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Ingegneria Chimica e dei Processi Industriali

INTEGRATION OF A MICROFLUIDIC PLATFORM WITH AN AUTOMATIC IMAGING SYSTEM FOR HIGH-THROUGHPUT SCREENING OF CELLULAR PHENOTYPES

Relatore: Prof. Nicola Elvassore
Correlatore: Prof. Camilla Luni

Laureando: DANIELE MARANGON

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Sommario

Questa tesi è stata realizzata per la maggior parte nel laboratorio di bioingegneria presso lo Shanghai Institute for Advanced Immunochemical Studies (SIAIS) della ShanghaiTech University (Shanghai, Cina) e in parte nel laboratorio BioEra a Padova. In questi laboratori lo screening di fenotipi cellulari in modo high-throughput è motivo di interesse per lo studio di processi biologici dove la ricerca e la successiva quantificazione di fenotipi cellulari è essenziale per capire i fenomeni che avvengono nelle cellule, per esempio in seguito a modifiche del genotipo.

L'obbiettivo di questa tesi è la combinazione di recenti tecnologie per sviluppare un metodo robusto e adatto allo screening di fenotipi cellulari in modo high-throughput utilizzando piattaforme microfluidiche.

Grazie alle tecnologie nel campo della microfluidica è stato possibile realizzare delle piattaforme microfluidiche compatibili con uno strumento che consente di fare screening in modo high-throughput. Successivamente è stato progettato un supporto compatibile con lo strumento high-thrpughput in modo da garantire una corretta lettura delle piattaforme microfluidiche. Per validare la metodologia sviluppata nello screening di fenotipi cellulari in modo high-throughput sono stati condotti degli esperimenti di tossicità su campioni cellulari coltivati in piattaforme microfluidiche valutando la mortalità cellulare e i cambiamenti sulla morfologia cellulare provocati dall'agente tossico. Le piattaforme microfluidiche progettate sono adatte per l'imaging di fenotipi cellulari assicurando una valutazione consistente ed accurata delle proprietà cellulari. Infine, l'efficacia della metodologia sviluppata è stata applicata in un caso reale dove è stato rilevato un marcatore di membrana espresso da cellule staminali pluripotenti, coltivate in piattaforme microfluidiche, attraverso uno strumento di imaging high-throughput, dimostrando che la metodologia è applicabile per lo screening di diversi fenotipi cellulari coinvolti in processi biologici di interesse.

Summary

This thesis was developed for the great part in the Biological Engineering Laboratory in the Shanghai Institute for Advanced Immunochemical Studies (SIAIS) at ShanghaiTech University (Shanghai, China) and in part in BioEra lab in Padova. In these laboratories the high-throughput screening of cell phenotypes is a matter of great interest for the investigation of biological process where the detection and quantifications of different cells features is essential to understand how cells respond to perturbations, such as after genome editing.

The aim of this work is to combine the available technologies and develop a robust method suitable for performing high-throughput phenotypic screening of cells by imaging within a microfluidic platform.

Thanks to the microfluidic technology the design of microfluidic platforms compatible with the high-throughput screening instrument was possible with the final goal of analysing cell phenotypes. Then a suitable frame compatible with the high-throughput screening instrument has been designed to provide a robust readout of cells in the microfluidic platform. To validate the methodology developed for high-throughput cell phenotypes screening a toxicity assay has been performed in the microfluidic platform to detect and quantify the mortality and the morphological changes of the cells. The designed microfluidic platforms are suitable for imaging of cell phenotypes, ensured by a robust protocol developed for the acquisition of the image samples and for the quantitative evaluation of the cellular properties. Furthermore, the effectiveness of the developed methodology was proved in a real case where a membrane marker expressed by pluripotent stem cells, cultured within the microfluidic platform, has been detected by a high-throughput screening instrument demonstrating that the methodology is ready to be applied to screen many cells phenotypes involved in relevant biological process.

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Introduction

The characterization of the cellular phenotype, the ensemble of observable traits of a cell, such as morphological and biochemical properties, is of great interest to understand how cells respond to perturbations of their environment or their genetic manipulation. The possibility of performing such analysis within a high-throughput screening enables the identification of relevant biological conditions, even with limited prior biological knowledge. The imaging technology gives the possibility to analyse cellular phenotypes at the molecular level, after fluorescence labelling of molecular targets, and, compared to other techniques, has the advantage of preserving the spatial information of the cell culture and gives the possibility of following the evolution of dynamic processes. On the other hand, microfluidic technology has been used to culture cells in vitro at microliter scale, mimicking the volume ratio between cells and inter-cellular fluids present in tissues in vivo. This aspect of miniaturization is particularly important for the study of cell self-regulated processes, because the small culture volume avoids the dilution of cell-secreted molecules Exploiting the benefits of the microfluidic technology for cell culture it is possible to develop cells assays to investigate biological processes by high-throughput imaging technology.

The aim of this work is to integrate the available technologies and develop a robust method suitable for performing high-throughput phenotypic screening of cells by imaging within a microfluidic platform.

In chapter 1 an overview of the state of the art of the technologies and techniques related to this thesis is provided, in particular the following topics are addressed: microfluidic technology, gene editing, phenotypic screening by high-throughput and high-content imaging. At the end of the chapter the motivation and the aim of this thesis is reported.
In chapter 2 the main methodologies of this thesis are described: from the microfluidics methods for the production of the platform used in this work to a description of the biological methods used for validation of the procedure, and to the imaging methods.
In chapter 3, the main results obtained along the development of an optimized microfluidic platform interfaced with a high-throughput imaging instrument are reported. Then, some decision making suggestion to perform the high-throughput screening are reported. Finally, the preliminary results of this optimized procedure applied to a real biological study are given. Future perspectives and conclusions of this work are described in a final section.

Chapter 1

State of the Art

In this chapter the background at the basis of the work of this thesis is described. This thesis is based on the integration of different technologies that will be described next: microfluidic technology, gene editing, phenotypic screening by high-throughput and high-content imaging. Finally, the motivation and the aim of this thesis is reported.

1.1 Microfluidic technology

In biological applications, the reduction of the scale through microfluidic (10^{-9}-10^{-12} L) is done to better mimic ratio of the cell volume and the volume of fluid present in the interstitial spaces between cells in tissues (*Duncombe et al.2015*). In particular, in microfluidics devices cells release molecules whose concentration in the medium is rapidly increased due to the small scale (Gagliano et al. 2016). Furthermore, the reduction of reagents volumes handled in the experiments reduce the reagent usage and the cost of the experiment compare to the conventional multiple-well plate based assays, increasing the number of tests that is possible to do that could be prohibitive expensive otherwise. (*Chun-Wei Chi et al. 2016*). Actually, microfluidic tools are principally developed for research purposes and they have been used in applications like protein crystallography, genetic analysis, amino acid analysis, high-throughput screening, bioreactors, chemical synthesis, single cell analysis, drug discovery and development (*Melin & Quake, 2007*). Coupled with spatial and temporal control of the chemical and physical environment, microfluidic live-cell imaging can offer insights into the dynamic cellular processes and can be used to study long-term developments such as cell ageing, inheritance and death. In addition, microfluidics tool can be automatized with integrated valves or pumps and real times control that allow the development of truly 'lab-on-a-chip devices' that improve the accuracy and throughput of biological assays by orders of magnitude (*Mehling & Tay, 2013*).

1.1.1 Physics of microfluidic device

The physics on microfluidic devices is developed in a low scale geometry(10^{-6}-10^{-9}m). Due to the small scale of the geometry and low velocities of the fluid inside the microfluidic chambers (1 µm/s–1 cm/s) Reynolds number are low and they can range between 10^{-6} and 10: the viscous forces are dominant respect to the inertial forces and the flow regime is laminar.

Furthermore, the large surface-to-volume ratios of microfluidic devices render surface effects increasingly important. Capillary forces are used to manipulate and transport fluids in microfluidic channels. Modifying the interfacial energies, the balance is compromised and it causes fluid motion. In the microfluidic system this is achieved modifying the solid-liquid surface tension by the plasma treatment, that make the surfaces exposed hydrophilic. (*Squires & Quake, 2005*).

1.1.2 Properties of polydimethylsiloxane(PDMS)

In biological applications that involve microfluidics the polydimethylsiloxane(PDMS) is the most used material. It is a compound that belongs to the silicon family. It shows an unique combination of properties due to the presence of inorganic siloxane backbone and organic methyl groups attached to silicon as it is possible to see in Figure 1.1.

