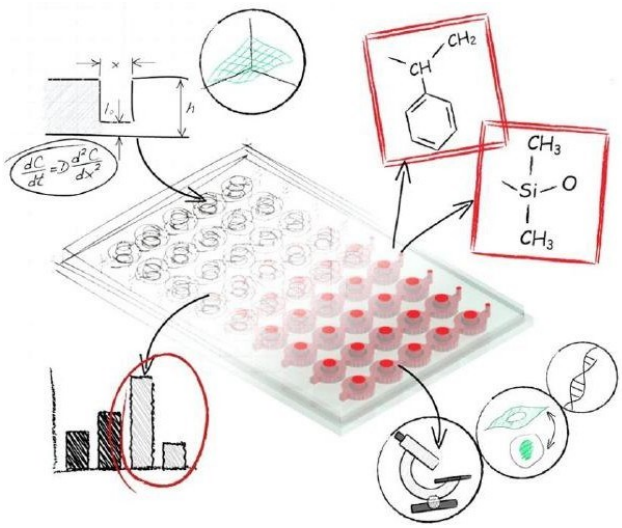


Figure 1.3 *Illustration of the principal features and functions of a "Lab-on-Chip".*

In the early 90s, the concept of LoC was used for the first time indicating a microfluidic device providing integration on a single chip of all those components necessary to carry out the different stages of a chemical and/or biological process in sequence. These miniaturized devices enabled to consider process in which thousands of reactions can be performed in parallel in an automated way. This creates high potential for use in molecular biology and combinatorial chemistry.

The real challenge, at this point of development, is to implement an automated system capable of processing and controlling different actions simultaneously to obtain high-throughput response in a single device. In fact, it is too complex for a single operator to manually manage all the required operations. The solution came from the concept of programmable microfluidics, a term introduced to indicate a microfluidic chip in which all device-specific actions can be managed via software through a control system.

To fabricate these complex devices, we must first carefully study the materials and the most suitable methods. In particular, the material must be as compatible as possible with the analysis methods used, allow the use of common and economical manufacturing methods, and ensure integration with functional components such as pumping systems, valves, and fluid mixing systems. During the past years various materials have been used for the manufacture of microfluidics system as for example silicon, glass, and polymers. Silicon thanks to his mechanical and semiconductor properties, glass thanks to his excellent optical transparency, and polymeric materials for their low cost, ease of manufacturing (by molding), and biochemical compatibility. The most used material for microfluidic devices is Polydimenthylsiloxane (PDMS).

1.2.1 Biomaterial: characteristics and properties of Polydimethylsiloxane

The biomaterials for a microfluidic perfusion culture system are essentially divided into two main categories: the microfluidic channel material and the material over which cell attach[6]. Materials like glass and silicon were abandoned and replaced by a new polymeric material call Polydimethylsiloxane (PDMS)[7].
This elastomeric material belongs to the siloxane's family; its molecular structure is constituted by the repetition of fundamental monomeric units as reported in Figure 1.4[7]. This monomeric unit is composed by silicon atoms linked to two atoms of oxygen and two methyl groups with covalent strong bonds.

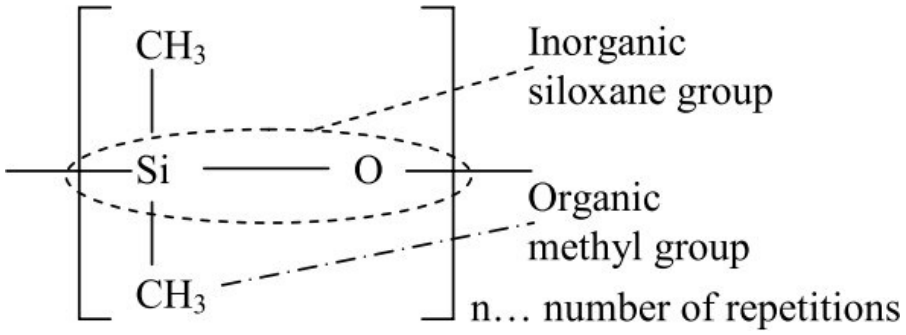


Figure 1.4 *The fundamental monomeric unit: the structure of Polydimethylsiloxane combines both organicand inorganic groups; adapted from[7].*

The choice of this material derives by the following advantages[5]:

1. Preparation is fast and easy and does not require special equipment;
2. If it is fully cured is not cytotoxic;
3. PDMS offers transparency and stable optical features providing excellent live cell imaging condition;
4. In combination with fluorescent live cell imaging, microfluidic cell culture devices made by PDMS allow powerful characterization of a multitude of cellular response.

However, PDMS is a hydrophobic and porous material that have a good permeability to gasses, and allows absorption of hydrophobic compounds such as lipids or other small molecules from the culture media to the PDMS bulk[4]. These properties also cause rapid evaporation. To avoid all this, it is necessary to implement some precautions: change the culture media frequently, place the device in an environment with high humidity, for example an incubator connected to a humidifier, or design an on-chip media reservoir.
The PDMS is obtained mixing a base with a curing agent in specific weight ratios

depending on the desired mechanical properties: the greater the percentage of curing agent in the mix, the harder and stiffer will be the final product. The most widely used commercial kit to obtain PDMS for biological application is the Sylgard® 184 kit (Dow Corning). After mixing, PDMS is cured to cross-link the polymer completely. The curing temperature affects the mechanical properties of the product: the greater the temperature, the greater the Young's modulus, the lower the compressive modulus and the higher the hardness of the material[8]. Lastly, if PDMS is not fully cured, can be toxic to cells, and this toxicity is even more pronounced for "off-ratio" mixtures used when fabricating multi-layer devices[5]. To avoid toxicity, chips should be taken at curing temperatures for extended durations to fully crosslink PDMS. This material can also be autoclaved to improve biological compatibility. Indeed, cells normally do no attach to native PDMS, but it needs a procedure called coating, that consist of deposit a substrate of proteins, such as fibronectin or various mixture like laminin or Matrigel.

1.2.2 Design and production of the platform

The production of a microfluidic device can be divided in four steps that will be described in the next section. Each is essential for the good realization of the final PDMS platform.

1.2.2.1 *Microfluidic platform design*

The first stage of the development of a microfluidic device is realized using a CAD software for creating a model with the desired geometry. Then, it is important to analyze the behavior of fluids inside the designed geometry to verify its capability to respect the experimental requirements. This step can be performed using multiphysics simulators (e.g., Comsol Multiphysics Simulator®). These software are fundamental tools to simulate the action of physical phenomena of a different nature (e.g.,mass and heat transport, mechanical stress) on the device. In case of multilayer platforms, the model is separated into all the different layers that compose the final device. In each of these layers, thus, it will be possible to find the set of all the components of the chip that have the same height. Once all the necessary tests have been carried out, the final design can be produced, starting from high resolution prints for all layers, used as photomasks for the following steps.

1.2.2.2 Soft lithography

Soft lithography is a technique introduced for the first time by Whitesides and his collaborators in 1998 that allows to create an embossed mold (also called master mold) for reproducing micro-structures on an elastomeric material (PDMS). Soft lithography can be divided into two macro categories: Single layer soft lithography and Multilayer soft

lithography that can be used for more elaborated systems. This technique begins with the deposition of a photoresist on a silicon wafer through the use of a spin coater. The photoresist is a polymeric substance that reacts to UV, X and ionic rays. Depending on the specific geometry, two types of photoresists could be used: negative or positive. They differ in the height and shape of the section of the channel they produce, and in the photo lithography workflow. This is due to their different chemical composition and especially to their opposite behaviors with respect to UV light. After the deposition of the photoresist with the spin coater, the photoresist will be exposed to a collimated beam of UV light through the previously created photomask. Finally, uncured areas will be removed in development phase using an organic solvent. The limit in dimension for this technique are between 500 nm and 500 μm of height and width. It is important to underline that between one step and the following there is a heat treatment that can last minutes or even hours. In Figure 1.5 a summary diagram of the *soft lithography* technique is reported.

1.2.2.3 Replica Molding

After the production of the master mold, the microfluidic platforms are fabricated via replica molding. This process permits to reproduce the shape of the mold by pouring and polymerizing a material such as PDMS. PDMS needs to be degassed and cured with precise time and temperature to crosslink the polymer, and then peeled from the mold. To complete the device, holes are created to connect the hydraulic circuit with external pumping systems. Thanks to this technique it is possible to realize microfluidic platforms in PDMS with easiness of operations, in short times and ensuring repeatability of the final product. This last aspect is very important in biological application since it allows to standardize the protocols reducing the number of variables which could affect the results.

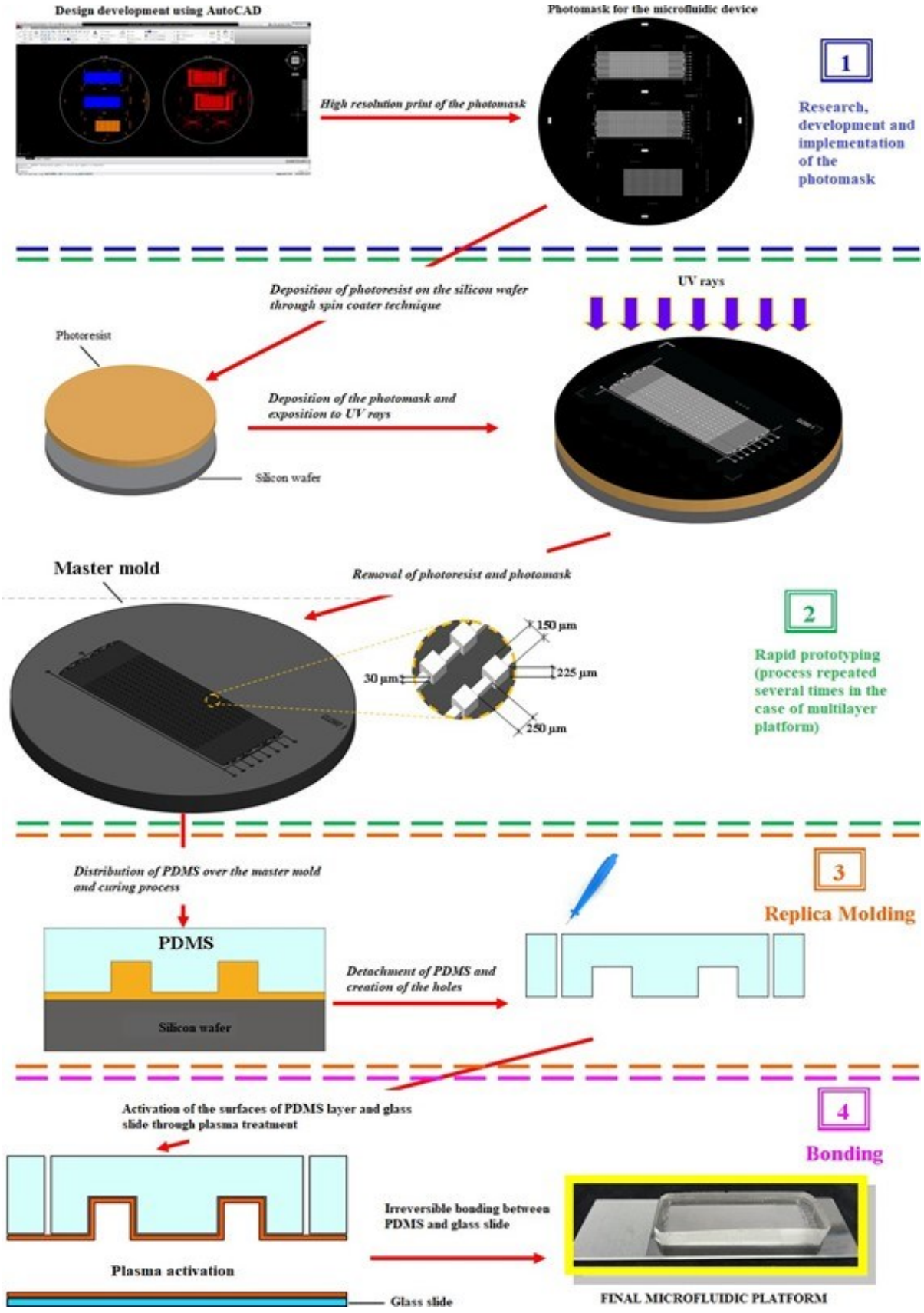


Figure 1.5: *Illustration of the phases of soft lithography technique necessary to produce a microfluidic platform.*

1.2.2 Plasma treatment

The device in PDMS is now complete and ready to be attached to a substrate that can be either a glass slide or another PDMS surface. Depending on the surface to which the chip is to be adhered, two types of bonding can be distinguished:

a. *thermal bonding*: to join the chip with a PDMS surface by heat treatment at different temperatures and time intervals;

b. *plasma bonding*: to join the chip with a PDMS surface or a glass slide.

Plasma treatment is the most used technique suitable for this kind of application since it allows the functionalization of surfaces and it permit their adhesion. Plasma treatment exploits the ionization of the low-pressure oxygen inside the chamber obtained using high frequency voltages: the ions react with organic species to form CO_2, CO, and H_2O. It is important to clean the surfaces for increasing the number of exposed hydroxyl groups (-OH) in all the surface (PDMS device and glass slide) that must be activated. The surfaces are attached together thanks to the exposed hydroxyl groups that form strong covalent bonds [Si-O-Si].

1.2.3 Programmable microfluidics

Some microfluidic platforms contain specific component able to introduce reagents and compounds, mixing chamber and system to improve the control of the fluids inside the microchannel[9]. To create these components inside a microfluidic device it has been introduced an innovative technique, reported in Figure 1.6[10], called *multilayer soft lithography*. This is given by the combination of *soft lithography* technique with the ability to join several layers of polymer with different functions.

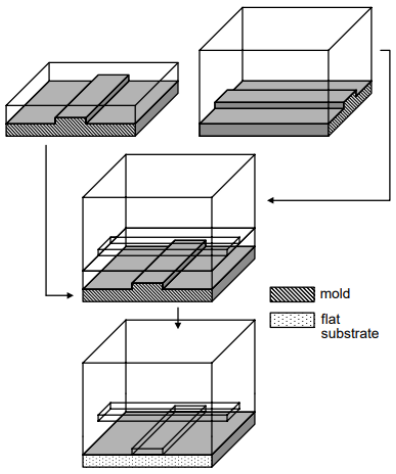


Figure 1.6 *Typical manufacturing scheme of multilayer soft lithograph; adapted from[10].*

The advantage introduced by this technique consists in the possibility to create inside the device itself automated systems of control and movements of the fluid, thus reducing the number of external devices needed. Moreover, the whole fluid handling inside the platform can be controlled through specific software, increasing the precision, and reducing the operations required by the human operator. All of this is possible thanks to the realization of mechanical microvalves[10], the key elements that allow fluid transport within the platform channels. Microvalves are produced starting from the mold in PDMS of two different rigid masters. The first is the one with the layer of fluid channels within which fluid flows and takes the name of flow layer. The second contains the channel which, if pressurized, will deflate the valve membrane capable of stopping the flow of fluid in the flow layer duct. These channels constitute the control layer. The elastic membrane will be formed when the two PDMS molds are joined by plasma treatment. In this way the control layer's own channels will intersect orthogonally with those of the flow layer. The state of the art proposes mainly three different types of valves in the microfluidic field:

3. Push-down valves: present in systems where the control layer is located above the fluidic layer. The deformation of the upper channels by a pneumatic or hydraulic pressure leads to partial or total occlusion of the underlying channels as reported in Figure 1.5 a[11].

4. Push-up valves: present in systems where the control layer is below the fluidic one. The pressurization of the control channel leads to the deflection of the membrane which tends to occupy the overlying structures as reported in Figure 1.5 b[11].

5. Sieve valves: they are very similar to the push-up valves as a conformation, but they differ from the fact that they consist of rectangular fluidic channel. In this way the fluidic channels will never be completely occluded by the deformation of the membrane channel.