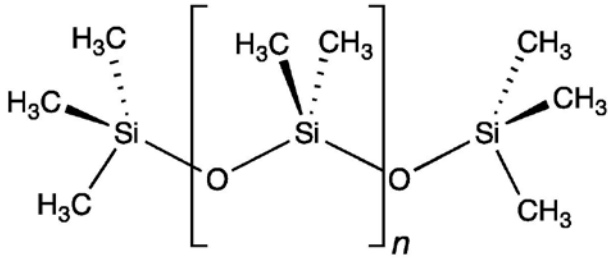


Figure 1.1. *Chemical structure of polydimethylsiloxane(PDMS).*

The main characteristics of the polydimethylsiloxane are listed below (Dietzel A, 2015):

1. it provides a good chemical stability and it is not hygroscopic, without swelling with humidity;
1. it is inert, nontoxic, biocompatible, nonflammable, optically transparent down to wavelength about 300 nm and can be exposed to UV light;
1. it is permeable to nonpolar gases like oxygen allowing the life of the cell cultures in the microfluidic devices
1. it is durable and has good thermal stability (up to 186 °C in air);
1. it is isotropic, homogeneous and inexpensive;
1. it can be bonded to itself and a series of other materials after oxygen or air plasma treatment;
1. it can be sterilized and can be used many times without a visible reduction of the performance.

1.1.3 Microfluidic platform construction

A microfluidic platform is built assembly a PDMS mould to a glass slide in order to create a set of chambers that constitute an independent environment suitable for cell culturing.
The main

techniques used to produce the platform are the photo lithography, the replica moulding and the plasma treatment.

1.1.3.1 Photo lithography

Photolithography is a microfabrication technique originally used to create microelectronics component where over a silicon wafer is deposited a photoresist that is coupled with a black and transparent photomask with the desire pattern to impress in the wafer. The process is showed in Figure 1.3. When the wafer is exposed to the UV light the photoresist starts to polymerize in the region where the photomask let pass the light, i.e. in the geometry region. After that the photoresist that is not crosslinked is washed with an organic solvent. A typical photoresist is the SU-2100®(Microchem) an epoxy based photoresist designed for micromachining and other microelectronic applications.

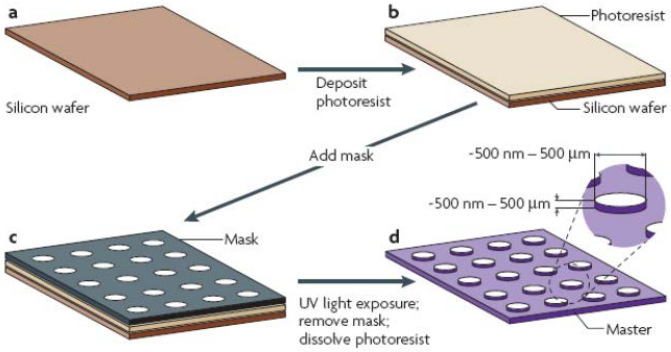


Figure 1.3. *Photolytography process to create the pattern; a) cleaned silicon wafer; b)deposition of the photoresist; c) coupling of the photomask; UV light exposure and wash by organic solvent.(www.elveflow.com)*

However, this technique can be adopted to create a master mould with the pattern of the microchannel of the microfluidic platform that can be replicated by pouring and baking the PDMS over the stamp.

1.1.3.2 Replica molding

The replica molding consists to the replication of the pattern impressed in the master mold in the PDMS many times and the process is showed in the Figure 1.4.

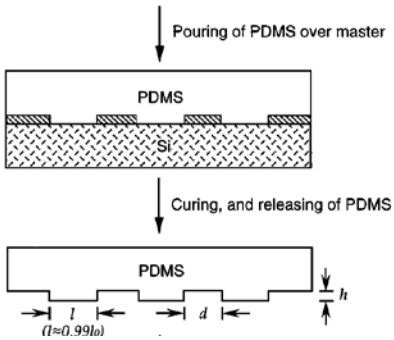


Figure 1.4. *PDMS replica molding. In a clean master mold is poured the liquid PDMS and then the wafer is baked to create the PDMS mold. The height h present in the master is reported in the PDMS, while there is a reduction of 1% of the length of the channel during the curing process. (Xia and Whitesides,1998)*

The surface of the master mold have to be clean to avoid defects in the final elastomeric mold. First the liquid PDMS solution composed by the base and the curing agent is mixed in the appropriate amount, then after a first row degassing step under vacuum the solution is poured over the surface of the master. At this point the wafer is placed under vacuum for the second degassing step to take out all the air bubbles from the surface and it is baked over an hot plate.

1.1.3.3 Plasma treatment

In order to create a microfluidic device, it is necessary to couple the PDMS mold with the glass slide. To stick the surfaces of the two materials it is necessary to activate them through a plasma treatment as it is showed in Figure 1.5. The main steps are the followed:

1. Surfaces of glass slide and PDMS layer are accurately cleaned to remove dust and other contaminants (Figure 1.5a);
2. 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 H2O, cleaning the surfaces and increasing the number of exposed hydroxyl groups (-OH) at the surfaces of the PDMS layer and of the glass slide (Figure 1.5b);
3. The surfaces of glass slide and PDMS layer are attached together: thanks to the exposed hydroxyl groups they can form strong covalent bonds [Si-O-Si] (Figure 1.5c).

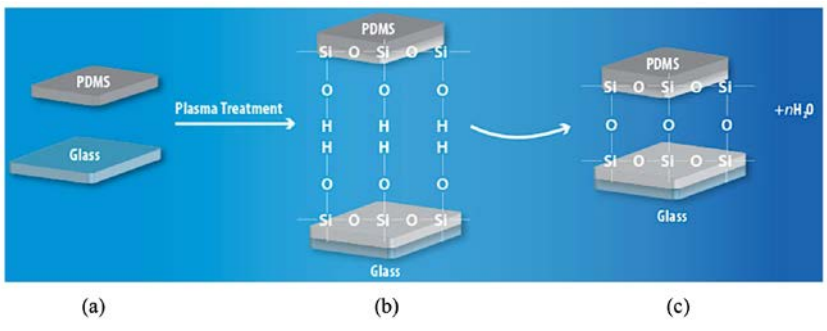


Figure 1.5. *Plasma treatment to build a microfluidic chip; a) surfaces cleaning; b) Surface activation by the formation of the hydroxyl group over the surfaces of the two materials; c) Surfaces coupling to form the covalent bond Si-O-Si. (plasmatreatment.co.uk)*

After the treatment, the part of the PDMS that are not attached to the glass became hydrophilic for the presence of the hydroxhil groups present over the free surfaces. This facilitate the liquid handling inside the channels exploiting capillary forces that are responsible to move fluids passively inside the channels.

1.2 Gene editing

Gene editing is a group of technologies that give to scientists the ability to change cells' DNA. The classical method for gene modification is homologous recombination. This approach has been used in mouse embryonic stem cells to generate germline cells without a gene or with extra gene (1,2). A disadvantage of this approach is that it takes more than a year to generate a genetically modified mouse. Nowadays the gene editing technique are fast, cheap and efficient and allow to introduce a variety of genetic alterations into mammalian cells, ranging from deleting a single nucleotide (the building block of the DNA) to inserting new genes, to deleting a chromosomal region.

The most recent technology for gene editing is known as CRISPR Cas9 (cluster of regularly interspaced palindromic repeats CRSPR-associated protein 9) which is the technique used in Shanghai Institute of Immunochemical Studies SIAIS) laboratory where part of this thesis was performed. In nature CRISPR are bacterial DNA loci, that is a position of a significance sequence inside the chromosome, containing short repeat segments that match foreign DNA elements. Together with Cas9 proteins, they form an adaptive immune system in bacteria and archaea, which can acquire sequence segments from foreign DNA and use these sequences to recognize and destroy the foreign target DNA (A. Dominguez et al, 2016). In 2013 this CRISPR-Cas9 system of *Streptococcus pyogenes* has been adopted for the genome editing of mammalian cells. In Figure 1.6 is represented how it works the CRISPR-Cas9.

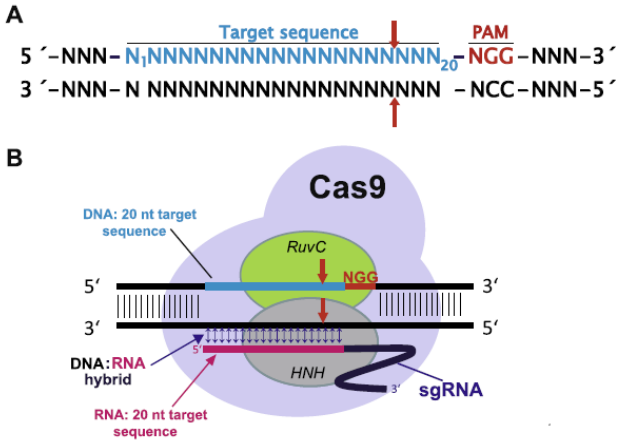


Figure 1.6 *a) DNA strands where is highlighted the 20-nucleotides target sequence to be cut by the Cas9 protein upstairs the PAM(50-NGG-30) sequence; b) Cas9 proteins is guided through the DNA sequence target by the sgRNA that binds the complementary strand of the DNA. When the PAM sequence is detected, a double-strand brake take place (red arrows) thanks to RuvC and NHN domains of Cas9 protein.(Wefers et al, 2017).*

The Cas9 protein has two domains (RuvC and HNH) that have the function to cut the specific DNA target sequence. A single guide (sg)RNA is an engineered molecule that combine the function of CRSPR RNA(crRNA) to guide the Cas9 protein through the DNA target sequence and the function of trans-activating crRNA (tracrRNA) for the maturation of crRNA in ribonuclease III and Cas9. This molecule has two features: the 20-nucleotide sequence at the 5′ end of the sgRNA that determines the DNA target site by Watson-Crick base pairing, and the double-stranded structure at the 3′ side of the guide sequence that binds to Cas9 (A. Doudna& Charpentier, 2014). The first twenty nucleotides of sgRNA guide Cas9 to a specific complementary DNA target sequence via RNA-DNA hybridization. If the target sequence is located upstream of an invariant PAM sequence (protospacer adjacent motif) required for the DNA target recognition, the Cas9 nuclease function is activated and creates a double-strand break (DSB) located upstream the PAM site (red arrows in Figure 1.6b), (Wefers et al, 2017). The formation of double-stranded breaks can activate one of the two main DNA-repair pathways in the cell: the non-homologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway. The NHEJ pathway repairs a break by randomly adding or deleting nucleotides, which results in changes to the DNA sequence, making it inappropriate for gene-correction purposes. Therefore, to enable a customized repair of the broken DNA strand it is better to follow the HDR pathway that use homologous sequences to repair the DNA strand. (Winblad & Lanner, 2017). One application of gene editing is the prevention and treatment of human diseases. Nowadays, most research on genome editing is done to understand diseases using cells and animal models and has not been demonstrated yet if this approach is safe and effective for use in people. Some diseases of which the research in gene editing is going through are single-gene disorders such as cystic fibrosis, hemophilia, and sickle cell disease. Furthermore, there is the prospection to use the gene editing for more complex diseases, such as cancer, heart disease, mental illness, and human immunodeficiency virus (HIV) infection. Most of the changes introduced with genome editing are limited to somatic cells that changes affect only certain tissues and are not passed from one generation to the next (www.ghr.nlm.nih.gov).

1.3 Phenotypic screening

A phenotype is a set of observable properties of a cell that are related to its appearance and behavior. Cell phenotype could be related to the morphology, cell or nucleus dimensions, presence of specific proteins on the membrane. The phenotype is determined by the set of genes that are present in the cell DNA, called genotype. The overall appearance and behavior of a cell depends on thousands of genes and it is difficult to understand how genotype and phenotype are related. Phenotypic screening is a high-throughput method for understanding how cell phenotype is affected by perturbations. For example, scientists use genome editing, described

in section 1.2, to perturb the normal genotype and better understand the function of the genes, observing how the phenotype is affected. This approach is particularly important for basic science research. Another application of phenotypic screening is in the field of drug discovery. In this case the perturbation is given by the administration of different types of drugs. Using this approach, it is not required any knowledge on the drug target or any hypothesis on the mechanism of the disease, in contrast to the target-based strategies that have been widely used in the pharmaceutical industry in the past years. However, also high-throughput phenotypic screening has some limitations. To effectively determine the phenotype, multi-parameter measurements are needed to discriminate the phenotypes. Usually there is an inverse relationship between the level to which an assay is multiplexed, in terms of number of parameters that compose the readout, and the throughput of the assay. In Figure 1.7 is shown the relation between multiplexity and throughput of phenotypic profiling technologies, expressed as number of samples analyzed per week. Although a high-throughput screen (HTS) with a single read-out can screen over a million compounds per week, highly multi-parameter assays with tens of thousands of read-out parameters are much more informative, but also much slower and costly.

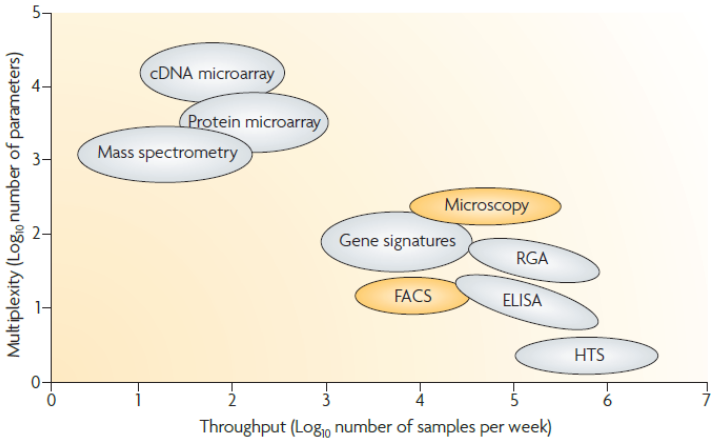


Figure 1.7 *Multiplexity and throughput of phenotypic profiling technologies. (Feng Y et al, 2009)*

Multiplexity is maximized for assays based on transcriptomic, and proteomic techniques (cDNA microarray, protein microarray, mass spectrometry), which analyze tens of thousands of molecules at once. On the other hand, techniques such as fluorescence-activated cell sorting (FACS) or enzyme-linked immunosorbent assay (ELISA) can measure only few tens of proteins at once. FACS technique is widely used because it measures 10-20 proteins preserving the information at the single-cell level. For FACS analysis, specific proteins in the cells are labeled with a fluorescent probe and analyzed after passing one-by-one in a capillary in front of a laser that have the proper wavelength for exciting the fluorophore. However, the cells need to be suspended in a buffer solution before the analysis, and any information about how cells with different properties spatially organize during cell culture is lost.

Cell imaging is a powerful alternative to FACS. Both techniques require staining the cells with fluorescent probes, but, for cell imaging, cells are analyzed by microscopy in culture, thus all cell morphological and spatial features are preserved. Moreover, cells can be captured at different time points to follow specific dynamics. An example of high-throughput cell-imaging with an automated microscope shown in Figure 1.8

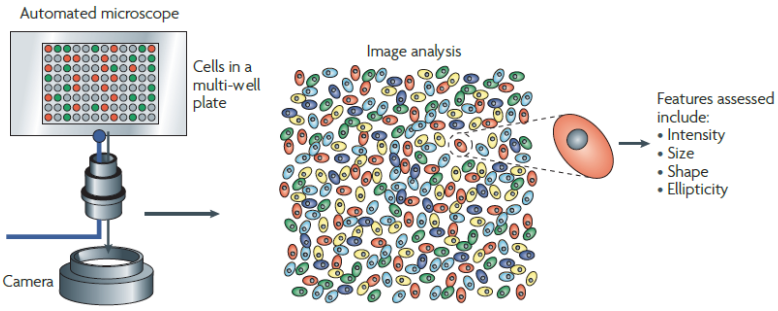


Figure 1.8 *Phenotypic high-content screening using an automated microscope. (Feng Y et al, 2009)*

Recent advances in fluorescence microscopy technology have enabled the automated collection of images from multi-well plates, in three to four fluorescence channels, as a function of time. With appropriate analyses, this number of channels is sufficient to obtain large amounts of phenotypic information from a single well: from the number of viable cells as a measure of toxicity response to a chemical, to the translocation of specific molecules within a cell over time. (Feng Y et al, 2009).

1.4 High-throughput imaging screening technology

In order to perform a phenotypic screening through a cell-imaging method as described in the previous section, an instrument that allow to perform high-throughput screening of many phenotypes namely Operetta® (Perkin Elmer) has been used for this work (Figure 1.9).

Figure 1.9 *a) Overall view of Operetta® joined with a desktop computer to control and to manage the analysis; b) view of the internal components of the instrument: 1 is the live chamber, 2 is the electrical connector and 3 is the temperature sensor. (Operetta Technical Manual)*

This instrument allows to screen many kinds of commercial microplates acquiring, analyzing and managing fluorescence and brightfield images. Furthermore, an image analysis sequence consisting in a set of building blocks is possible to implement to detect and measure the interested cells properties. The main features of the instrument are listed below.

- The instrument is provided to a high power xenon lamp (300 W) capable to cover the entire visible spectrum.
- Up to 8 excitation and emission filters can be chosen.
- Up to 4 objectives lens can be installed in a rotative turret and they can be exchangeable. The objectives is compatible with objectives with different fields of view and resolution, from 2X to 100X of magnification and high numerical aperture and long working distance feature.
- A fast laser based autofocus unit is installed in the instrument to detect the samples of the microplates.
- The focal plane thickness ranges between 1.5 to 145 µm.
- Simple experiment set up thanks to Harmony® software thanks to its user friendly interface that guarantees fully automated image acquisition and data management.

- Possibility to perform live cells image analysis thanks to the installed live chamber that control the environmental conditions (temperature and CO_2 percentage) constant during the analysis.

Furthermore, the instrument is provided by a confocal disc that is possible to use in confocal imaging to eliminate background, and protect live cell samples minimizing photobleaching and phototoxicity. The size of the spinning disk pinhole is optimized for a good confocality of a wide range of objectives.