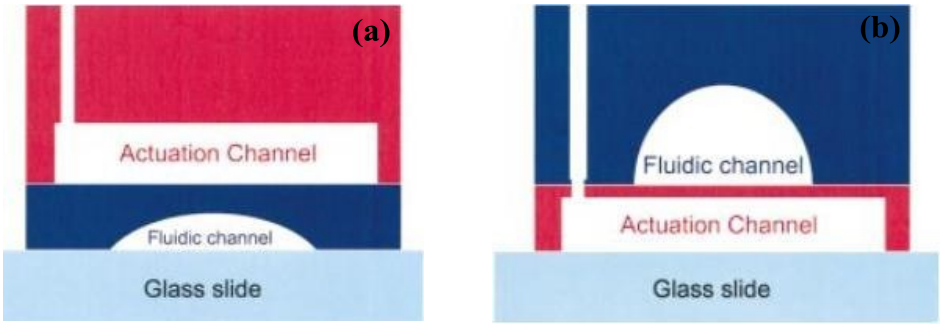


Figure 1.7 *(a) Schematic diagram of push-down valve geometry; (b) Schematic diagram of a push-up valve geometry; adapted from[11].*

1.3 Neuroblastoma

Neuroblastoma is a pediatric tumor that originates from neuroblasts, cells of the sympathetic nervous system that control part of the involuntary activities such as digestion, breathing and heartbeat[12]. The reason why this tumor can arise in many locations of the body is that neuroblast are present in the neck, thorax, spinal column, and adrenal glands. Most cases of Neuroblastoma occur in the adrenal glands or in the nerve ganglia (agglomerations of nerve cells) of the abdomen, while almost all the remaining cases affect the ganglia on the spinal column[13]. Neuroblastoma is an extremely aggressive disease indeed data from the Associazione Italiana Registri Tumori (AIRTUM) said that Neuroblastoma ed other tumor of the sympathetic nervous system represent 7% of all the tumor in the children within 14 years.

1.4 Aim of this thesis

The aim of this thesis is the development of an automated cancer-on-a-chip platform to mimic the progression of Neuroblastoma, and screen for anticancer drugs responses in controlled microenvironments. The creation of cost-effective and reliable *in vitro* models that can be used for accurately screening anticancer drug effects, as well as overcoming the drawbacks of conventional models, is of great importance for improving the clinical management of primary tumors and metastatic cancers. The need for an improvement in the available devices derives mainly from the increasing usage of automation in biological applications. Therefore, the development of a new multilayer platform is thus mandatory to have the possibility to implement new ideas and improvements.

This new microfluidic device should have the following characteristics:

1. Simplicity and reproducibility: the platform must have a straightforward geometry, being simple to fabricate, assemble and reproduce.
2. Reliability and resistance: the platform and its micro-components must be used for long-term applications without incurring in problems such as lamination of the platform or failure of the microvalves.
3. Possibility to be easily integrated with different analysis techniques, especially imaging systems.

The use of an automated microfluidic platform represents a novelty in this research field because what has been done, up to now on a large scale, has been reproduced not only on microscale but also in an automated way. The device is produced using multilayer soft lithography to obtain integrated microvalves for the co-culture of different types of cells simultaneously in a controlled way. The laboratory where this work has been performed, BIAMET (Biomedical Applications of Multiscale Engineering Technologies) is a laboratory

where biology, engineering, medicine, and biotechnology meet to study human cancer disease and in particular Neuroblastoma. The work of this thesis is divided into two parts: the first is based on the design and the development of a device and the second part that refers to the biological validation.

Chapter 2

Materials and methods

This chapter reports the main procedures and protocols for the fabrication of an automated multilayer platform with a microvalve integrated system for controlling culture of multiple cell types. In this section are also explained the design and the production of the controlling microvalve system and the validation of their proper functioning. At the end of the chapter, all the protocols regarding the biological experiments and validations on this platform like seeding, immunofluorescence and cell viability assay are described.

2.1 Design and production of a multilayer platform

To correctly design a microfluidic platform, it is important to respect some constrains on mechanical stability and choose a geometry appropriate to the objectives to be achieved. The platforms' automation bring advantages and disadvantages; the positive aspects are those listed in paragraph 1.2, while the difficulties arise from the need of a multilayer platform. Automation requires the fabrication of a device having two different layers: one for the flow and one for its control. The first constrain is that the chip must be attached on a 75x50 mm glass slide, so the maximum surface of the device must be 60x40 mm (considering a factor of 0.8). The design must also to satisfy the general rules concerning the design of microfluidic platforms:

- The minimum spacing between the edges of different structures (not between the channels) shall be equal to 2 mm;
- All devices must have standard "device alignment marks", they are useful to align different PDMS layers to each other;
- No structure (control or flow) can be fabricated having an aspect ratio lower than 1:10 and greater than 10:1 (height:width), aspect ratio extremely high or low could produce deformation in the PDMS structure, as reported in Figure 2.1[14];
- All the dimensions of the final design have to be increased of 1.5% in order to take into account the shrinkage of the polymeric material after the extraction from the mold.

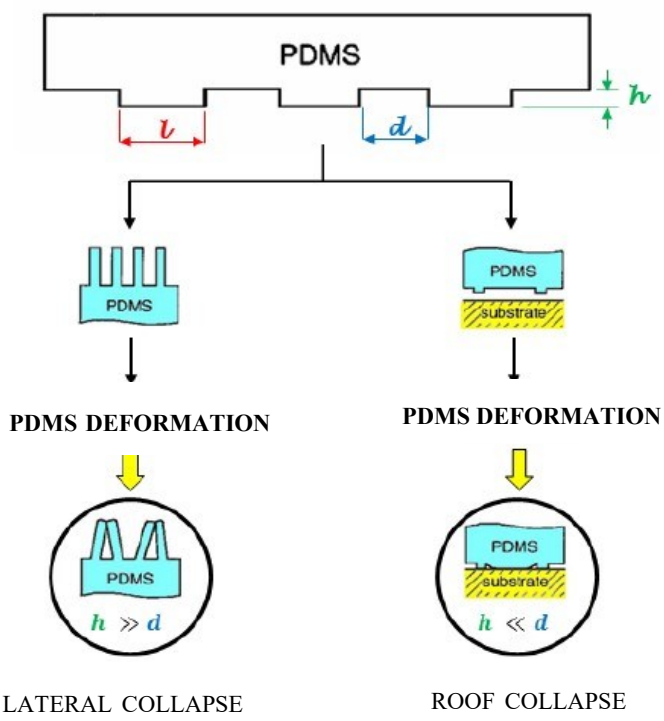


Figure 2.1. *Limit cases occurring in the presence of aspect ratio values far from the optimal value. On the right there is the representation of a case of too high aspect ratio that leads to a lateral collapse of the structures. On the left, however, too small aspect ratio causes a "ceiling failure" of the structure*[14].

In particular the design of a microfluidic device is done using AutoCAD® software that permits to create the desire geometry; then, the file is modified with Adobe Illustrator (Adobe®) in order to obtain the photomask. The photomask is used during the procedure of soft lithography to obtain the desired pattern as explain in the next paragraph.

2.2 Soft lithography

In Paragraph 1.2.2.2 the general principles of soft lithography were explained, this section will describe the details for all procedures for soft lithography fabrication of the flow and control mold.

2.2.1 Production of flow mold with soft lithography

The production of the flow mold is considerably more complicated with respect to the control, since it requires the use of both the positive and negative photoresist. This is essential for the creation of microvalves integrated inside the devices.

2.2.1.1 Design of photomask

The design of photomask is the starting point to fabricate the mold and is essential to obtain the desired microfluidic structure. First, using software like AutoCAD® and respecting the constraints described in section 2.1 two geometry are obtained, one for the negative layer and one for the positive. Then, the design is imported in Adobe Illustrator (Adobe®) which allows to color the photomask. The color black is used to selectively block UV rays at, in enabling to transfer a specific pattern onto the photoresist layer.

2.2.1.2 Cleaning and HDMS treatment of silicon wafer

A silicon wafer (diameter equal to 4 inches) is cleaned with Acetone (Sigma-Aldrich), Methanol (Sigma-Aldrich), and distilled water to promote the photoresist adhesion in the following steps. The wafer is then dried using compressed air to remove possible impurities. After the cleaning, the silicon wafer is treated with Hexamethyldisilazane (HDMS, Sigma- Aldrich) vapor for almost 10 minutes.

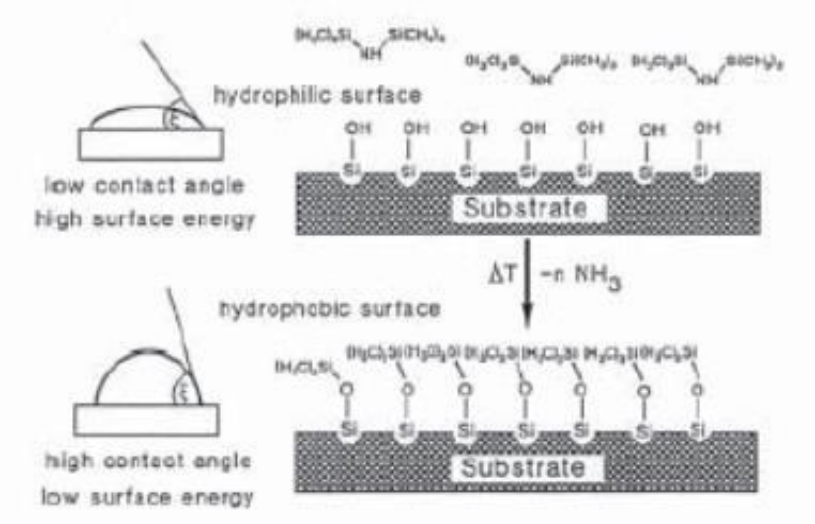


Figure 2.2 *Adhesion of resist to a surface coated with HDMS*[15]

HDMS is a primer which acts as an adhesion promoter for photoresist, more precisely transforming the surface of silicon wafer from hydrophilic to hydrophobic, as reported in Figure 2.2[15].

2.2.1.3 Positive photoresist's layer

The wafer is put in a spin coater like the one reported in Figure 2.3 and secured in the chuck by vacuum generated by a pump.

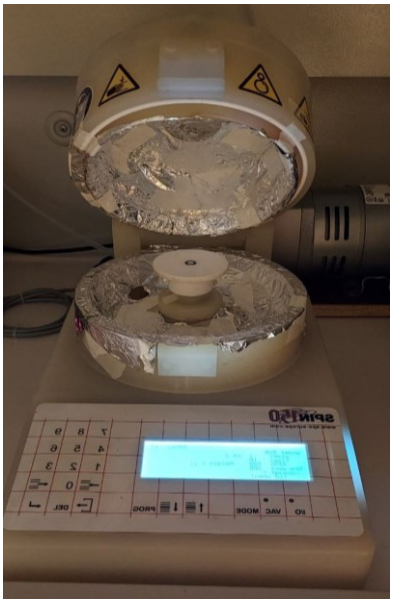


Figure 2.3 *Spin coater used in "clean room" facility.*

The spin coater now needs to be programmed using a protocol adequate to reach the desire thickness of the photoresist layer. The correlation between spin speed in rpm and the photoresist thickness is shown in figure 2.4[16]. To reach about 15-20 µm of thick the spin steps are:

- 1st step: 250 rpm for 15 seconds with an acceleration of 1064 rpm/s.
- 2nd step: 560 rpm for 75 seconds with an acceleration of 1064 rpm/s.

At this point SPR-220-7 positive photoresist (Rohm and Haas, Dow Corning) is distributed and spin-coated over the wafer.

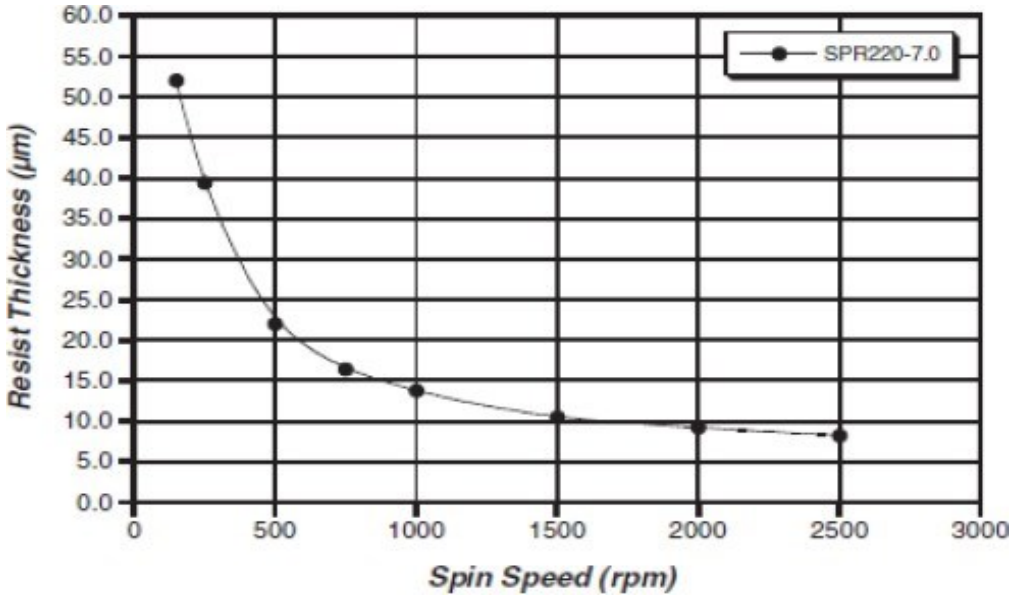


Figure 2.4 *Film thickness [µm] vs spin speed [rpm] for the SPR220-07 positive photoresist[16].*

2.2.1.4 Soft Bake

The Soft Bake is a heating treatment used to guarantee the right properties to the photoresist. This is a critical step because the failure to sufficiently remove the solvent will affect the resist profile, and an excessive baking could damage photoactive compounds and reduce their sensitivity. Soft Bake consists in placing the wafer immediately after the spin on a hot plate set at 90°C for 10 minutes[16]. The temperature and duration of heating depend on the height of the photoresist layer.

2.2.1.5 HDMS and second spin coating of positive photoresist

The next step is an additional treatment with Hexamethyldisilazane (HDMS, Sigma-Aldrich) vapor for almost 10 minutes and then spinning a second layer of the same photoresist over the first one. The spin protocol to obtain a final thickness of 35-40 µm is as follows:

- 1st step: 250 rpm for 15 seconds with an acceleration of 1064 rpm/s.
- 2nd step: 560 rpm for 105 seconds with an acceleration of 1064 rpm/s.

At this point a second heating treatment at 90°C for 60 minutes[16] is necessary, followed by a rehydration phase of at least 3 hours where the wafer is maintained covered to avoid light exposure.

2.2.1.6 Exposure of positive photoresist

In this step, the photo-sensitive compound of the photoresist is activated by the UV light and the photomask needs to be aligned over the wafer to polymerize the positive photoresist in the areas not exposed to UV light. For the exposure step, a UV lamp with wavelength to 365 nm is used, setting the right time calculated with Equation 2.1. The lamp power P [mW/cm^2] is known, while the exposure energy E_{exp} in [mJ/cm^2] depends on the thickness of resist layer.

$$t_{exp} = \frac{E_{exp}}{P} \tag{2.1}$$

In this case the needed exposure energy is 2000 mJ/cm^2 [16].

2.2.1.7 Development and Hard Bake of positive photoresist

After exposure, the wafer is developed in a solvent composed by water (97-98%) and tetramethylammonium hydroxide (2,45%), (Microposit MF-319, Microchem) to dissolve not cross-linked polymer. A glass container is filled with solvent and the wafer is immerged into it, then the glass container is placed over an oscillating plate for about 15 minutes[16]. Then the wafer is rinsed with distilled water to stop the development, and dried with compressed air. The

final step is the Hard Bake, a thermal treatment in which the remaining photoresist is evaporated, and the mechanical and thermal properties of the mold are improved. Hard Bake is done placing the wafer on a hot plate for 10 minutes at 65 °C and then setting a ramp of 10 °C/h for 15 hours to reach the final temperature of 190 °C/h [16].

2.2.1.8 Negative photoresist's layer

After the 15 hours Hard Bake, the next step consists on spin coating a layer of SU8-2100 negative photoresist (Microchem) over the same wafer to obtain a thickness of 250 µm. In Figure 2.5[17] is reported the relation between spin speed and the final thickness of the material.

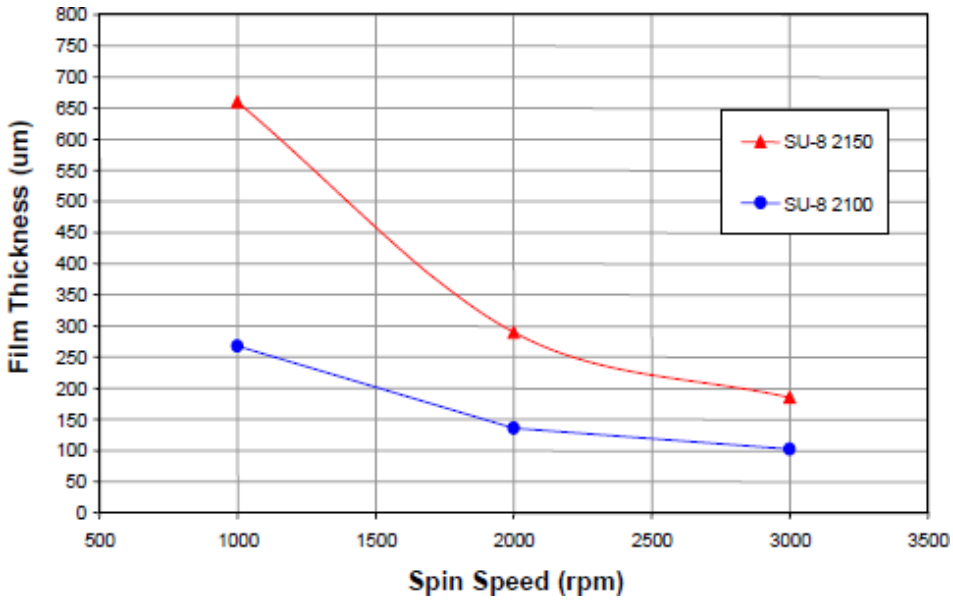


Figure 2.5 *Film thickness [µm] vs spin speed [rpm] for the SU-8 2100 negative photoresist[16].*

So, to obtain a final thickness of 250 µm the spin coater protocol is:

- 1st step: 500 rpm for 10 seconds with an acceleration of 100 rpm/s.
- 2nd step: 1000 rpm for 30 seconds with an acceleration of 300 rpm/s.