To understand how the samples are acquired and how the image that represent the section of the sample detected is displayed in the computer monitor, the optical path of the light is showed in Figure 1.10. The light beam is filtered with an appropriate selector from the excitation filter wheel. Then the light is carried through the samples with two mirrors that are mounted on a slider. In case of confocal mode, the slider can move and guide the light through a spinning pinhole disc. This device scan in the same time an array of diffraction limited points across the sample and as result it is possible to get better quality images with a low background noise. Then the light pass through an objective, where is mounted in a rotative support where up to four objectives can be mounted and can be selected in an automated way. Up to the sample carrier is mounted a transmission light source to acquire brightfield images, either separately or in combination with the fluorescence image acquisition. Fluorescence emission and transmission light is collected through a bandpass filter mounted on an emission filters wheel and detected by a high sensitivity Peltier cooled CCD camera that elaborate an image to display on the computer monitor. (Operetta Technical Manual).

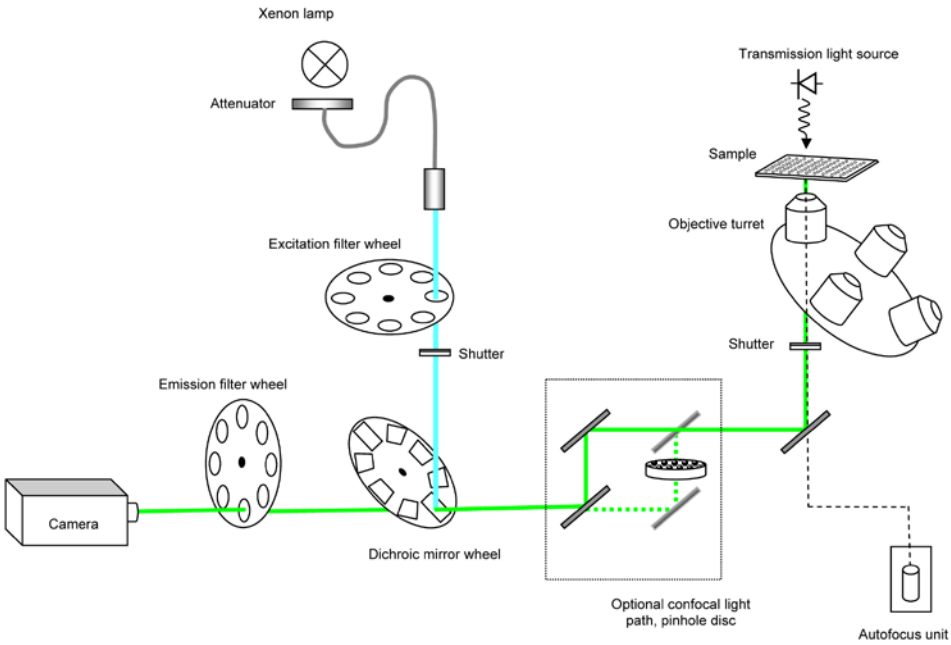


Figure 1.10 *Optical path of the light. The cyan lines represent the excitation light while the green lines are the emitted light from the sample. (Operetta Technical Manual)*

Several analyses to evaluate cells properties can be done with this high-throughput screening instrument like cell cycle, cell differentiation, cell morphology, cytotoxicity, neutrite outgrowth, protein expression, RNAi screening, signaling pathway analysis, and transcription factor.

All of these properties can be evaluated thanks to an high-content image analysis sequence using the tools of Harmony software implemented with powerful algorithms that are capable to detect single cells in the images acquired and it is able to calculate the properties at single cell level. The images that are provided as input to the image analysis tool are segmented, i.e. they are break down in discrete object like individual cells or individual nuclei. The image analysis sequence can be performed with a set of building blocks available in the software tool where giving as input the fluorescence channels used to detect the target components of a cell and a method that provide that is able to segment the image in each single object. It is possible to select many methods between a list of them available in the software and designed to be adapted to various image type, e.g. high contrast nuclear staining or low contrast. The method is chosen by a trial and error procedure using the visual feedback display and tuning the parameters of which the methods is designed to obtain the desire properties of the cells.

1.5 Motivation and purpose of the thesis

The traditional culture systems at macro-scale show some limitations at reproducing the behavior of cells when they are in-vivo in tissues while this microfluidic technology has already shown its ability to preserve important self-regulatory processes that occur in real tissues. However, high-throughput instruments for analyzing cell phenotype have been specifically developed to be coupled with conventional culture systems.

At Shanghai Institute for Advanced Immunochemical Studies (SIAIS), where part of this thesis was performed, both microfluidic and high-throughput imaging systems are available. An ongoing project is using a CRISPR-Cas9 sub-library to perform gene editing deleting ~100 genes from cell genome to study the phenotypic effect of these genes in pluripotent stem cells. However, an efficient imaging-based method to perform an automatic quantitative screening of these cellular phenotypes was needed.

Within this context, the aim of this work was to combine the available technologies to develop a robust method suitable for performing phenotypic of cells by imaging within a microfluidic platform.

Chapter 2

Materials and methods

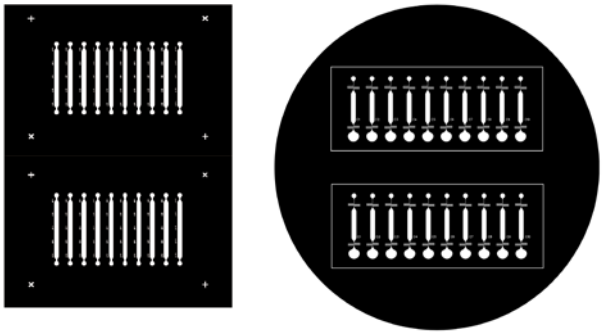
In this chapter the methods to design and to produce the microfluidic platforms are reported. In addition, the procedure of the biological experiments and the acquisition methods with a high-throughput imaging system for screening are explained. At the end the modelling methods are reported.

2.1 Microfluidic methods

It is described the main procedure to obtain a microfluidic platform from the photomask design to the silicon replica moulding. However, the wafers have been already produced in SIAIS lab (Shanghai Institute of Immunochemical Studies) and BioERA lab (Biological Engineering Research and Application) and the work concerned only to the production of the silicon replica mould from the wafers.

2.1.1 Photomask design

The first step to obtain the silicon mould is to design the geometry of the microfluidic platform. Initially the draft is designed with AutoCAD® (Autodesk). The photomask is characterized by a region of dark polarity and light polarity depending on whether the UV light have to hit the region of interest where the negative photoresist has to start the polymerization (Melinda A. Lake et al, 2015). This means that the regions that correspond to the channels are left transparent, instead the other regions that surround the channel that do not polymerize are coloured in black. To create this file to build the photomask is used Adobe Illustrator(Adobe®) and the design it is showed in Figure 2.1. During the printing phase of the photomask it is important to set a high resolution to keep all the features of the design in the final silicon mold. Usually the photomasks are printed in a flexible, inexpensive plastic film with a resolution of 5-10 μm (Melinda A. Lake et al, 2015)



a) b)

Figure 2.1 *Design of the photomask used in this work generated using Adobe Illustrator(Adobe®). a) Ten-chamber microfluidic platform. b) Upgraded version of the ten-chamber microfluidic platform*

2.1.2 Wafer fabrication

In this section it is explained the procedure to obtain the mold of the microfluidic platform through the photo lithography techniques. On a silicon wafer it is deposited a photoresist, where the desired geometry will be impressed. The photoresist used (SU-8 2100) is a negative and photosensitive epoxy resin provided by Microchem.

2.1.2.1 Wafer pretreatment

A silicon wafer with a diameter equal to 4 inches is cleaned rinsing consecutively with acetone (Sigma-Aldrich), methanol (Sigma-Aldrich) and distilled water to help the adhesion of the photoresist in the next steps. Then, the wafer is dried using compressed air and placed on a hot plate at 105 °C for at least 10 minutes to remove humidity. Finally, the wafer is treated with Hexamethyldisilazane vapor for 10 minutes.

2.1.2.2 Deposition of photoresist

After the pretreatment the wafer is placed in the spin coater and it is deposited an amount of photoresist suitable to cover roughly 30% of the wafer surface paying attention to include less air as possible. From this point, all the steps have to be done in a dark ambient to avoid the premature reticulation of the photosensitive polymer.

As reported in the SU-2000 data sheet the photoresist (SU-8 2000 Negative Photoresist Processing and Datasheet) is available in twelve standard viscosities that allow to obtain a thickness from 0.5 to more than 200 µm with a single process deposition. To obtain a structure 200 µm high is selected the SU-8 2100 photoresist where it is reported the relation between the thickness of the polymer that it is deposited and the spinner velocity.

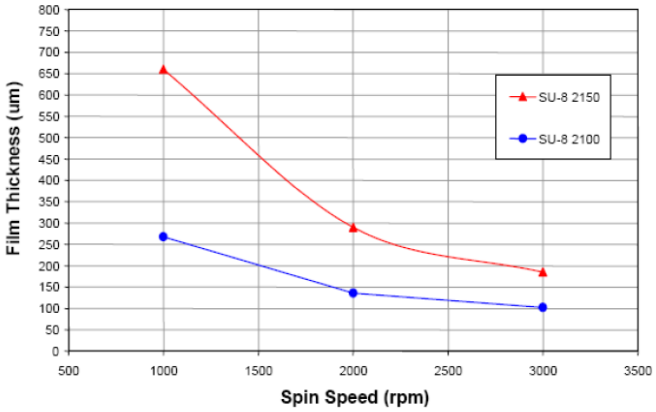


Figure 2.2. *Film thickness [µm] vs Spin Speed [rpm] for the photoresist SU-8 2150 and SU-8 2100. (SU-8 2000 Negative photoresist processing and datasheet)*

To obtain a photoresist with a thickness of 200 µm it is used a spin coater where it is set up the following program based on Figure 2.2:

- Step 1: 500 rpm for 5-10 seconds with an acceleration of 100 rpm/sec.
- Step 2: 3000 rpm per 30 seconds with an acceleration of 300 rpm/sec.

2.1.2.3 Soft bake and exposure

This procedure is done to remove the internal stress of the material and to partially evaporate the solvent contained in the photoresist. A first optional preheat phase at 65°C and a heat phase at 95°C are done in a hot plate. In Table 2.1 the baking times for the two temperatures are reported depending on the thickness of the photoresist.

Table 2.1. *Soft Baking times [min] at 65°C and 95°C depending on the thickness photoresist [µm]. (SU-8 2000 Negative photoresist processing and datasheet)*

Thickness [µm]	**Soft Bake Times [min]**	
	(65°C)	**(95°C)**
100-150	5	20-30
160-225	5-7	30-45
230-270	7	45-60
280-550	7-10	60-120

To start the polymerization a UV lamp is used with a wavelength of 365nm. The exposition energy E_a depends on the thickness of the photoresist and the value are reported in Table 2.2 and need to be set before the exposition.

Table 2.2. *Exposure energy mJ/cm^2 of the UV lamp based on the thickness of the photoresist [µm]. (SU-8 2000 Negative photoresist processing and datasheet)*

Thickness [µm]	Exposure Energy mJ/cm^2
100 – 150	240 - 260
160 – 225	260 - 350
230 – 270	350 - 370
280 – 550	370 - 600

Knowing the power of the lamp P, it is possible to calculate the time of exposure as

$$t = \frac{E_a}{P} \tag{2.1}$$

Before to start the exposition the photomask have to be placed up to the wafer in order to expose and polymerize only the interested part. It is noticed that an underexposition of 20% respect the suggested time increase the success of the operation, especially for complex geometry.

2.1.2.4 Post exposure and bake development

A second heating is necessary to complete the polymerization and to evaporate the solvent from the wafer and should take place after the exposure. Even in this step, there is an optional heating at 65°C and a heating at 95°C where the time depend on the thickness of the photoresist as it is showed in Table 2.3 below.

Table 2.3. *Post bake time [min] at 65°C and 95°C for different photoresist thickness. (SU-8 2000 Negative photoresist processing and datasheet)*

Thickness [µm]	Post Bake Times [min] (65°C)	(95°C)
100-150	5	10-12
160-225	5	10-15
230-270	5	15-20
280-550	5	20.30

In the developing phase a solvent it is needed to wash the area of the photoresist that have not been crosslinked. As solvent it is used the Propylene glycol monomethyl ether acetate (Sigma-Aldrich). The wafer is placed in a glass plate filled by solvent where it is agitated using a rocking shaker. The developing time is reported in Table 2.4 below and it depends on the thickness of the photoresist.

Table 2.4. *Development time [min] for differents thickness of the photoresist [µm]. (SU-8 2000 Negative photoresist processing and datasheet)*

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

After the development step the wafer is cleaned with isopropanol (Sigma-Aldrich) and dried with compressed air.

2.1.2.5 Hard bake

The last step is a heat treatment to maintain the thermal characteristic and to ensure the durability of the wafer when it will be used in thermal processing to produce the silicon replica molding. It is done putting the wafer on a hot plate for 10 minutes at 65 °C and then setting a ramp of 120 °C/h for 2 hours to reach the maximum temperature of 160 °C. After the treatment it is good to wait that the hot plate reaches the room temperature before to remove the wafer to avoid thermal shocking.

2.1.3 PDMS replica moulding

Replica moulding is an efficient technique for the duplication of the information present in the surface like morphology, shape and structure (Xia & Whitesides, 1999). Thanks to this procedure it is possible to obtain the PDMS moulds and reservoirs with the desire geometry that will compose the microfluidic platform.

The first step is to treat the wafer with Chlorotrimethylsilane (Sigma-Aldrich) vapour for 60 minutes to facilitate the detachment of the PDMS from the wafer surface.

The PDMS is made by two components, a base and a curing agent (Sylgard®184, Dow Corning) in a weight ratio of 10:1. The two chemicals are poured in a cup and weighted over a scale (Mettler Toledo) and under chemical hood they are mixed for some minutes manually until the mixture will turn white. Then the cup is covered by an aluminium foil and it is placed under vacuum for 20 minutes for the first degassing step to take out the air inside the liquid mixture. Then the PDMS is poured into the wafer and it is placed under vacuum for 40 minutes to remove the last air bubbles. When all the air bubbles are disappeared, the wafer is baked in the hot plate where three different heating programs reported in Table 2.5 it is possible to set.

Table2.5 *Baking time[min] of the wafer at different temperature [°C]*

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

Each heating program gives the same mechanical properties to the solid PDMS. Usually the baking is carried on at 75°C for 75 minutes. When the wafer is cooled until the room temperature, the edge that correspond to a chip are cut with a scapel and the inlets of the PDMS mould are punch with a 1mm puncher.

The reservoirs need to prevent the evaporation of the medium solution from the culture chambers. They are made pouring the PDMS in a 45×45mm surface plate, and after a degassing step of 40 minutes it is placed in a hot plate to bake at 75°C for 75 minutes. A solid piece of PDMS 3 mm height is punched with a 3 mm puncher and shaped in order to fit it in the silicon mould.

The PDMS mould are attached to the glass through a plasma treatment, using as instrument the plasma cleaner (PDC, Harrick Plasma). The glass have to be cleaned before with a cleaning solution made by a soap that removes the impurities from the surfaces and then dried by compressed air. Two quartz glass with different sizes are used to create the microfluidic platform: one is a 26×75mm microscope glass slide and the other is a 110×75mm glass plate. A last cleaning is done covering with the tape the surfaces of the glass that will be treated and the side of the PDMS where it is impressed the geometry mould. For both glasses the following procedure is the same:

- the silicon mould and the glass are placed around the centre of the chamber of the plasma with the surfaces to be treated pointed up;
- through a vacuum pump it is reached inside the chamber a pressure equal to $9.6*10^{-1}$ mbar;
- the inlet valve is opened to let the air blow inside the chamber until to reach a pressure equal to 1.2 mbar;
- at this point the power of the plasma cleaner is switched on for 15 second to let the plasma activate the surfaces;
- the power of the plasma is switched off and the inlet valve is opened until to reach a pressure equal to $1.0*10^{3}$ mbar inside the chamber;
- the surfaces of the PDMS mould and the glass are put in contact to attach and make the microfluidics platform.

The microfluidics platforms are placed in a hot plate preheated at 100°C to promote the adhesion of the two surfaces at least for 15 minutes. To attach the reservoirs in the chip it is followed the same procedure in the plasma treatment

The coupling of the surfaces after the plasma treatment is a crucial step because the channels of the microfluidic platform have to respect the proper distances in the glass in order to be faced correctly with the imaging system, as described below. An example how a microfluidic platform mounted in a microscope glass slide (76×26 mm) appears after the replica moulding procedure is showed in Figure 2.3.

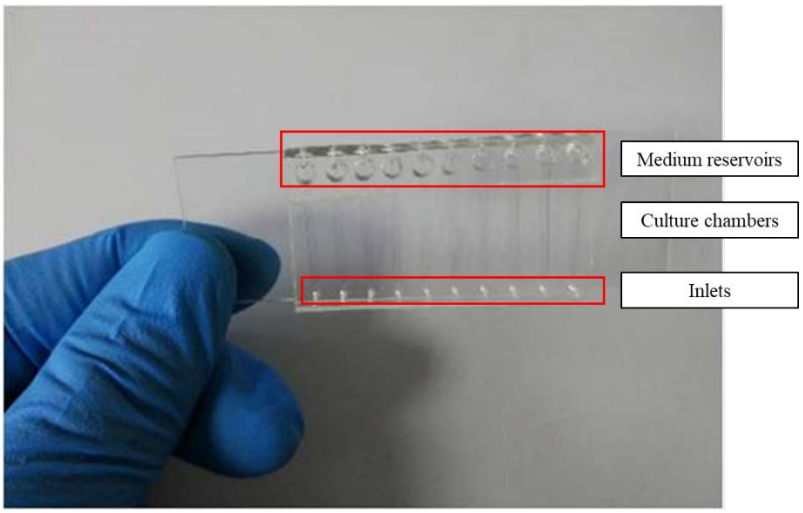


Figure 2.3 *Microfluidic platform fabricated in a microscope glass slide*

Before to use the microfluidic platform for any biological application, the chambers of the platform are washed with isopropanol (Sigma-Aldrich) and water and the device must be sterilized with vapor at 121 °C for 27 minutes in autoclave with a pressure of 2.2 bar.