The wafer must then be left for at least 10 minutes on a flat surface to better homogenize the height of photoresist layer and to avoid the presence of air bubbles.

2.2.1.9 Soft Bake, exposure and Post Exposure Bake of negative photoresist

Soft Bake is done placing the wafer on a hot plate at 95 °C for 45 minutes. The time necessary to perform this step is reported in Table 2.1 and depends on the height of the photoresist layer[17].

Table 2.1 *Soft Bake times [min] in relation to the thickness [µm] of SU-8 2100 photoresist; adapted from*[17].

Thickness [µm]	Soft Bake time @95 °C [min]
100-150	20-30
160-225	30-45
230-270	45-60
280-550	60-120

Before the exposure step, the photomask must be placed over the wafer and aligned with the positive photoresist layer. This step is helped by the presence of device alignment marks. The necessary exposure energy E*exp* depends on the thickness of the photoresist layer as reported in Table 2.2[17].

Table 2.2 *Exposure energy [mJ/cm²] vs thickness [µm] of SU-8 2100 photoresist*[17].

Thickness [µm]	Exposure Energy [mJ/cm²]
100-150	240-260
160-225	260-350
230-270	350-370
280-550	370-600

In this case, the exposure energy is 350 mJ/cm^2 and the corresponding t_{exp} is calculated with equation 2.1. The next step, the Post Exposure Bake is the last heating treatment of the process and is done on a hot plate at 95 °C for 15 minutes. It is needed to ensure the complete reticulation of the polymer after the exposure.

2.1.1.10 Development and Hard Bake of negative photoresist

The development procedure is the same described in section 2.2.1.7, changing the solvent with propylene glycol monomethyl ether acetate (Sigma-Aldrich) and developing for approximately 17 minutes as reported in Table 2.3[17].

Table 2.3 *Development times [min] in relation to the thickness [µm] of SU-8 2100 photoresist*[17].

Thickness [µm]	Development time [min]
100-150	10-15
160-225	15-17
230-270	17-20
280-550	20-30

Then the wafer is rinsed with isopropyl alcohol (Sigma-Aldrich) and dried with compressed air. Hard Bake step is done putting the wafer on a hot plate at 65 °C for 10 minutes and then setting a ramp of 120 °C/h for 2 hours to reach the final temperature of 160 °C [17].

2.2.2 Production of control mold with soft lithography

In this section will be described the protocol for soft lithography fabrication of the control mold. It is very similar to the negative photoresist layer procedure described in the previous sections.

2.2.2.1 Design of photomask

As before, the starting point is the design of the photomask to obtain the desire microfluidic structure. The photomask is obtained using the software AutoCAD® and then the design is imported in Adobe Illustrator (Adobe®) to realize the photomask.

2.2.2.2 Pretreatment of silicon wafer and deposition of photoresist

A silicon wafer (diameter 2 inches) is cleaned and dried following the same procedure described in section 2.2.1.2. The wafer is then inserted in a spin coater and secured in the chuck by vacuum generated by a pump. At this point, a layer of SU-8 2050 negative photoresist (Microchem) is distributed over the wafer and spin coated. To obtain a thickness of 45 µm, observing the relation between spin speed and the desired thickness reported in Figure 2.6[18], the spin protocol is the following:

- 1st step: 500 rpm for 10 seconds with an acceleration of 200 rpm/s.
- 2nd step: 3000 rpm for 30 seconds with an acceleration of 200 rpm/s.

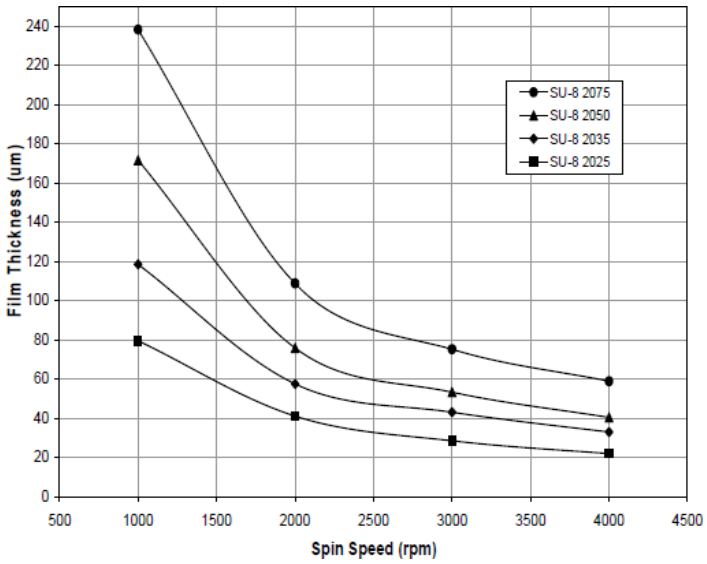


Figure 2.6 *Film thickness [µm] vs spin speed [rpm]for the SU-8 2050 negative photoresist*[18]

2.2.2.3 Soft Bake and exposure

In this step the Soft Bake lasts 7 minutes at 95°C, as shown in Table 2.4[18].

Table 2.4 *Soft Bake times [min] in relation to the thickness [µm] of SU-8 2050 photoresist; adapted from[18].*

Thickness [µm]	Soft Bake time @95 °C [min]
25-40	5-6
45-80	6-9
85-110	10-20
115-150	20-30
160-225	30-45

The energy needed for the exposure step again depends on the thickness of material as reported in Table 2.5[18]. For the thickness of 45 µm it is necessary an exposure energy of 160 mJ/cm^2.

Table 2.5 *Exposure energy [mJ/cm^2] vs thickness [µm] of SU-8 2050 photoresist[18].*

Thickness [µm]	Exposure Energy [mJ/cm^2]
25-40	150-160
45-80	150-215
85-110	215-240
115-150	240-260
160-225	260-350

2.2.2.4 Post Exposure Bake, development, and Hard Bake

The conditions for Post Exposure Bake and development depend both on the thickness of the photoresist layer as reported in Table 2.6[17] and Table 2.7 [18].

Table 2.6 *Post Exposure Bake times [min] in relation to the thickness [µm] of SU-8 2050 photoresist[18].*

Thickness [µm]	PEB time @95 °C [min]
25-40	5-6
45-80	6-7
85-110	8-10
115-150	10-12
160-225	12-15

The PEB step is done putting the wafer over a hot plate at 95 °C for 7 minutes. The development is done with propylene glycol monomethyl ether acetate (Sigma-Aldrich) and with the same procedure described in section 2.2.1.10. The development time is 6 minutes. The final step is the Hard Bake, done putting the wafer on a hot plate for 10 minutes at 65 °C and setting a ramp of 120 °C/h for 2 hours to reach the maximum temperature of 160 °C[18].

Table 2.7 *Development times [min] in relation to the thickness [µm] of SU-8 2050 photoresist[18].*

Thickness [µm]	Development time [min]
25-40	4-5
45-80	5-7
85-110	7-10
115-150	10-15
160-225	15-17

2.3 Plasma treatment protocol

The plasma treatment is done to create an irreversible hydraulic sealing between different surfaces. This technique is used on PDMS and glass slide or on two layers of PDMS. In both cases the steps are the same: it starts cleaning the surface of the materials to remove impurities and placing them on the Plasma Cleaner (PDC-002-CE by Harrick Plasma, reported in Figure 2.7a) with the surface to be activated facing upwards. Then the door and the valve are closed and the vacuum pump activate to reduce the pressure inside the plasma chamber. After 4 minutes the plasma cleaner is switched on, and the valve slightly opened to allow the entrance of air, until the color in figure 2.7b is reached. This phase last for 1 minute after which vacuum pump and plasma cleaner are switched off and the valve opened to repristinate the atmospheric pressure. The last step consists in joining the two pieces creating irreversible chemical bonds.

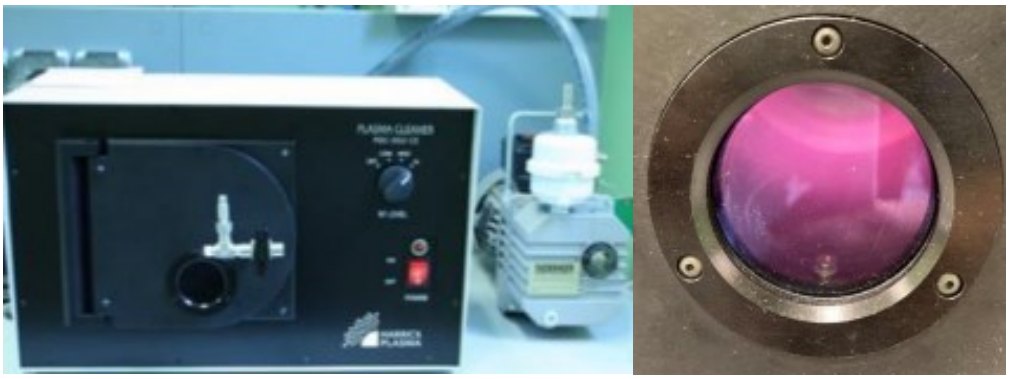


Figure 2.7 *(a) Plasma cleaner and (b) the color of the plasma inside the chamber in the right configuration.*

2.4 Replica Molding

The protocol to produce a PDMS multilayer platform is composed by the following steps:

- PDMS (Sylgard®184, Dow Corning) (Figure 2.8a) is prepared by mixing a base (siloxane) with a curing agent (cross-linker) in a weight ratio of 10:1. The solution is stirred to obtain a homogeneous mixture and placed under vacuum in a desiccator (Figure 2.8b) to remove all air bubbles trapped inside the material.

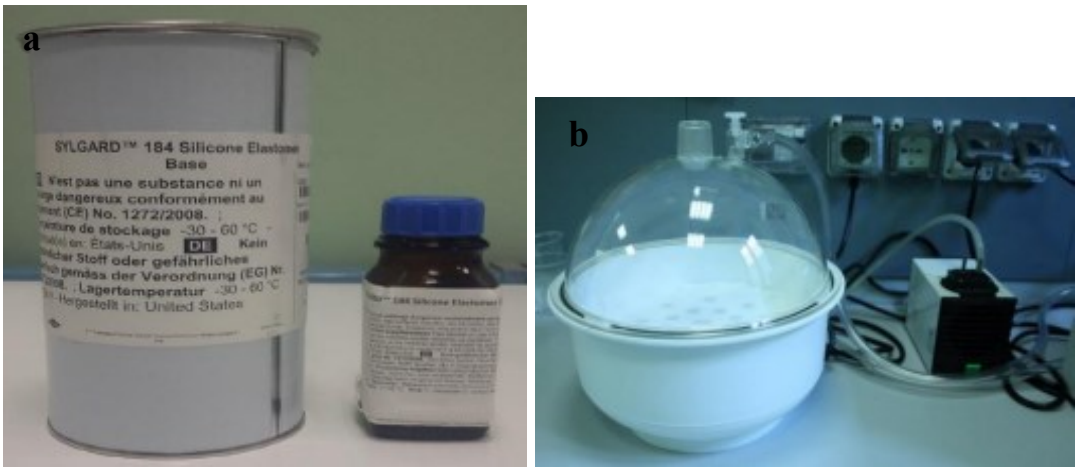


Figure 2.8 *(a) PDMS Sylgard® 184 (Dow Corning), two-part kit: silicone elastomer base on the left, curing agent on the right. (b) Desiccator connected to a vacuum pump.*

- "Silanization" of the wafer for control and flow mold using vapor of chloro-trimethylsilane for 60 minutes. This step improves the durability of the mold.
- Production of PDMS layers from the two molds. For the flow mold the PDMS is poured slowly in the center of the wafer, degassed and put in an oven for 60 minutes following the protocol reported in Table 2.8. For the control mold, the PDMS need to be spin coated over the wafer following this protocol: one single 75 seconds step with acceleration 300 rpm/s and a velocity of 1300 rpm to obtain a thickness of 80 μm; baking in the oven for 60 minutes to cure.

Table 2.8 *Times [min] and temperatures [°C] to cure PDMS.*

Time [min]	Temperature [°C]
60	80
75	75
120	60

- The PDMS layer on flow mold is cut and removed gently. At this point the inlets and the outlets of the microfluidic device are punched with the punch of 1 mm of diameter.

- At the end of the heating treatment, the flow layer is attached with plasma treatment over the control layer without peeling the control layer from the mold. In this step great precision is needed to correctly align the two layers. At this point the control layer is cut and peeled from its mold and the layers are holed to create the inlets for the control layer with a dispense tip (Nordson EFD 21G) with internal diameter 0.51 mm.
- The final step consists in assembling the chip with a glass slide. The slide is first covered with a layer of PDMS with a thickness of 10 μm using the same spin protocol for the control layer but increasing the velocity to 2300 rpm. The coated slide is put in the oven for about 60 minutes prior to the final plasma binding to the chip.
- The assembled microfluidic platform is further baked in the oven at 80°C for 10 minutes to favor the complete adhesion of the control layer.

2.5 Automation

As described in the previous section, this microfluidic platform is designed to guarantee the possibility to automate it. The automation is achieved by using solenoid valves controlled via a custom program, developed using Arduino.

2.5.1 Instrumentations for the microfluidic platform automation

In figure 2.10 is reported the instrumentations used to automate and control the multilayer platform. The main system is mounted inside a box to make easier the moving of the system in the laboratory.

The main system is composed by three parts:

1. Electronic unit for the generation of the signal (Figure 2.9a), this part is connected to an external computer to generate the digital input;
2. Solenoid valves (Figure 2.9b): each is fixed to a tube connected to the control's inlets of the microfluidic platforms; each tube ends with a needle to be easily inserted in the holes. The valves control the flow of compressed air that passing through the tube open/close the microvalves inside the platforms;
3. Pressure regulator (Figure 2.9c): the pressure of solenoid valves can be easily controlled on the manometer and changed by a pressure regulator (CDK Corporation).

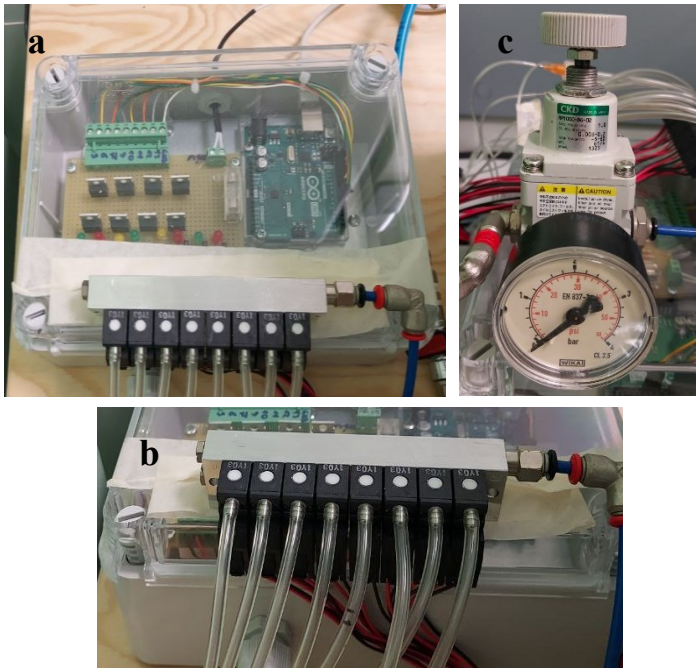


Figure 2.9 *(a) Electronic system of the automation instrument composed by hardware Arduino and solenoid valves, (b) detail of the solenoid valves, (c) manometer.*

All the on-chip valves are driven by solenoid valves (24V, CDK Corporation) which are in turn controlled by an electronic unit for the generation of digital signals (Arduino Uno Rev3 SMD) connected to the USB port of a computer. An Arduino software is needed to create an interface controlling the instrumentation from a computer. Each solenoid valve can switch the on-chip valve from atmospheric pressure (on-chip valve open) to the pressure that permit its closure through compressed air. In addition to the main system described above, a syringe pump controls fluid flow on the order of microliters per second. This pump could be used to seed the cells inside the platform and to continuously perfuse fresh culture media inside the device as reported in Figure 2.10.