2.1.4 Description of different microfluidic platform configurations

The microfluidic platforms designed for the high-throughput screening are based from two kinds of geometry: one is a ten chambers geometry and the other one is an upgraded version of the first.

2.1.4.1 Microfluidic platform features

A single chamber in the first type of platforms design is represented in Figure 2.4. It has two openings with a diameter of 1.5 mm, of which one is used as inlet and one as outlet, as previously shown (Figure 2.3). The cell culture chamber has a length of 19.84 mm, a width of 1.5 mm, a surface of 26mm^2 and a 5.4 µL.

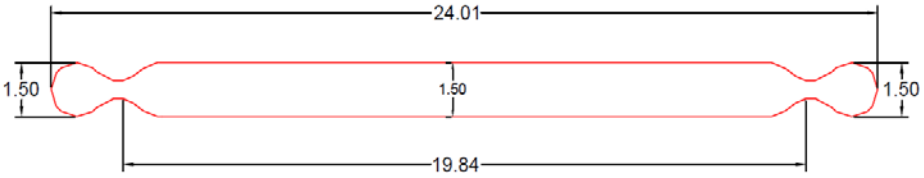


Figure 2.4 *Single chamber of a standard microfluidic platform. Dimensions are reported in [mm].*

The microfluidic platform is composed by ten chambers all independent from each others where on the right hand are reported the numbers and letters along the chamber to identify the position of it. The total length of the silicon mould around 50 mm and a width of 26 mm. The reservoirs attached in one side of the end of the channel and they have the same length of the mould with a width around 6.5 mm and a diameter of the holes of 3 mm (Figure 2.5).

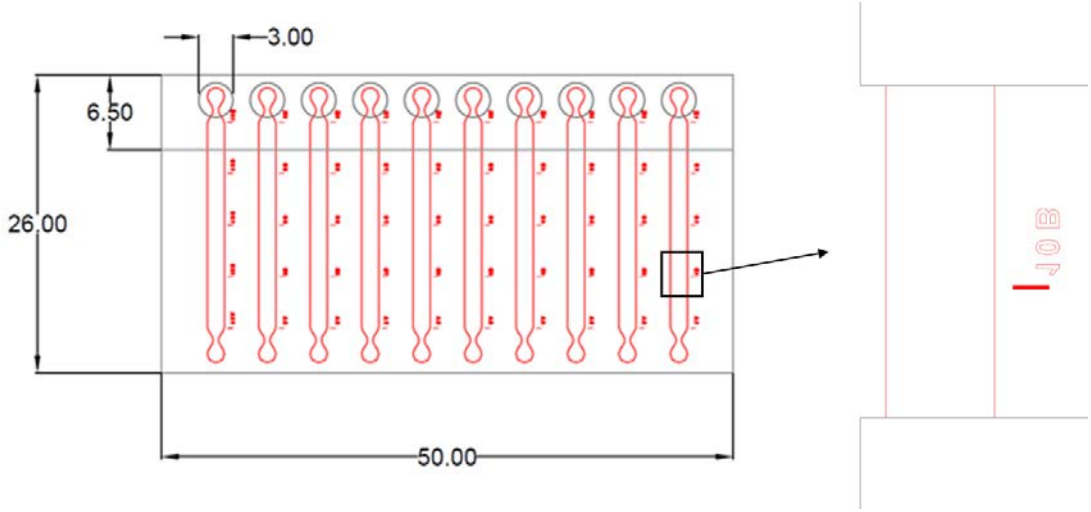


Figure 2.5 *PDMS mould with ten channels in a standard design. In red are represented the chambers of the platform where the number identify the chamber in the platform and the letter identify the section of the chamber. In grey are represented the edges of the silicon mould. Dimensions are reported in [mm].*

In the upgraded version of the first, a single channel reported in the new design reported in Figure 2.6 can be divided into five parts:

1. Inlet with a diameter of 1.5mm.
2. Serpentine channel with a width of 100µm and it has a high of 200 µm and a length of 15 mm
3. Cell culture chamber, with a length of 11.2 mm, a width of 1.5 mm, a height of 200 µm, a surface of 14.7 mm^2 and a volume of 3 µL.
4. Reservoir which has a diameter of 3.5mm

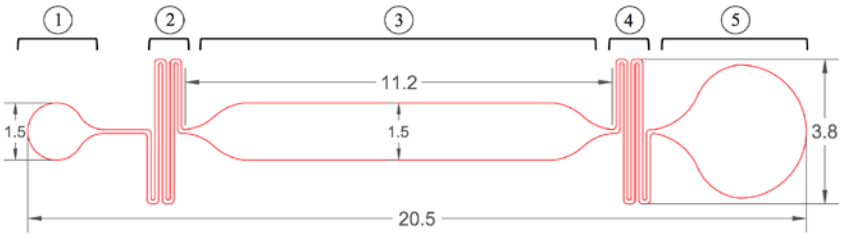


Figure 2.6. *Single chamber of a new design microfluidic platform. Dimensions are reported in [mm].*

The complete microfluidic platform (Figure 2.7) have a length of the silicon mould around 66 mm and a width of 24 mm.

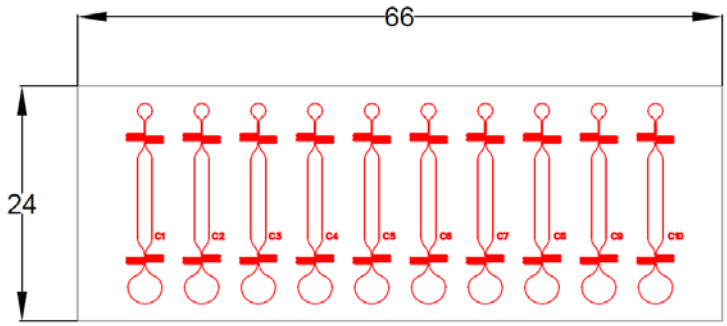


Figure 2.7. *PDMS mould with ten channels in a new design. In red are represented the chambers of the platform and in grey are represented the edge of the silicon mould. Dimensions are reported in [mm].*

2.1.4.2 Configurations to interface with the high-throughput imaging

Operetta is an imaging system for high-throughput screening, whose software includes preset acquisition positions according to the American National Standards Institute (ANSI).

The microfluidic platforms to be faced in Operetta need to respect geometrical constrains for the correct visualization of the chambers. The first approach was to adapt the microfluidic platform to a multi-well plate where the chambers fit the wells in order to detect the platform with the software. After the plasma treatment the PDMS mould have been coupled with a 26×76 mm glass slide and 110×75mm glass plate taking as reference a 6-well plate to match the channel with the well (Figure 3.9 in section 3.3) , instead taking as reference a 96-well plate five chambers have been matched in one well using both type of glasses (Figure 3.11 in section 3.4).

A developed approach was to design a configuration to simulate a multi-well plate where each chamber of the platform mimics a well. To create the platform a sketch of the designed from AutoCAD® is printed and is taken as reference to attach the PDMS to the glass.

2.1.5 Design

The support that hold the microfluidic platform in the instrument have been built from the commercial plate CELLSTAR® OneWell Plate™ (Greiner) reported in Figure 2.8

Figure2.8 *One-well plate used to build the support to hold the microfluidic platform in the high-content screening instrument. (www. shop.gbo.com)*

In addition, it's external dimensions comply with ANSI standards and can be easily interfaced with a wide range of automated cell culture system, liquid handling system and screening system. The plate is made by high grade polystyrene. The bottom of this plate was cut and a PDMS frame was built inside it to hold the glass of the microfluidic platform. This guarantees compatibility of the geometry for interfacing with Operetta®, flexible design of the microfluidic platform integrated with this holder, and direct exposure of the bottom glass of the microfluidic platform to the objective of the imaging system.

2.2 Biological methods

In this section it is reported the main passages for the cell culturing and the procedure to develop the toxicity assay and the immunofluorescence assay.

2.2.1 Cell culture

The cell type used in this work is human skin fibroblast. Two kinds of cell lines have been used: one is the BJ (ATCC® CRL-2522™) and the other one is the HFF-1(ATCC® SCRC1041™). These are cells that growth attached to the bottom surface. The cultivation is done both in microfluidic devices and in a 96-well plate. The main steps followed were the cell thawing, cell seeding, cell passaging and cell freezing. The method is the same for both cell lines.

2.2.1.1 Medium solution preparation

The medium solution contains the principal nutrients like proteins, vitamins and carbohydrates required to the cells to live and to reproduce. It is composed by Dulbecco's Modified Eagle's medium (DMEM, ThermoFisher) and the Fetal Bovine Serum (FBS, ThermoFisher) in different concentration depending on the type of the cells:

- for the BJ (ATCC® CRL-2522™) a DMEM solution with 10% of FBS is prepared;
- for the HFF-1(ATCC® SCRC1041™) a DMEM solution with 15% of FBS is prepared.

Media solution are stored at 4°C.

2.2.1.2 Cell thawing

Cells stocks are stored in cryovials at -80° or in liquid nitrogen. When the vial of a cell line is taken out from the cold storage it is placed rapidly in a thermostatic bath at 37°C. When only a little piece of ice is left inside, the content of the vials is transferred in a 15mL tube and is added 10mL of warm medium. Because the cells are frozen in a solution containing Dimethyl sulfoxide (DMSO, Sigma-Aldrich), as explained below it is important to do this step quickly to avoid the toxicity effect of this chemical on the cells at room temperature. Then after a centrifugation at 1100 rpm for 5 minutes the supernatant containing DMSO is aspirated and it is added 950 µL of medium solution. After the resuspension of cells inside the tube they are counted and seeded in a Petri dish at the desire density.

2.2.1.3 Cell passaging

This step is done when the cells reach the confluency inside a dish and cannot expand anymore. The cells are split in a new dish at lower densities where they can growth and replicates. The procedure is the same for both cell lines. The medium solution is aspirated with a vacuum pump and then the cells are washed with phosphate buffer saline (PBS) to remove the dead ones. Then it is added trypsin ethylenediaminetetraacetic acid (EDTA) 0.05% to detach the cells from the bottom and the dish is put in the incubator at 37°C for 5 minutes. After checking in the microscope if a great part of the cells detached from the bottom of the plate the action of the trypsin EDTA have to be stopped adding an equal amount of medium solution. Then the cell suspension is collected in a 15mL tube and it is centrifugated at 1200 rpm for 5 minutes. The pellet of cells remain in the bottom of the tube and the supernatant is aspirated with the vacuum pump. Then the cells are resuspended in an appropriate amount of media solution (usually 1 mL) to count them or to seed them directly in a new dish. Finally, the cells can be placed in the incubator at 37°C and 5% of CO_2. Usually the medium solution has to be changed every two or three days in the dish.

2.2.1.4 Cell seeding in microfluidic chips

Before to seed the cells inside the microfluidic chips the channels have been coated with fibronectin, a protein that allows the cells to attach to the surface of the glass. The following procedure is the same for both microfluidic platforms built in a 76×26mm glass slide and 110×75mm glass slide.

First the devices mounted in a glass slide are placed in a 100 mm diameter Petri dish, instead the ones mounted in a big glass plate are placed in a 500 mm diameter Petri dish. Then every channel is filled with 12µL of fibronectin solution at 25 µg/mL in PBS and they are left to adsorb on the glass surface for at least 30 minutes at room temperature. It is added inside in the dishes around the microfluidic platform an antibiotic (Penicillin Streptomycin, from Thermofisher, diluted 1/100 in ultrapurified water) to reduce the evaporation during the incubation and to reduce the potential contaminations in the dish. After 30 minutes the chambers coated are rinsed with fresh medium solution to remove the exhausted fibronectin solution.

Meanwhile the cells cultivated in a dish are resuspended as described in Section 2.2.1.3 and counted with an automatic cell counter. To count the cells, a small volume of 10µL is withdraw from the cells suspension and it is put in a vial where it is added 10 µL of Trypan Blue (Sigma-Aldrich) a dye that highlights dead cells. Then two chambers of a plate counter are filled with 10 µL of solution (cells suspension + Trypan Blue) and the plate is inserted in an automated cells counter. The instrument gives as output the number of cells over volume [cells/mL] and the number of cells in the suspension is obtained by the arithmetic mean of the value for the two chambers measured by the instrument.

The seeding density [cells/mm^2] in terms of area is decided a priori and to know the total number of cells to seed in the chambers it is necessary to convert this quantity to a density in terms of volume according to the following equation (2.2):

$$\rho_{seedingV} = \frac{\rho_{seedingA} \cdot A_{chamber}}{V_{chamber}} . \tag{2.2}$$

Where $\rho_{seedingV}$ is the volume seeding density [cells/µL], $A_{chamber}$ is the area of a single chamber [mm^2] and $V_{chamber}$ is the volume of a single chamber [µL]. The area and the volume of a single chamber for the two geometries is reported in Section 2.1.4.1. The total volume V_{TOT} [µL] of the microfluidic platform is calculated with the following equation (2.3):

$$V_{TOT} = V_{seeding} \cdot N_{chambers} + V_{safe}. \tag{2.3}$$

Where $N_{chambers}$ is the number of the chambers to seed, $V_{seeding}$ [µL] is the volume of cell suspension to seed in each channel and it is equal 12µL/channel in the classic geometry and 6µL/channel in the new geometry: these values include the filling of reservoirs and the

serpentines of every channel in the platform; V_{safe} [µL] is a safe volume that is included to take into account medium losses due to inaccuracies during liquid handling. To be safe a volume of 100 µL is taken into account. The total number of cells $N_{cells\ tot}$ that are required to be seeded in the platform are calculated from equation 2.4:

$$N_{cells\ tot} = V_{TOT} \cdot \rho_{seedingV} . \tag{2.4}$$

The volume of cell suspension to withdraw V_{susp} [µL] is given by equation 2.5:

$$V_{susp} = \frac{N_{cells\ tot}}{\overline{\rho_{cell\ counter}}} .1000 \tag{2.5}$$

where $\overline{\rho_{cell\ counter}}$ [cells/mL] is the average density if cells from measured from the cells in terms of volume measured by the automatic cell counter. If the value of V_{susp} is lower than the volume that it is added to resuspend the cell it is required to add a volume of medium giving by the difference between V_{susp} and the volume of the resuspension.
When all the chambers are seeded the platform are moved in the incubator at 37°C and 5% of CO_2. The medium is changed after 24 hours the day after seeding and then every 12 hours.

2.2.1.5 Cell seeding in multi-well plate

The cells are seeded in a 96-well glass bottom plate (Cellvis). The surface of a single well in this plate is 32mm^2 and the working volume is 200 µL. To guarantee the attachment of the cells in the bottom surface in each well a layer of fibronectin is coated before to seed the cells. In this case the coating agent have to be diluted with a volume ratio 1:5 in PBS and it is deposited a layer 2 mm height. The plate is left at room temperature for 30 minutes to let the fibronectin adsorb on the glass surface and in the wells not coated around are filled with 200 µL of PBS to avoid the evaporation during the incubation.
The procedure to seed the cells in the wells is the same described in Section 2.2.1.4 where the seeding volume and the volume of a single well is replaced by the working volume and it is considered the surface of a single well to calculate the seeding density in volume units.

2.2.1.6 Cell freezing

Cells can be frozen in cryovials to keep a backup of them or to store them to perform future experiments. To avoid the mortality of the cells due to an increasing of volume for the formation of ice in their structure it is added the DMSO in the medium solution. Furthermore, it is added FBS to guarantee cell viability and cell recovery after thawing it. So first it is prepared a freezing solution with 400µL of FBS and 100 µL of DMSO. At the same time the cells are suspended in 0.5mL of media solution in a 1.5mL vial and then it is added dropwise 0.5mL of freezing

solution. Then the cryovials are stored at -80°C up to six months or under liquid nitrogen for longer period of time.

2.2.2 Toxicity assay

The cells cultures are treated with a solution made by the medium and the DMSO (Dimethyl sulfoxide, Sigma-Aldrich) in different concentrations volume/volume in a range between 0% and 10%. Two kinds of toxic assay have been developed based for the two cells line:

- for the HFF-1(ATCC® SCRC1041™) a set of five solutions made by the medium and the DMSO at concentrations of 1.25%, 2.5%, 3.5%, 5% and 10% have been prepared;
- for the BJ (ATCC® CRL-2522™) a set of three solutions made by the medium and the DMSO at concentration of 0.5%, 5% and 10% have been prepared.

The assays are carried on both in the multi-well plates and in the microfluidic platforms. When the cells are confluents, in the microfluidic chambers or the wells the medium is replaced by the DMSO solutions. Chambers and well are filled with DMSO solutions in random known positions to make the results statistically meaningful. Then the devices are incubated at 37°C and 5% of CO_2 for 24 hours.