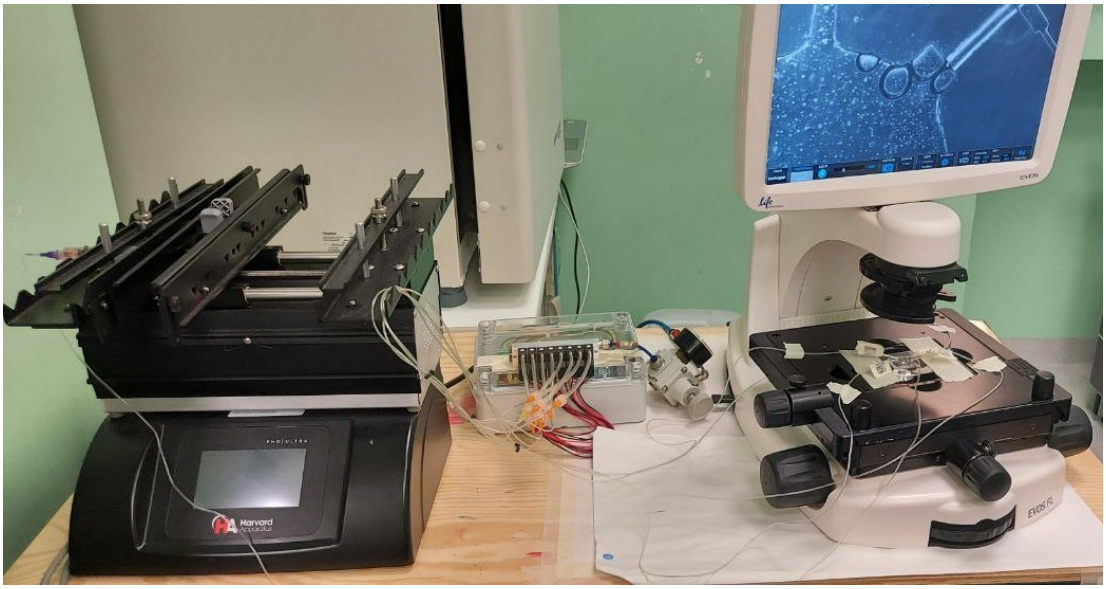


Figure 2.10 *Overview of the automated system set up.*

2.5.2 Arduino code and automation procedure

Arduino is an open-source electronics platform based on easy-to-use hardware and software. Arduino boards read inputs messages (like light on a sensor or a finger on a button) and turn them into an output activating a motor, turning on an LED, or in this case opening a valve. For doing this it is possible to use the Arduino programming language (based on Wiring) and the Arduino Software (IDE), based on Processing. In this case Arduino is needed to create the interface to control the instrumentations from a computer. Through the software we can control the solenoid valves properly and consequently the on-chip valves. It is necessary to develop a proper Arduino code depending on the specific application. In this case we need to control four valves, that permit to separate the four chambers of the platform from the central chamber. The system is composed of eight independent solenoid valves, thus enabling simultaneous control of two separate microfluidic devices.

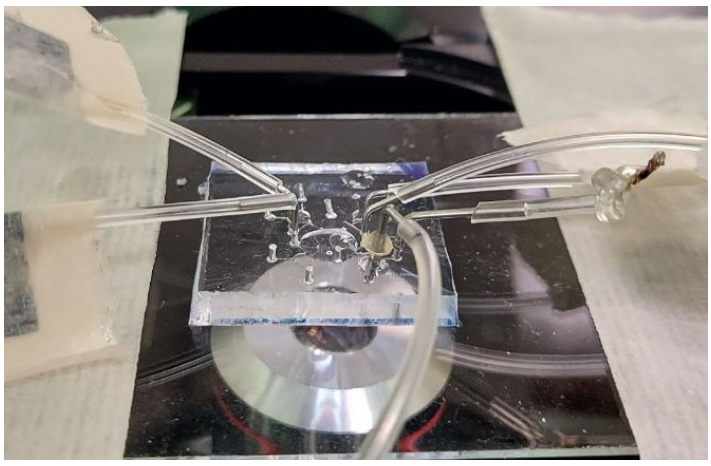


Figure 2.11 *Detail of the tube connected to the control layer.*

The first step is the interface between the automation system and the computer, using a custom software develop with Arduino Software (IDE). At the end of this operation, it is possible to control the solenoid valves manually with the user interface in the computer. At this point 4 tubes connected to the solenoid valves are fixed inside the holes of the control layer (Figure 2.11) and a check of the proper working of the valves is done, under a microscope, including a control of the minimum closing pressure for all the valves.

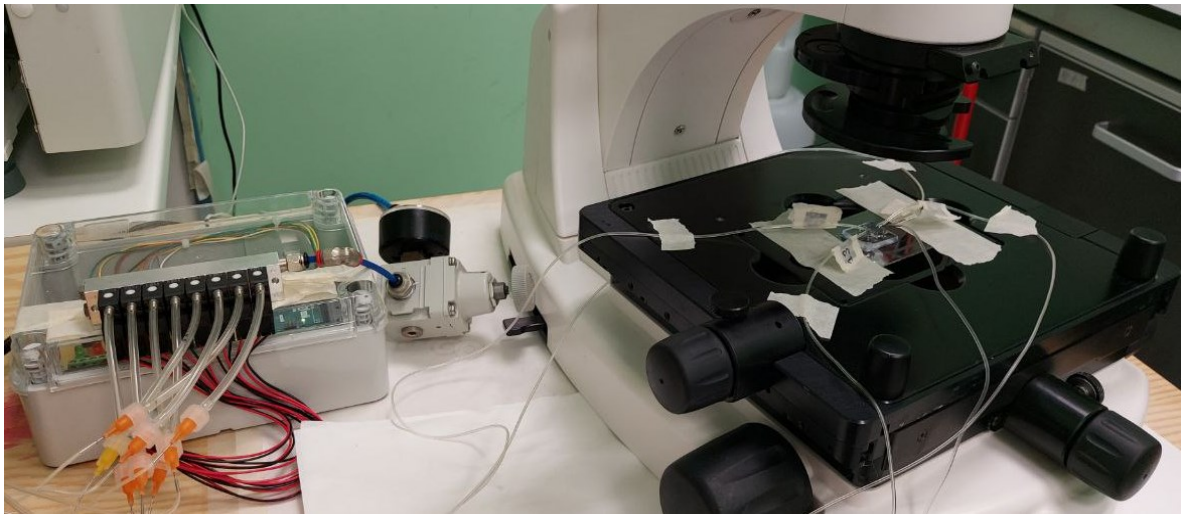


Figure 2.12 *Overview of solenoid valves connected to microfluidic platform.*

2.5.3 Leakage test

The aim of this test is to check the mechanical resistance of the microfluidic platform and of its valves for a long period of time.

The platform is automated with the procedure described in Section 2.5. The test is performed using different pressure values and repeated several times for each condition. The pressure values tested range from 6 to 25 psi. Each test is performed by setting an automatic program that opens the valves for 2 minutes every hour for 1 week.

2.6 Biological validation

To verify if the multilayer platform designed is suitable for cell culture applications, a set of biological validations were performed using different cell lines.

2.6.1 Cell lines

The cells lines use to perform the biological validations of the microfluidic device are provided by ATCC and are:

- SK-N-AS derives from bone marrow of a child with poorly differentiated embryonal Neuroblastoma. This cell line represents the main objective of the study on cancer cells behavior;
- Mesenchymal stem cells (MSC), also known as mesenchymal stromal cells or medicinal signaling cells, are multipotent stromal cells that can differentiate into a variety of phenotypes: osteoblasts, chondrocytes, myocytes and adipocytes, for example. MSCs can be found in bone marrow, cord cells, adipose tissues, and amniotic fluids. This cell line can be considered a target metastatic site of Neuroblastoma;
- Human umbilical vein endothelial cells (HUVECs) are derived from the endothelium of veins from the umbilical cord. They are used as a laboratory model system for the study of the function and pathology of endothelial cells (e.g., angiogenesis).

2.6.2 Biological protocol for cells

Cells are typically kept in culture in 75 cm^2 flasks and maintained in an incubator at 37°C with humidified 5% CO_2. Every two or three days, depending on the cell lines, the culture medium must be changed to give fresh nutrients to the cells. The solution for the medium for MSCs is composed by:

- 90% MesenCult™ MSC Basal Medium (Human) that is a standardized basal medium designed for the in vitro culture of human mesenchymal stem cells;
- 10% MesenCult™ MSC Stimulatory Supplement (Human).

For SK-N-AS the composition is:

- 90% of base medium DMEM (Dulbecco's Modified Eagle Media provided by Thermofisher Scientific);
- 10% of Fetal Bovine Serum (FBS), 1% of PenStrept (an antibiotic to prevent possible contaminations) and 1% of MEM that is an Amino Acids Solution used as

a growth supplement for cell culture medium, to increase cell growth and viability. MEM Amino Acids Solution is formulated to contain 50X the essential amino acids (except L-glutamine) found in the standard Minimum Essential Medium (MEM).

For HUVECs:

- Endothelial Cell Growth Medium 2 (PromoCell®) that is a low-serum (2% V/V) medium that contains Insulin-like Growth Factor (Long R3 IGF), Vascular Endothelial Growth Factor (VEGF), specific supplements and Penicillin-Streptomycin mixture.

2.6.3 Cell splitting protocol

The splitting protocol summarizes the procedures required to maintain cell cultures over time and avoid contact inhibitions when cell numbers increase above a certain threshold. Splitting also allows to count cells and seed them at the correct density in both a new flask and in the microfluidic devices. Almost all operations are carried out under a biological hood to maintain sterility. The starting point is to warm inside a water bath at 37°C all the culture media for the different cell lines used and the Trypsin/EDTA solution. Then the flask is taken from the incubator and placed inside the biological hood to work in secure and sterile conditions. At this point the steps for the splitting procedure are:

- Remove the old medium from the flask and store it in a 15 mL Falcon tube;
- Use 5-7 mL of Phosphate Buffered Saline (PBS, Thermofisher Scientific) to wash the surface of the flask to remove culture media residues that can reduce the Trypsin action. The PBS is then aspired and discharged;
- Add 3-4 mL of Trypsin-EDTA (Thermofisher Scientific) to the flask and incubate for 5 minutes to detach the cells from the flask surface. Trypsin-EDTA is made from trypsin powder, an irradiated mixture of proteases derived from porcine pancreas. Due to its digestive strength, trypsin is widely used for cell dissociation and routine cell culture passaging;
- Add the old medium in the flask to inhibit the action of Trypsin-EDTA and to collect the cells ditched from the surface. By gentle pipetting, the cells are aspirated from the bottom surface and placed in a 15 mL Falcon tube;
- An aliquot of this cell suspension is collected and used to count cells following the protocol described in the next paragraph;

- Centrifuge the Falcon using soft deceleration and setting a protocol adequate for the specific cell types. This induces the sedimentation of the cells that form a dense pellet, while the residues remain in the supernatant. The liquid is aspired and discarded.
- After cells count, an appropriate aliquot of cells suspension is added to a new 75 cm^2 flask, with 7-8 mL of fresh culture medium.

This procedure is usually repeated every 3-4 days accordingly to the specific cell lines culture protocol.

2.6.4 Cell count

The cell count is done using the Bürker chamber, this device has two cells that have a depth of 0.1 mm, and the plane of each cell is divided into 9 squares of 1 mm side. Each of the nine squares includes: 16 large squares with a surface of 0.4 mm^2; each large square is divided into 9 small squares with a surface of 0.0025 mm^2. The main steps for cells count are here reported:

- A volume of cell suspension obtained after flask splitting is collected and diluted with fresh medium accordingly to the approximate number of cells of the starting flask. After that the solution is gently pipetted to avoid cell sedimentation and clustering;
- 10 μL of Trypan Blue (Invitrogen) are added to an equal volume of the cell suspension. Trypan blue is a cell stain that colors in blue only dead cells and thus enabling counting of the living cells alone;
- 10 μL of cell suspension and Trypan blue are introduced between the chamber and the glass slide;
- The chamber is observed at the microscope with a 10x magnification. At least 5 large squares are considered (Figure 2.13), and the cells are counted.

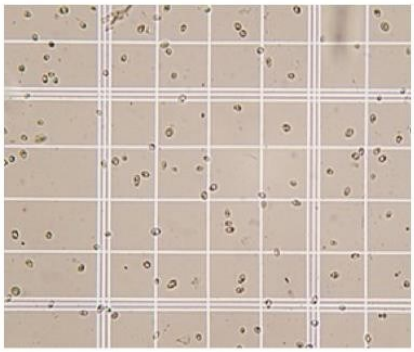


Figure 2.13 *One of the 9 squares divided by three parallel lines which are present on each of the two chambers; the small point visible inside squares are cells.*

To calculate the total number of cells the following Equation (2.2) is used:

$$N_{Cell} = N_{Average} * d_1 * d_2 * K * V_{sosp} \quad (2.2)$$

Where $N_{average}$ is the average number of cells counted using the Bürker chamber, d_1 is the dilution factor of cell suspension, d_2 is the dilution factor for the use of Trypan Blue, K is a constant related to the geometry of the chamber and V_{sosp} is the total volume of cell suspension after the detachment process. This procedure permits to know the total number of cells in a certain volume of solution and its essential to calculate the cell density for seeding the microfluidic platform.

2.6.5 Cell seeding and culture inside automated microfluidic devices

In this paragraph is explained the procedure for seeding the cells in an automated way using the set up shown in section 2.5. First, the automated workstation must be prepared by configuring the PC, incubator, syringe pump, and microscope as in section 2.5.2. The microfluidic platform must now be placed inside petri dishes and UV-sterilized before debubbling. For multilayer platforms, the use of autoclave sterilization is not recommended because of the high temperatures that can damage the control layer. After that, the microfluidic devices are connected to the automation system to verify the minimum pressure to close completely all the valve. The "debubbling" step is necessary to eliminate all the air trapped inside the device. For doing this, a 5 ml syringe is filled with PBS and connected through a small tube to one inlet of the microfluidic device. The pump flow rate is set to 4 µl/min and the solution is perfused (keeping open the microvalves) until all the platform is completely full of liquid. After proper debubbling, a coating treatment is performed to improve the attachment of cell inside the device. The cells used in all the experiments require a coating with Fibronectin (Thermo Fisher Scientific), a glycoprotein of the extra-cellular matrix, at a concentration of 25 µg/ml. A syringe is filled with 1 ml of Fibronectin solution that is injected with a flow rate of 2 µl/min and incubated for 1 hour at room temperature.

At this point all microvalves must be closed to start the cells seeding phase. The aim is to seed the SK-N-AS cancer cells only in the central well, and hMSCs and/or HUVEC in the lateral wells, depending on the experimental requirements. After the procedure of cells splitting described in Section 2.6.3 the different cell lines are seeded inside the device to obtain a final density equal to:

- 200 cells/mm^2 for SK-N-AS cells;
- 100 cells/mm^2 for hMSCs;

- 150 cells/mm^2 for HUVEC.

Different syringes are filled with the cells solutions and are connected to the appropriate inlets (SK-N-AS in central chamber, hMSCs and HUVEC lateral chambers), the pump flow rate is set to 2 µl/min and the perfusion start for 10 seconds. This operation is done for all the wells that compose the device. The procedure is completed by placing the devices inside an incubator keeping microvalves closed until the all the cells inside the device are completely attached on the well surface. After 24 hours from seeding, each inlet is connected to the syringe pump keeping the valves closed for the medium change. Valves are then switched to the open position to permit the communication between the different wells.

2.6.6 Live and dead assay

The Live&Dead assay consists in staining cells using fluorescent substances that have specific affinity to one substrate, in this way they mark a specific target selectively. In this way it is possible to check the cell viability inside the automated microfluidic device. To perform this test, microfluidic devices are seeded using the different cell lines, cells are cultured for 24 hours and then the device are disconnected form the automation system and stained with different cells markers. The staining solution was prepared diluting two cell markers in PBS:

- Calcein-AM: used in volume ratio of 1:1000; it is a green, fluorescent dye which marks the living cell cytoplasm. Its excitation wavelength is equal to 495 nm while the emission one is equal to 515 nm.
- Hoechst: used in a volume ratio 1:500; it is a blue, fluorescent dye which marks all cell nuclei to make easier the operation of counting. Its excitation wavelength is equal to 350 nm while the emission one is equal to 461 nm.

The chemical structures of these substances are reported in Figure 2.14.

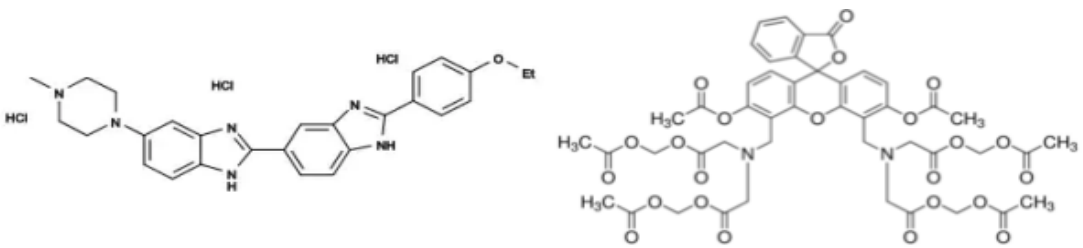


Figure 2.14 *Chemical structures of (a) Hoechst and (b) Calcein-AM.*

After washing the chip with PBS to remove the medium containing Fetal Bovine Serum (FBS) that interferes with Calcein and Hoechst's staining, the previously prepared solution is manually pipetted inside the microfluidic platforms and incubated for 15 minutes. Then the devices are analyzed with a fluorescence microscope (INVITROGEN EVOS FL) and images are taken for later counting and calculating cell viability by image analysis with ImageJ.

2.6.7 Fixation and staining with Phalloidin and DAPI

In the experiments of co-culture, the cells inside the devices are analyzed after PFA fixing with DAPI and phalloidin. The cells are maintained in culture for 72 hours and then disconnected from the automation system and analyzed. Phalloidin is a high-affinity F-actin probe conjugated to the red-orange fluorescent dye, tetramethylrhodamine (TRITC). Phalloidin binds F-actin with high selectivity while TRITC provides red-orange fluorescence of unparalleled brightness and photostability. DAPI is a blue fluorescent dye that fluoresces selectivity for DNA with high cell permeability that allows efficient staining of nuclei. Its selectivity for DNA and high cell permeability allows efficient staining of nuclei with little background from the cytoplasm. The steps for this type of staining procedure are the following:

- The microfluidic device is washed with PBS 3 times;
- Under the chemical hood, 1 mL of 4 % Paraformaldehyde (PFA) is prepared, starting from a solution of 37% PFA, stored at 4°C. The solution once prepared is pipetted inside the chip;
- After 25 minutes the device is rinsed with PBS 3 times and stored at 4°C until final use;
- A solution of 0.1 % of Tryton X-100 in PBS is prepared and injected inside the device (being careful to not generate bubbles). The platform is left for 15 minutes at room temperature to permeabilize cells membrane. Then the platforms are washed several times using PBS;
- A solution containing Phalloidin at a 1:400 dilution in PBS is prepared and pipetted inside the devices. After 50 minutes, the devices are rinsed with PBS;
- A solution containing DAPI at a 1:1000 dilution in PBS is prepared, injected, and left at room temperature for 10 minutes and then washed 3 times with PBS.

At this point the microfluidic platform can be observed and analyzed using a fluorescence microscope.

2.6.8 Immunofluorescence (IF) staining

To verify if there is interaction between different cell lines in co-culture experiments and if this interaction causes any, an immunofluorescence staining analysis is performed. The cells inside the microfluidic device were fixed in 4% formaldehyde (Sigma-Aldrich, F8775) for 25 minutes at room temperature. After formaldehyde removing, cells were washed three times with PBS 1X. After that the cells were incubated for 1 hour with the blocking solution composed by PBS with 5% of BSA (Sigma-Aldrich) and 0.1%

After 1 hour, the devices are washed three times with a solution of PBS-/- with 0.1% of Triton X-100 (PBST). At this point the primary antibodies are applied. Based on different analysis made in our laboratory, it has been decided to analyze Vimentin, the major constituent of the intermediate filament family of proteins, expressed in normal mesenchymal cells maintaining cellular integrity and providing resistance against stress. Several studies confirm that vimentin is overexpressed in various cancers and that its overexpression correlates with accelerated tumor growth, invasion, and poor prognosis[19]. This antibody was used only for hMSCs at 1:100 dilution.

Each primary antibody is prepared in blocking solution (PBS-/- containing 5% of BSA and 0.1% of Triton X-100). Primary antibodies were applied overnight, then cells were washed with PBST and incubated for 1 hour with secondary antibody. Alexa Fluor 488 Goat Anti-Rabbit was used as secondary antibody at dilution 1:1000. After 1 hour, cells are washed with PBST twice and once with PBS-/-. Cell nuclei are then counterstained with 4',6-diamidino-2-phenylindole (DAPI) solution for 10 minutes. DAPI emits blue fluorescence upon binding to AT regions of DNA, marking all cell nuclei. Cells are then washed with PBS-/- and all the devices are filled with mounting medium composed by 80% of Glycerol and 20% of Milli-Q water. Finally, the stainings are analyzed with a confocal fluorescence microscope.

The analysis of the images was performed using ImageJ software. This method allows to quantify IF data collected on a confocal microscope quantitating the relative levels of a molecule of interest by measuring mean fluorescent intensity (MFI) across a region of interest.

The cell samples were observed with a 40× objective and two different dyes were set: Alexa fluor 488 for green channel (Antibodies), DAPI for blue channel (cell nuclei). First, some acquisition parameter must be adjusted. The laser intensity must be as low as possible to minimize photobleaching and saturation of fluorophores, and at the same time maintain an adequate signal-to-noise ratio for image analysis[20]. This can depend on the properties of the fluorophore, objective, laser wavelength, age of the laser, and wattage. For quantitation, it is essential that the laser power be kept constant across the analysis, and it is recommended to leave the laser power at 2% or below. Another option to adjust is the pinhole diameter which is

often expressed in Airy units (AU) defined as 1 Airy Unit = (0.61× wavelength of light x total Magnification)/ Numerical aperture of the lens used for imaging. The recommended setting for the pinhole is usually 1 AU. The Master gain and the digital gain are other adjustments that set the threshold of detection and the dynamic range of the detectors, key for reliable quantitation[20]. The first setting is 'Gain (Master)'; this setting adjusts the sensitivity of the detector, however, increasing the sensitivity can also increase the noise. It is recommended to not go above 800–850 for gain master. Digital Gain', adjusting this setting reamplifies intensities from master-gained images. This amplifies the signal at the same rate as it amplifies noise. It is recommended to leave this value set to 1[20].

For ImageJ quantitation, each image must be converted in 16 bit and the fluorescence intensity measured keeping constant the threshold for all the data collected for every antibody staining. Thresholding is a technique for segmenting an image into two classes of pixels, typically called "foreground" and "background". It works by choosing a cut-off value, so that every pixel below that value is the background, while every pixel greater than that value is considered foreground. After the MFI calculation it is preferable to subtract the background from the MFI. This step consists in selecting and measuring a non-fluorescent area of the same image or a negative control prepared during the same experiment and imaged under the same confocal parameters as the image being quantitated. MFI of that non-fluorescent area/negative control image is then subtracted from MFI.

The statistical analysis was performed with GraphPad Prism software. All data in graphs are from at least three independent experiments. In the box-and-whiskers graphs, box margins are the 25th and 75th percentile, the center line is the median, and the whiskers the minimum and maximum values. Statistical significance was determined using unpaired Student's t-test. Asterisks indicate a significant difference between the treated and the control group, unless otherwise specified (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Chapter 3

Results

In this chapter are reported the results of the construction procedure of the multilayer platform and of the experiments performed following the protocols explained in Chapter 2. In detail, the final design of the device and the construction of the mold followed by all the biological experiments are reported.

3.1 Design

Following the procedure explained in Section 2.1, the final design of the microfluidic platform is shown in Figure 3.1. This design was chosen to have a good compromise between the objective for which the platform was designed and the ease of building and automating it.

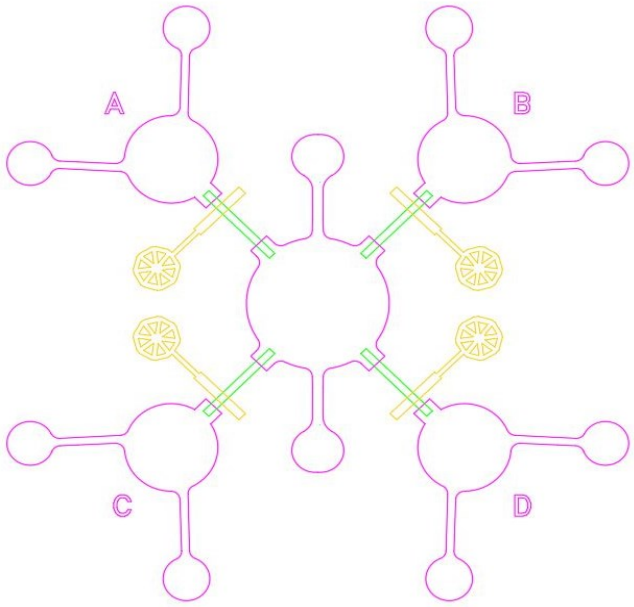


Figure 3.1 *Final design of the multilayer platform, composed by 3 layers. In this figure the 3 layers are represented with 3 different colors: green for the flow layer done with positive photoresist, violet for the flow layer done with negative photoresist and yellow for the control layer.*

In Figure 3.1, it is possible to appreciate three different layers, represented by three different colors: the flow layer done with negative photoresist in violet, the flow layer done with positive photoresist in green and the control layer in yellow. The main structure consists of five

chambers: a central one with a larger diameter and four smaller external ones, each chamber is connected by short channels to its inlet and outlet (punched as explained in Section 2.4), ensuring greater control and ease when seeding cells automatically. Going into the details of the two layers, the flow layer is composed by the structure reported in Figure 3.2, already described above.

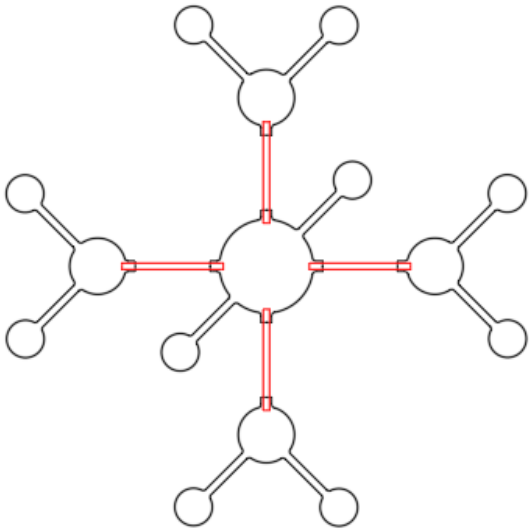


Figure 3.2 *Final design of the flow layer.*

The mold for this layer is formed by two different types of photoresist to allow the construction of the push-up valves formed by the intersection of the green layer (flow layer with positive photoresist) and the yellow layer (control layer). The positive photoresist is used to give round shape to the channels ensuring, required for a proper closing of the valves. A negative photoresist is used for all the other components of the flow geometry because positive photoresist could be stiffer after heat treatment and generate high resistance to the flow.
The positive layer channels have a width of 170 µm and a thickness of 35-40 µm, all other dimensions of the flow layer are reported in table 3.1.

Table 3.1 *dimension of the flow layer chambers.*

Dimension	Value	Unit of measure
Small chamber diameter	1.5	mm
Small chamber height	250	µm
Small chamber surface	1.77	mm^2
Central chamber diameter	2.5	mm
Central chamber height	250	µm
Central chamber surface	4.9	mm^2

Each external chamber is marked with a letter (from A to D) to facilitate operations of culture chamber identification during analysis under microscope.
There are also squares positioned all around the edges of the platform called device alignment marks (Figure 3.3*)*; they are useful to align the different photomasks (reported in Figure 3.3) during the procedure of photolithography. The overall dimension of the platform is approximately 60x40 mm, ensuring easiness in fabrication procedures.

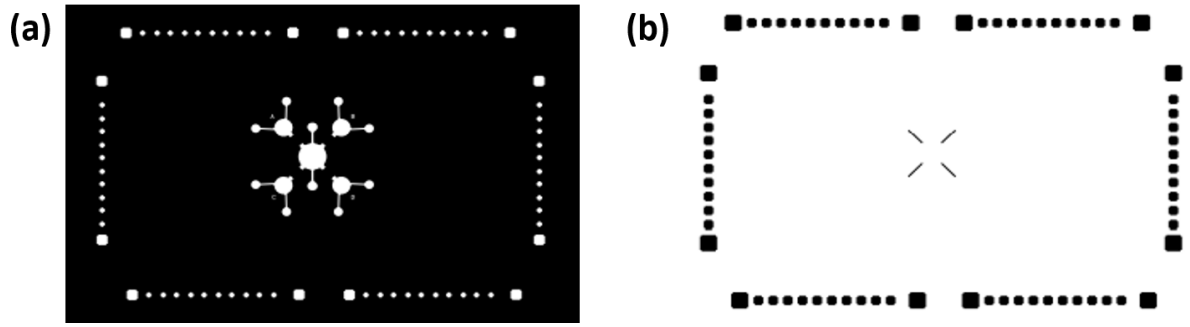


Figure 3.3 *Photomasks of the flow layer. (a) Photomask of the negative flow layer, (b) Photomask of the positive flow layer.*

The control layer design is reported in Figure 3.4. This layer is essential for the automation of the platform. All lateral wells are equipped of a specific microvalve to individually control the seeding procedure for each chamber and the connection with the central well.
To ensure the correct functioning of microfluidic valves during operations, a minimum surface of the valve must be guaranteed; this value is given by the product between the width of the channels of flow and control calculated in the section of orthogonal intersection.

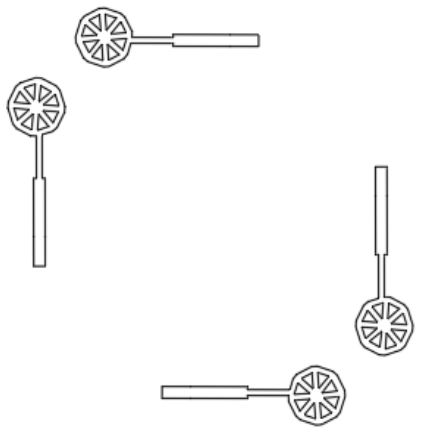


Figure 3.4 *Final design of the control layer.*

The control channel width is 200 µm, thus giving a surface of the valve of 170x200 µm (the minimum surface for closing properly the valve must be at least 150x150 µm). The main design characteristics of the multilayer microfluidic platform are reported in Table3.2.

Table 3.2 *Main design characteristics of the multilayer platform.*

Dimension	Value	Unit of measure
Control channel width	200	µm
Control channel height	35	µm
Flow channel width	170	µm
Flow channel height	250	µm
Valve Area	170x200	µm
Flow inlet	5	-
Flow outlet	5	-
Control valves	4	-

3.2 Soft lithography and Replica molding

The molds of the microfluidic platform build through photolithography technique are reported in Figure 3.5(a) and 3.5(b). One is for the control layer and the other for the flow layer. The height of the photoresist of the two molds are reported in Table 3.3.

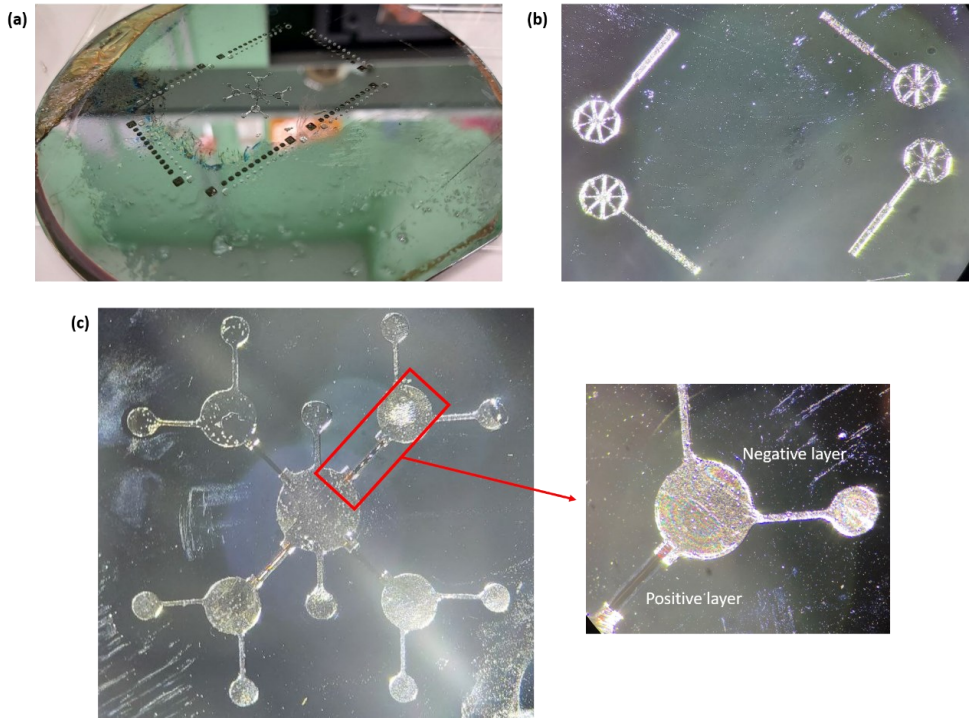


Figure 3.5 *Shape of the molds of microfluidic platform observed under stereomicroscope: (a) Flow mold; (b) Control mold. (c) Entire molds geometry of the flow layer with alignments marks.*

Table 3.3 *Height of the structures of the two molds for microfluidic device.*

Structure	Height [µm]
Negative flow layer	250
Positive flow layer	35-40
Control layer	35

A representative result of the replica molding procedure described in Section 2.4 is shown in Figure 3.6. The PDMS structure is attached on a 75x50 cm glass slide.

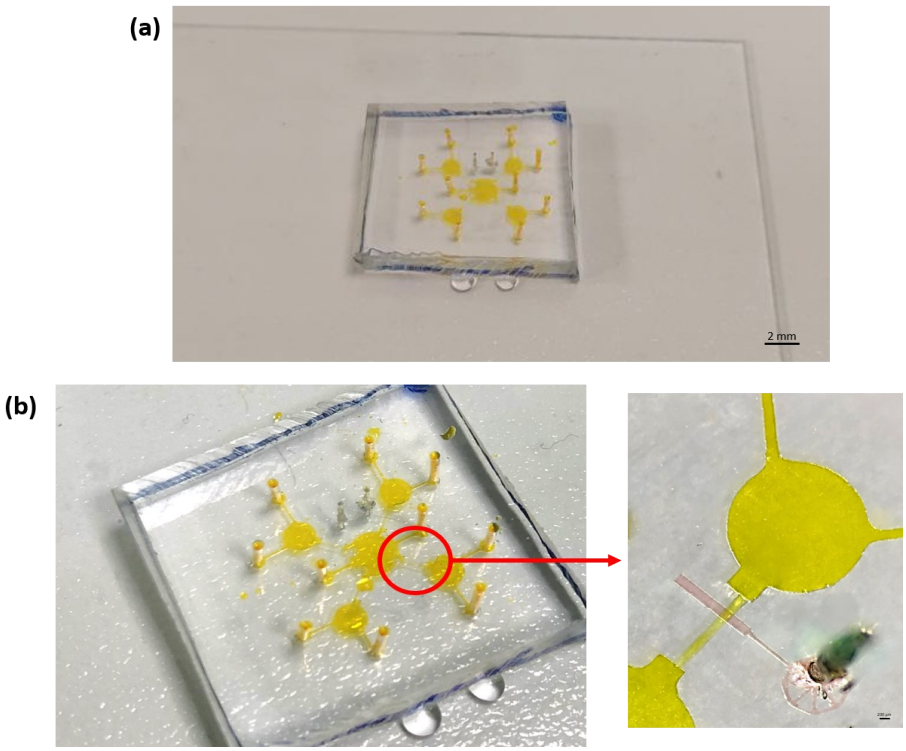


Figure 3.6 *Shape of the microfluidic device after the process of replica molding. (a) Microfluidic device attached to a glass substrate; (b) Zoomed view of the platform and of the intersection between flow (yellow) and control layer (red).*

3.3 Automation: Arduino code

The first step in automating the device is to develop a computer code to control the solenoid valves installed in the device shown in Figure 2.10. In this section, the final source code is described (Figure 3.7), although several variants were written to perform different experiments. The specific Arduino software programming language (IDE) was used to control the Arduino hardware, and the resulting code is composed of three parts.

The first consists on the definition of the constants and variables used, in particular the variable

'state' which indicates if the valve is open or closed. The second in which the variables just defined are connected as output to a pin on the Arduino hardware platform, and the third part in which communication begins with the serial monitor, which is used as the interface. At this point, it is possible to open and close the micro-valves manually by typing the value of the corresponding valve into the on-screen interface or use the full automation of the valves by implementing the code.

chip_multilayer_arduino_4_valvole

```
const int  valve1= 10 , valve2 = 11 , valve3 = 12 , valve4= 13 , valve5= 5 , valve6= 6 , valve7= 7 , valve8= 9 ;
int val1 = 0 ; // usiamo la variabile val per salvare i valori provenienti da tastiera

int stato1 =0 ;//usiamo la variabile stato per salvare lo stato in cui si trova la valvola 0= spento , 1 = acceso
int stato2 =0 ;
int stato3 =0 ;
int stato4 =0 ;

void setup() {
  pinMode(valve1,OUTPUT); //impostiamo il pin della valvola come output
  pinMode(valve2,OUTPUT);
  pinMode(valve3,OUTPUT);
  pinMode(valve4,OUTPUT);

  Serial.begin(9600); //iniziamo comunicazione con monitor seriale
}
 void loop ( ) {

  val1 = Serial.read(); //leggiamo il valore da tastiera e lo salviamo per valvola 1
//-------------------------------------------------------------------
if ( val1 == '1' ) {
  stato1 = 1 - stato1; }
if ( stato1 == 1 ) {
  digitalWrite( valve1 , HIGH); //accendiamo la valvola numero 1
  }
else {digitalWrite(valve1 , LOW); //spegniamo la valvola numero 1
  }

 //-------------------------------------------------------------------
```

Figure 3.7 *Section of the code written through Arduino IDE.*

3.4 Leakage test results

The leakage test allows to verify the mechanical resistance of the platform and of the microvalves for long periods of time. The experiments are performed following the procedures reported in section 2.5.4 (open of the microvalves 2 minutes each hour over 7 days). The testing air pressures start from 6 psi and increase with 2 psi steps until reaching 22 psi. For each condition the test is carried out 5 times. Results prove that below 10 psi the microvalves remain open, while above 14 psi the pressure it's too high and causes damage to the structure hindering the correct functioning of the microvalves. The correct operating conditions are around 10/12 psi, with 4 out of 5 tests carried showing that the valves remained intact and properly functioning. Figure 3.8 reports the fluid dynamic validation of the multilayer device using a blue colored tracer during the switch close-open-close of the microvalves. When the valve is closed, the liquid is confined only in the lateral well, when the valve is switched to the open position the liquid immediately enters in the central well, and when the valve is closed again immediately the flux of liquid tracer stop.

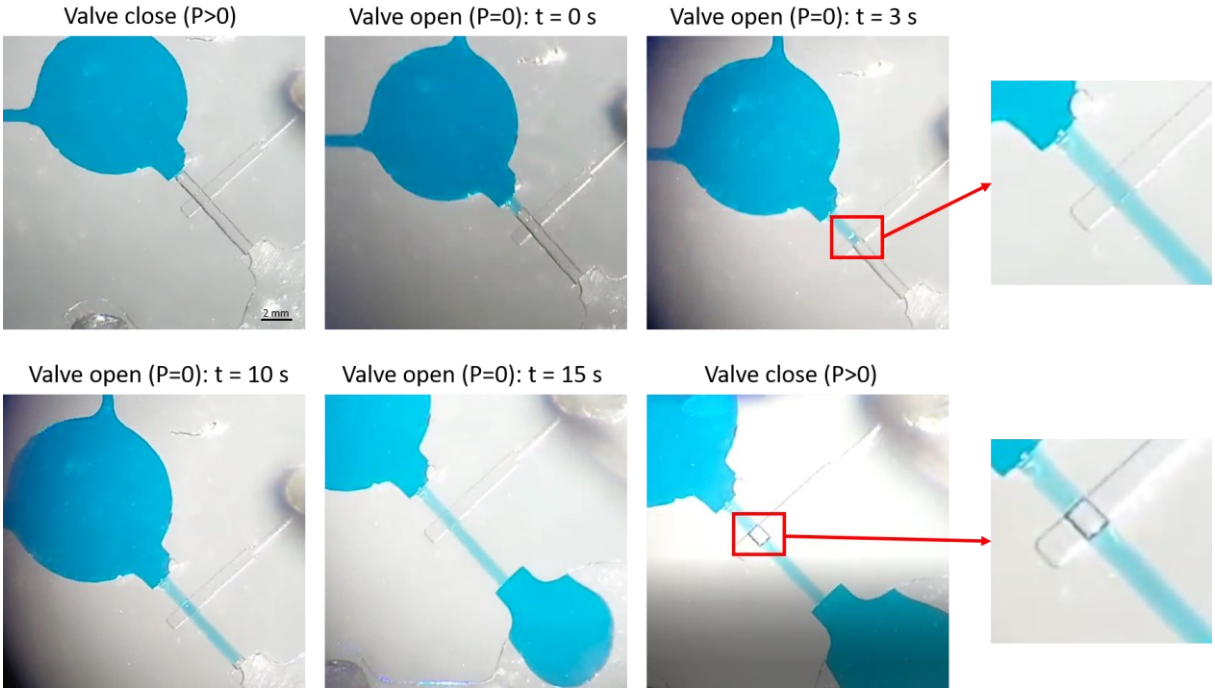


Figure 3.8 *Representative frames of a generic validation video: initial situation where the valve is closed and only the lateral well is full of liquid; intermediate situation where the valve is opened, and the liquid start to fill the microchannel and enter in the central well and the final situation where the valve is again closed.*

3.5 Biological validation

The biological validation of the microfluidic platform has been done using different cell lines as mentioned in Section 2.6.1 and following the protocols described in the previous chapter. Subsequently, key preliminary experiments were performed to search and highlight possible interactions between different cell types in the context of cancer. Specifically, to mimic Neuroblastoma (NB) metastasis to multiple distant organs, the corresponding cell lines were used to reconstitute *in vitro* both NB primary tumor (SK-N-AS) and its metastatic sites microenvironments (MSC and HUVEC). To perform these tests each cell line is seeded at an optimized density: for SK-N-AS 200 cells/mm^2, for HUVEC 150 cells/mm^2 and for MSC 100 cells/mm^2 using the automation system reported in Figure 3.9.

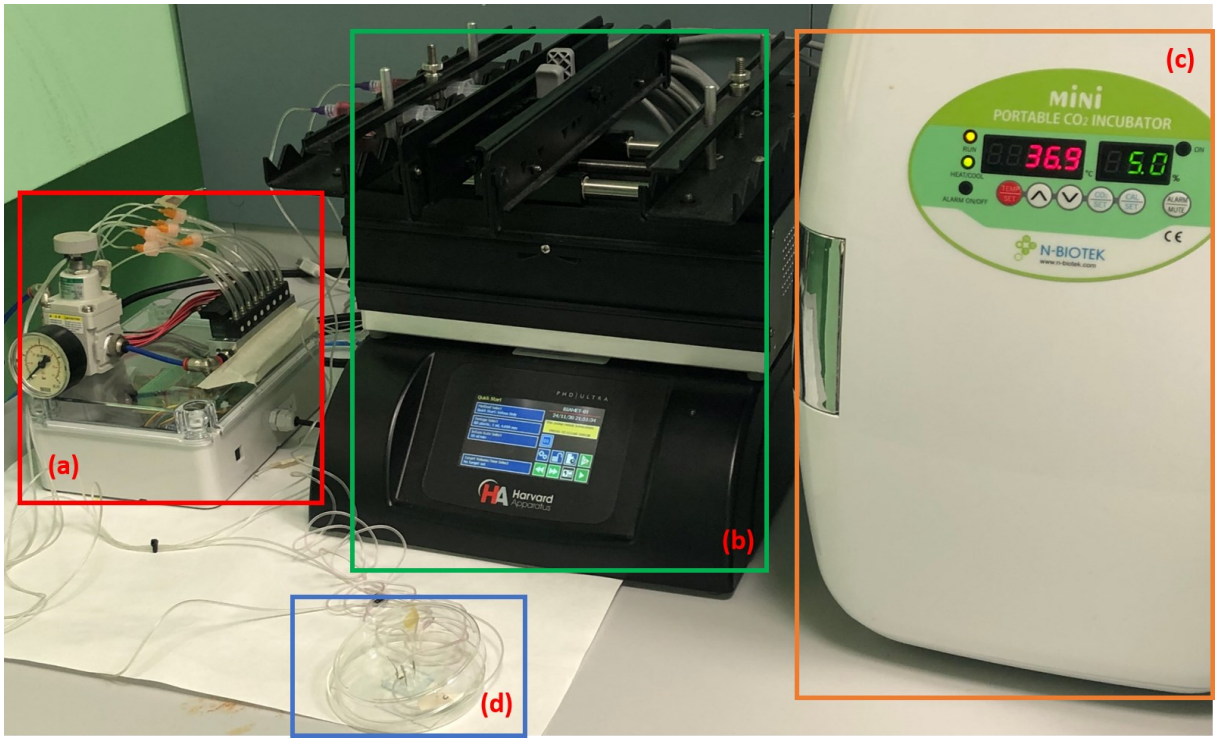


Figure 3.9 *Experimental set-up with all assembled components: (a) Microvalve control system (red box); (b) Syringe pump (green box); (c) Incubator (orange box) and (d) Microfluidic device in a Petri dish (blue box).*

3.5.1 Live&dead assay results

Fluorescence tests with Calcein-AM and Hoechst make it possible to distinguish live cells from dead ones to demonstrate that the cells adhered to the surface of the device and proliferated as *in vivo*. The images obtained from these fluorescence tests (Figure 3.10) are used in the estimation of cell viability with the results reported below.

For each cell line (SK-N-AS, hMSCs and HUVEC) the viability test was conducted at day 2 after seeding in the microfluidic device by taking a series of pictures in which total cells (marked in blue with Hoechst) and live cells (marked in green with Calcein-AM) were counted. With these results, the percentage of viability and the respective standard deviation was calculated, shown in Table 3.4.

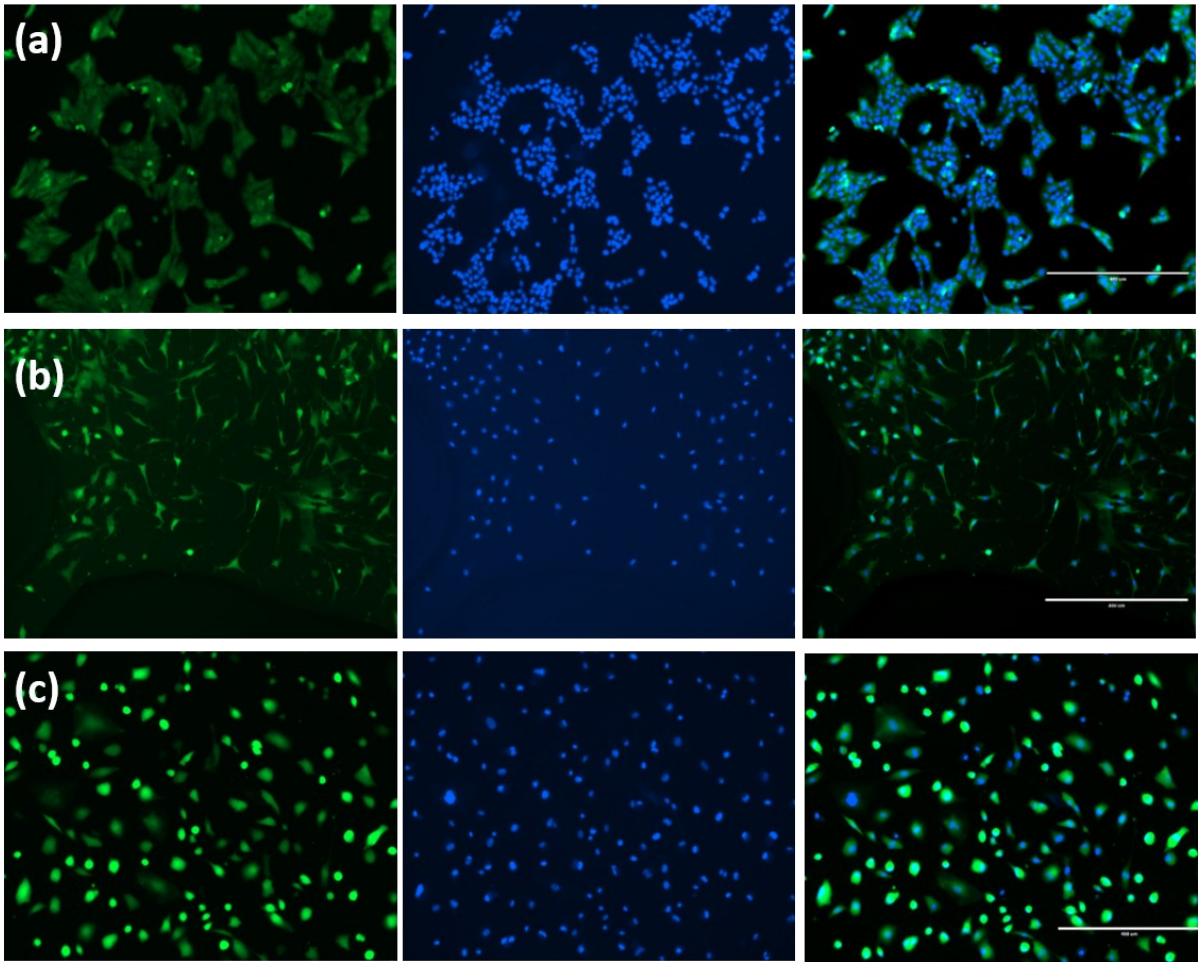


Figure 3.10 *Live&Dead assay results. (a) Cells staining of SK-N-AS after 48 hours from seeding in the multilayer device (10X of magnification): cell nuclei marked in blue with HOECHST; cell cytoplasm marked in green with Calcein and overlay. (b) Cell staining for hMSCs and (c) Cell staining for Huvec.*

Table 3.4 *Viability test results for each cell line.*

Cell lines	Viability %	Standard Deviation
SK-N-AS	85.83	± 3.97
hMSCs	94.38	± 3.28
HUVEC	97.05	± 1.43

It can be noticed that all the cell lines attach and grow inside the wells of the microfluidic device. Therefore, the microfluidic device can provide the optimal growth conditions for the cells. So, the design and the fabrication method of the device are suitable for cellular experiments with different cell lines.

3.5.2 Coculture experiment results

In this section, the results of coculture experiments inside the microfluidic device are presented. These experiments are performed by seeding in automated mode different cell lines inside the microfluidic device (as reported in Figure 3.11) and then after 3 days, using the protocol explained in Section 2.7.9 the cells are fixed and stained with fluorescent dyes.

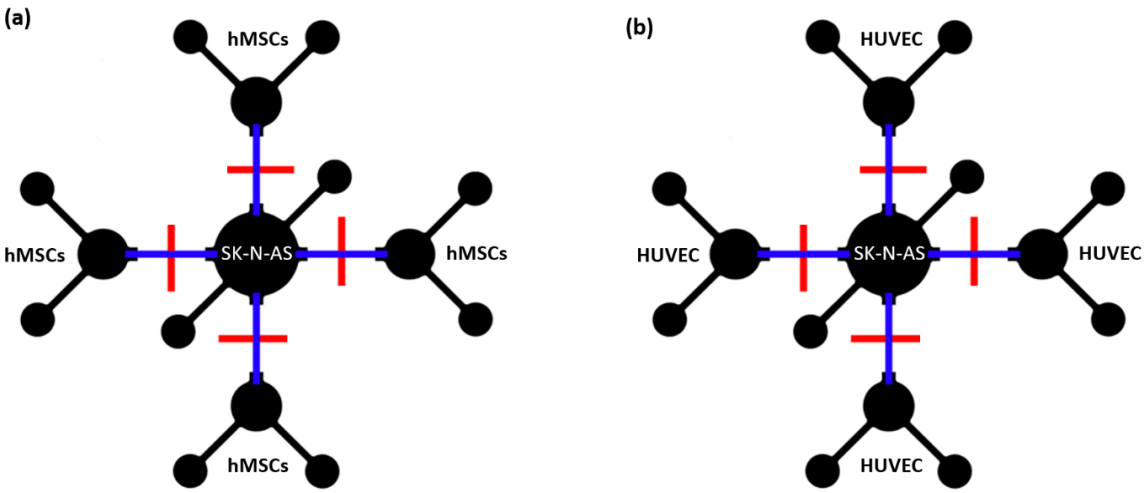


Figure 3.11 *Experimental set-up for automated cells co-culture. (a) Experiment SK-N-AS and hMSCs; (b) Experiment SK-N-AS and HUVECs.*

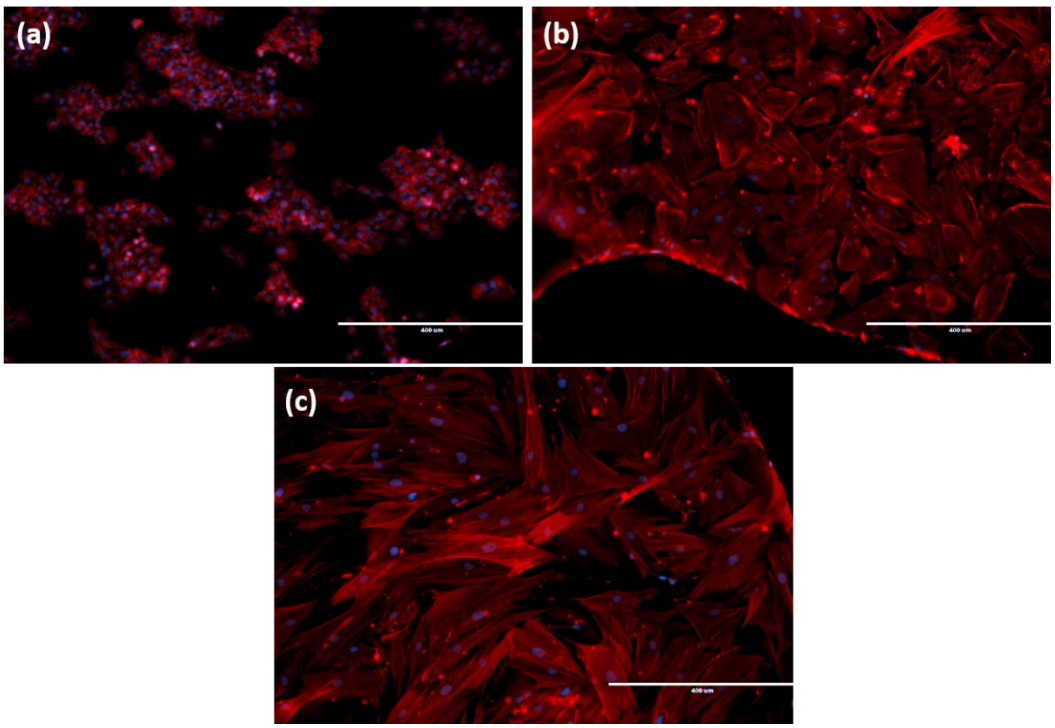


Figure 3.12 *Cells staining after 72 hours from seeding in the central well: cell nuclei marked in blue with DAPI and F-actin marked in red with Phalloidin and overlay. Scale bar 400 µm. (a) SK-N-AS lines; (b) HUVEC and (c) hMSCs.*

In this case, DAPI and Phalloidin are used, the former staining the nucleus blue and the latter staining the F-actin filaments. The first series of images (Figure 3.12) shows the images of the single cell type seeded alone inside the device; in this way it is possible to see how the individual cell line grows within the device.

The next series of images shows the coculture within the microdevices of the two different combinations reported in Figure 3.11: SK-N-AS with HUVEC (Figure 3.13) and SK-N-AS with hMSCs (Figure 3.14).
As already mentioned, SK-N-AS NB cells are seeded in the central well (and represent the main tumor), while HUVECs or hMSCs occupy all the lateral wells to recreate the tumor microenvironment and a target metastatic site. From the images it can be observed how Neuroblastoma cells after 72 hours of co-culture seem to form and grow in clusters. Interestingly, we observed that the growth of SK-N-AS as clusters occurs both for NB cells that reach and home in the lateral wells (due to the convective flux and/or their migration), and for the NB cells present in the central well whose behavior is affected by the chemical and biological signals of the other cells co-cultured in the same device (Figure 3.15). The cancer cells microenvironment can be affected by the interactions with other cells that is mediated by secreted chemokines, and other proteins (e.g. proteases). The action of secreted factors on the microenvironment may also facilitate the survival and progression of tumor cells.

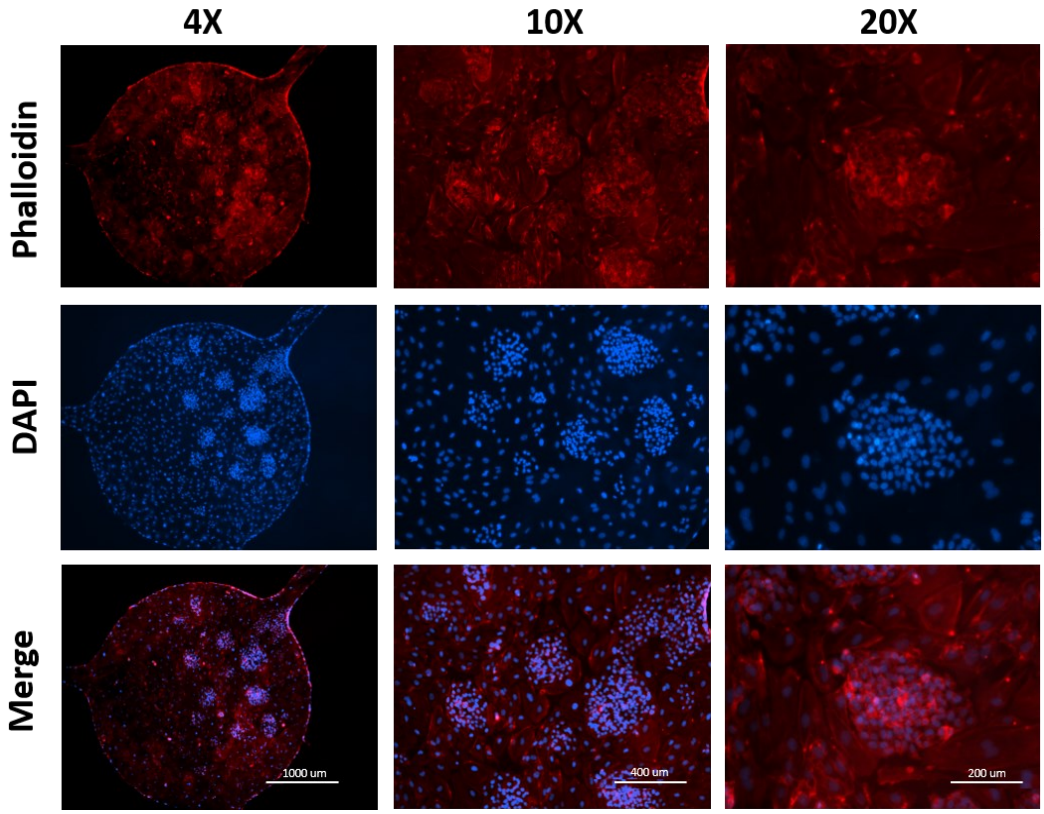


Figure 3.13 *Cells staining of SK-N-AS and HUVEC cells after 72 hours from seeding in the lateral wells: cell nuclei marked in blue with DAPI and F-actin marked in red with Phalloidin and overlay. Scale bar 1000, 400 and 200 µm.*

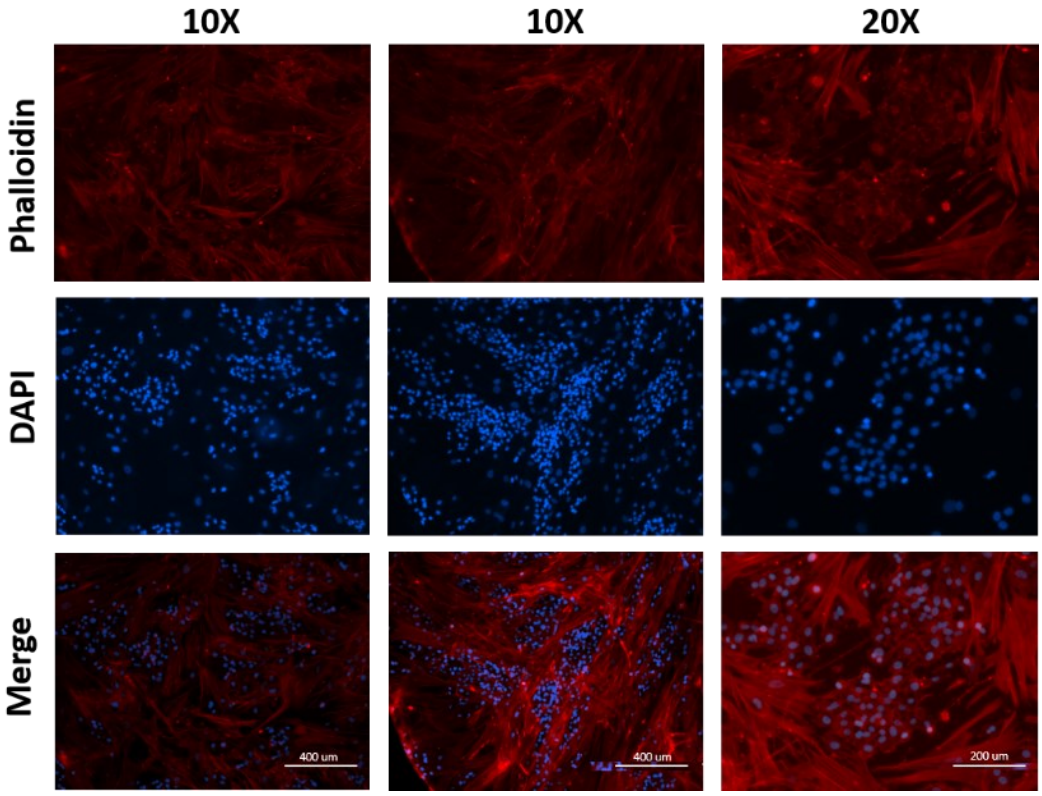


Figure 3.14 *Cells staining of SK-N-AS and hMSCs after 72 hours from seeding in the lateral wells: cell nuclei marked in blue with DAPI and F-actin marked in red with Phalloidin and overlay. Scale bar 400 and 200 µm.*

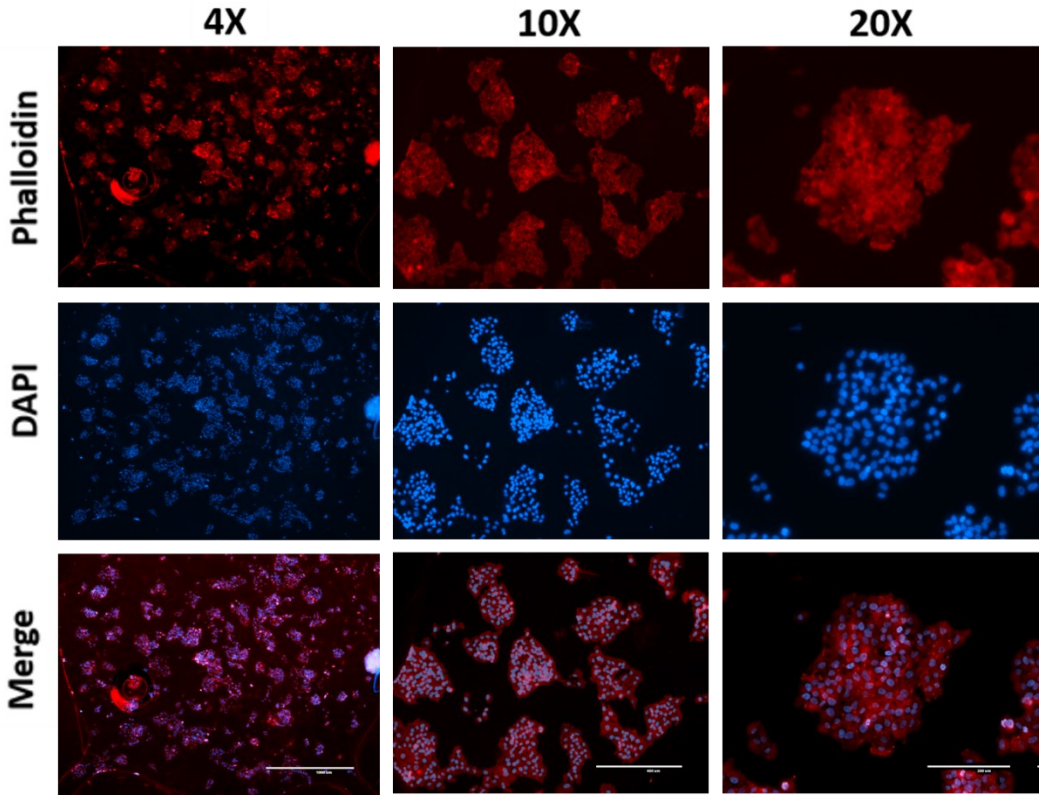


Figure 3.15 *Cells staining of SK-N-AS after 72 hours from seeding in the central well: cell nuclei marked in blue with DAPI and F-actin marked in red with Phalloidin and overlay. Scale bar 1000, 400 and 200 µm.*

3.5.3 Immunofluorescence staining

After obtaining these results, immunofluorescence tests were carried out to obtain more information on specific cell behavior by highlighting Vimentin protein expression in hMSCs. The experiments were carried out following the procedure explained in Section 2.7.10, as a result various images were taken using Confocal microscopy (a fluorescence microscope that offers a better resolution and a higher signal-to-noise ratio compared to traditional fluorescence microscopes). In the experiments with MSCs, a primary and a secondary antibody was used to highlight Vimentin, a protein present in the cell membrane, and DAPI, a fluorescent dye used to stain the cell nucleus. In the images shown in Figure 3.16 representing SK-N-AS cells and MSCs in coculture at 20x magnification, the protein is expressed in green and the nucleus in blue. The three images represent in order from the left: the cell membrane highlighted by Vimentin, the cell nucleus highlighted by DAPI and an overlay of the two images.

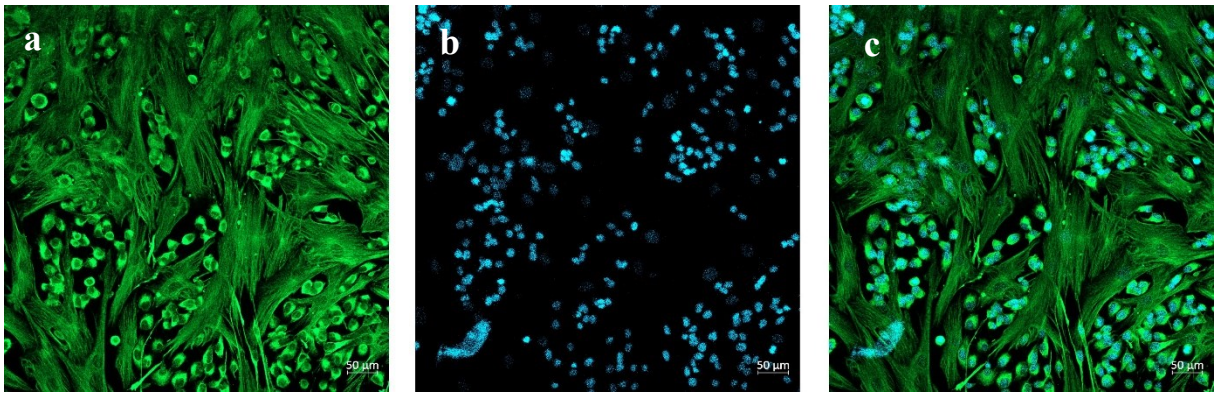


Figure 3.16 *Co-culture images at 20x magnification using DAPI and antibodies to express Vimentin. (*a*) Cells membrane. (*b*) Cells nuclei*. **(**c*) Overlay of* a *and* b *images.*

Using immunofluorescence analysis, it is possible as explained in Section 2.7.10 to conduct a quantification test of Vimentin protein expression in hMSCs and SK-N-AS cells from confocal images taken at 40x magnification. The images in Figure 3.17 show hMSCs under two different conditions: Figure 3.17(a) hMSCs were cocultured with SK-N-AS cells while in Figure 3.17(b) hMSCs were seeded alone, this condition being used as a control for the quantification results. From a set of around twenty such images, quantification was carried out using ImageJ and GraphPad Prism as explained in detail in Section 2.7.10, the results obtained are shown in Figure 3.18.

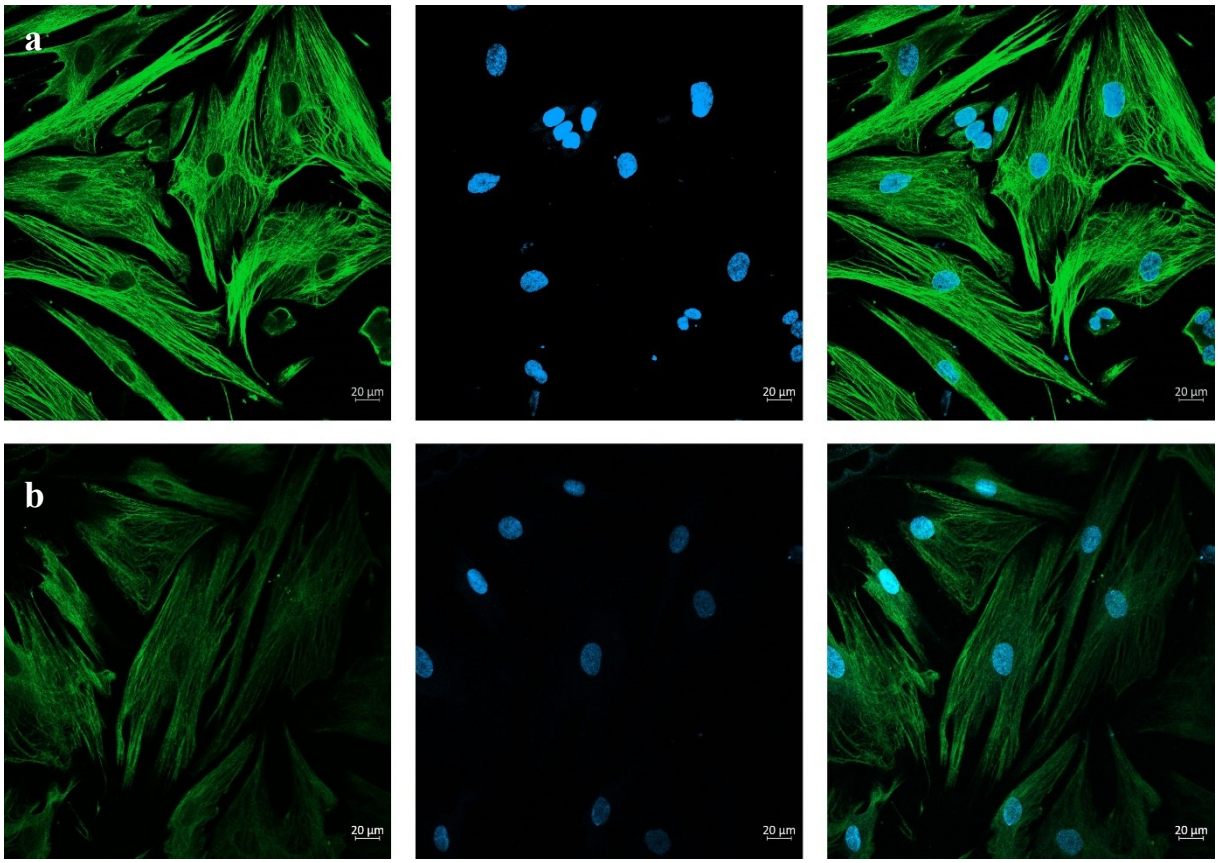


Figure 3.17 *Immunofluorescence images at 40x magnification, in the images in blu the nuclei of the cells and in green the Vimentin protein. (a) hMSCs co-coltured with SK-N-AS and (b) hMSCs seeded alone as experiment control.*

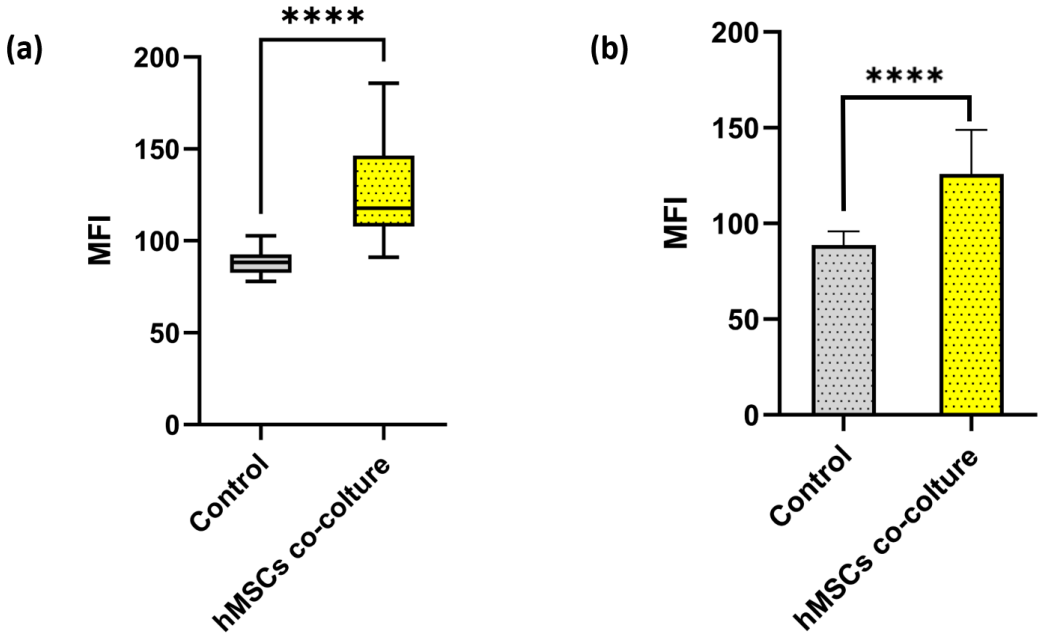


Figure 3.18 *Results for Vimentin MFI calculation. (a) Box plot for the MFI calculation for hMSCs test. Box plots represent values from at least n=3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs control; (b) Column plot for the results of MFI analysis. All values are mean ± SEM from at least 3 independent experiments.*

The graphs were obtained by performing a T-test on the collected data to find the p value. The results show the quantification of Vimentin expression and Vimentin is overexpressed in the co-cultured hMSCs compared to the control (hMSCs alone). The asterisks above the graph indicate the p value and, in this case, **** indicates a p< 0.0001. A p value with this value shows that the results are statistically significative. The same procedure was carried out for SK-N-AS and similar results were obtained. Below are representative confocal images taken at 40x (Figure 3.19) and the graph resulting through the quantification analysis (Figure 3.20).

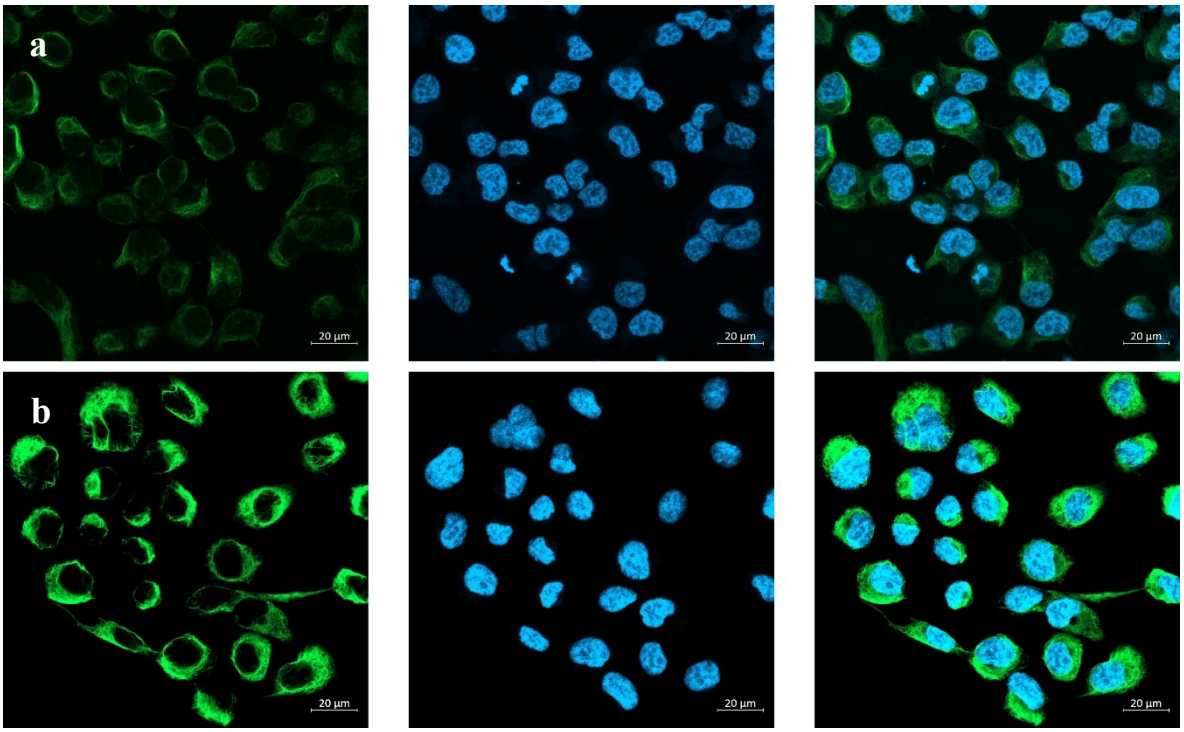


Figure 3.19 *Immunofluorescence images at 40x magnification, in the images in blu are reported the nuclei of the cells and in green the Vimentin protein. (a) SK-N-AS co-coltured with hMSCs and (b) SK-N-AS seeded alone as experimental control.*

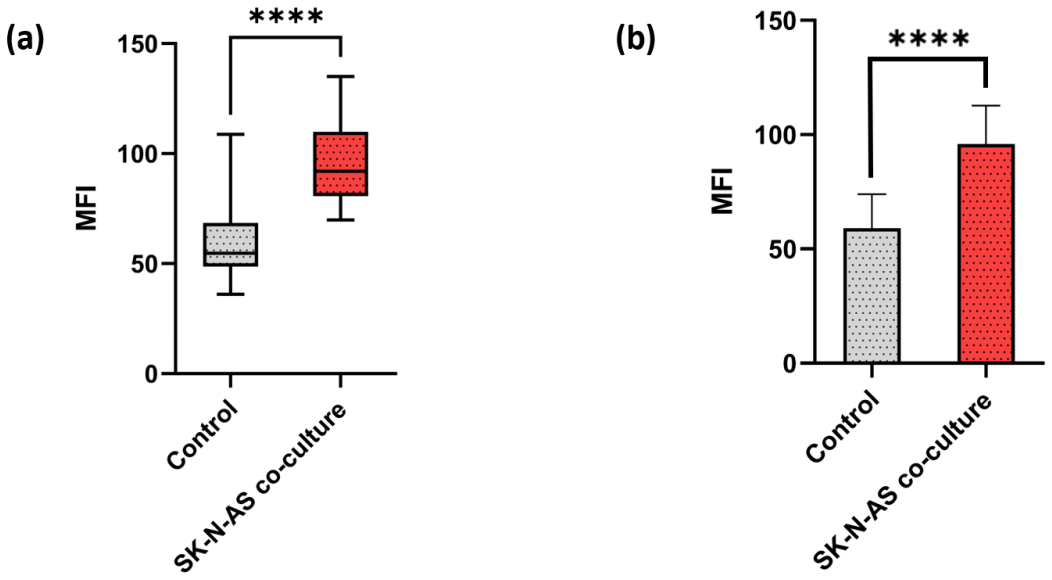


Figure 3.20 *Results for Vimentin MFI calculation. (a) Box plot for the MFI calculation for SK-N-AS cells test. Box plots represent values from at least n=3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs control; (b) Column plot for the results of MFI analysis. All values are mean ± SEM from at least 3 independent experiments.*

These results were obtained two days after cell seeding, indicating a fast interaction between the different cell lines within the microfluidic platform. In this specific case, tumor cells influence the activity of hMSCs with which they are in contact by inducing overexpression of Vimentin, which is involved in cancer progression and aggressiveness in both SK-N-AS cancer cells and hMSCs. Moreover, the results highlight that the microenvironment for co-culture conditions induce an upregulation of Vimentin, a protein that is directly involved in EMT and proliferation.

Conclusions

The main objective of the thesis is the design and development of an automated cancer-on-a-chip platform that allows greater control of the operating conditions and enables a wide range of long-term biological applications with the specific goal of mimicking the progression of Neuroblastoma.

This new microfluidic device should have the following features: simplicity and reproducibility, reliability and resistance, easiness of automation, and easy integration with different analysis and imaging techniques.

The production of the microfluidic device begins with the fabrication of the mold by multilayer soft lithography, and is completed by the creation of the platforms via replica molding in PDMS. Multilayer lithography is required to properly fabricate the on-chip microvalves. This process allows for fast and easy reproducibility of the device.

The protocol to automate the platform introduces more complicated steps that have to be performed carefully, compared to standard single-layer microfluidic devices production, but brings great advantages, and the possibility to perform complex experiments better reproducing in vitro what happens in vivo inside our bodies. The automation software is developed using Arduino, which guarantees flexibility and easy implementation of the desired working conditions, while maintaining relatively low costs compared to other possibilities such as LabVIEW®. After testing the mechanical resistance of the device, it is possible to conclude that the platform and its microvalves can function properly a for long period of time, thus meeting the required standard of resistance and reliability.

Biological validation of the platform shows that cells can grow and proliferate within the device, thus enabling its use in biological applications and further experiments. The microfluidic platform is used for cell co-culture experiments of tumor and non-tumor lines simultaneously, obtaining some promising results.

Our on-chip technology is effective in enabling the simultaneous culture of viable and physiologically active cell populations that constitute the main tumour and its metastatic target sites. The device is specifically designed to study Neuroblastoma tumor behavior and interactions with its main metastatic target sites within a single multilayer platform, but can easily be used to study other different type of cancers.

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