2.2.3 Immunofluorescence assay

To asses the viability of the cells after the treatment with the toxic agent it is performed the Live&Dead assay using the Viability/Cytotoxicity Kit (Thermofisher). The test consists to inject the fluorophores to the samples that bind specifics molecule inside the cells that determine the viability. Three kind fluorophores are used in this experiments:

- *Hoechst 33342* in a volume ratio of 1:5000 for live cells. It binds specifically the DNA of the cells, both live and dead. It is important to identify the cells from other particles in the final image. Its maximum excitation wavelength is 351nm and its maximum emission wavelength is 452 nm.
- *Calcein AM(acetoxymethyl)* 2µM concentrated. The molecule it is not fluorescent for the presence of esters group but when the reagent is transported inside the cytoplasm of live cells it becomes fluorescent for a reaction with esterases. Its maximum excitation wavelength is 494nm and its maximum emission wavelength is 517nm.
- *Ethidium homodimer* 4 µM concentrated. Usually this reagent does not enter in the live cells, but when they are in the final stage of apoptosis (dead cells) it can enter through the cellular membrane and binding the nucleic acids it becomes fluorescent. Its maximum excitation wavelength is 528nm and its maximum emission wavelength is 617nm.

A working solution made by PBS and these three fluorophores is injected in the samples after a wash with PBS to remove the medium that may inactivate the Calcein AM for the presence of serum. The samples are placed in the incubator for 40-45 minutes and then they are analysed with Operetta®.

2.3 Imaging methods

The samples are interfaced in Operetta® where the main parameters have been set up to perform the analysis based on the type of platform screened and on the toxicity assay developed. First it is performed the acquisitions of the images of the samples and then in a second time the analysis of them.

2.3.1 Images acquisition

The acquisition of the samples in the microfluidic platforms or in the multi-well involve some steps that are discussed in this section.

2.3.1.1 Software description

The software dedicated to Operetta® is Harmony® (Perkin Elmer), capable to do high-throughput imaging and analysis. All the parameters to acquire high quality images can be set in the software based on the type of devices that is interfaced and on the type of cells that have to be analyzed. After the measurements the images are collected and store them in a database. In Figure 2.9 it is showed a screenshot of Harmony during in the setup phase.

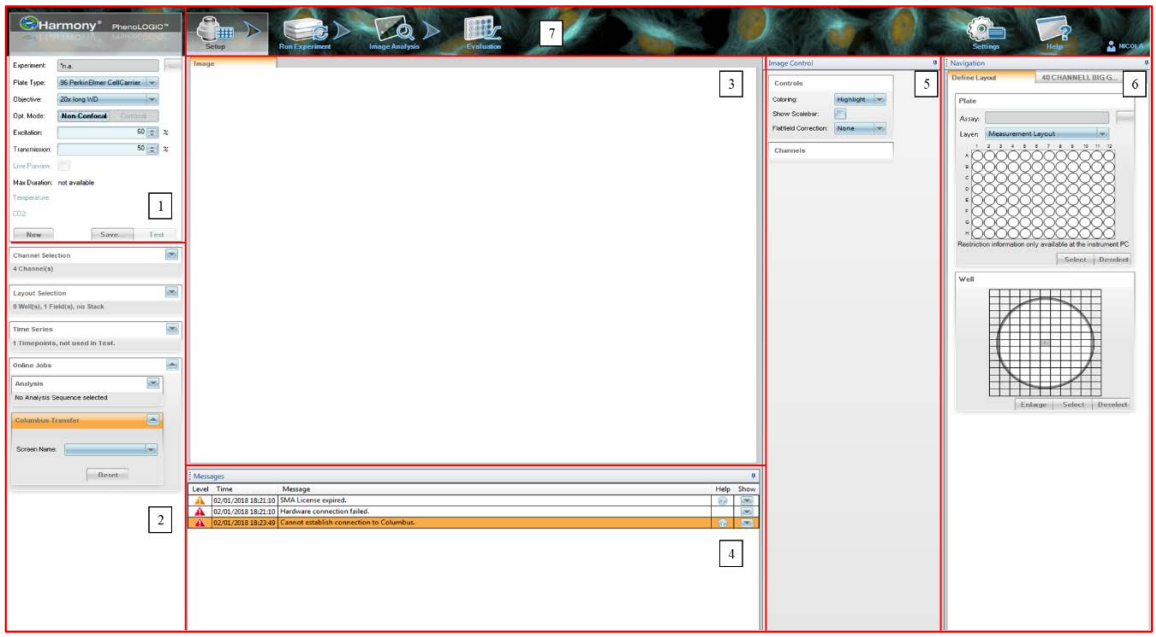


Figure 2.9. *Screenshot of Harmony interface with the main sections of the software highlighted in the setup phase. In order: 1. Global Control, 2. Settings, 3. Content Area, 4. Messages, 5. Image Control, 6. Navigation, 7. Analysis Navigation*

The workflow interface can be divided in the following parts (numbered in Figure 2.9):

1. *global control* where it is possible to select the first parameter as the plate type, the objective, the lamp power, the optical option and eventually saved settings in the database;
2. *setting part* where it is possible to select the emission/excitation filters for the light channels, time points for the analysis and number of field to select;
3. *content area* where it appears the image of the snapshot on the field and light channel selected;
4. *messages* as eventually errors of the instrument or notifications while the analysis is being performing;
5. *image control* where it is possible to control the image parameter of the snapshot in the content area selecting different type of colors exposition and tuning the gamma correction of the image;
6. *navigation where* it is possible to select the wells and the field inside where to acquire the images
7. *analysis navigation* where it is possible to switch between the measurements steps of the software like setup, experiment, image analysis and evaluation.

2.3.1.2 Plate type definition and selection

The plate type may be selected among different options stored in the database based on the type and the brand of the plate. If the model of the plate is not present in the list it is necessary to define a new plate, specifying the number of the wells organized by rows and columns and the shape of each wells, usually rectangular or circular. Furthermore, it is required to insert the measurements of the plate showed in Figure 2.10.

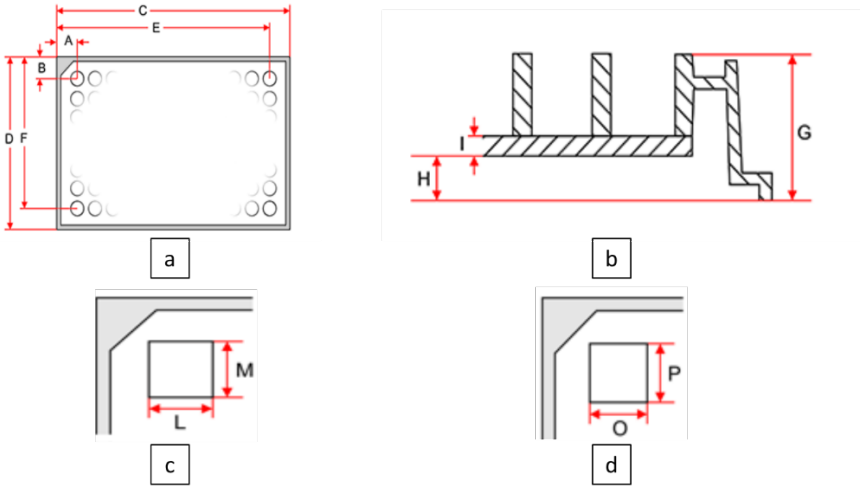


Figure 2.10. *Measurements [mm] of a new plate to to provide to the software. a) Positions of the wells. b) Plate bottom dimensions. c)Top well dimensions. d) Bottom well dimension. (Operetta Operation Manual)*

In Figure 2.10a the distances [mm] of B and F allow the instrument to recognize the positions of the wells along the y-axis, instead giving the distances [mm] of A and E the instrument detects the position of the wells along the x-axis. The value of D and C are the width[mm] and the length[mm] of the plate. The dimensions of the bottom of the plate showed in Figure 2.10b are the most important ones to provide because these are used by the autofocus unit to detect the samples and to display the image: H is the plate bottom height [mm], I is the thickness [mm] of the bottom and G is the total height [mm] of the plate. The values of L and M in Figure 2.10c are respectively the length[mm] and the width[mm] of the top of a well, instead the value of O and P in Figure 2.10d are respectively the length[mm] and the width[mm] of the bottom of a well. If the well is circular, the diameter [mm] on top J and the diameter [mm] on bottom K of the well is provided instead of the length and the width.
The 96-well plate (Cellvis) was not present in the database and the dimensions showed in Figure 2.10 were reported in Table 2.6 below

Table 2.6. *Dimensions [mm] of the 96 Well Plate (Cellvis). (https://cellvis.com/96-well-glass-bottom-plate)*

A	B	C	D	E	F	H	I	G	J	K
14.30	11.36	126.60	85.75	112.30	74.39	2.67	0.17	14.30	6.80	6.21

The dimensions of the bottom of both the multi-well plate and the frame to support the microfluidic platforms are measured by a caliber. Instead the dimensions of the custom configurations were determined in AutoCAD® by joining the draft of the frame and the draft of the custom configuration.

2.3.1.3 Objectives selection

Up to four objectives can be mounted on the objective turret of the imaging instrument and they can be easily changed between the ones available. The selection of the objective is done according to different considerations:

- the size of the object to be evaluated;
- how many cells per wells are required for a significant statistical analysis;
- how important is the imaging speed and how prone is the sample to the photobleaching due to prolonged exposure;
- type of microplate interfaced in the instrument.

The main characteristics of the objectives available are showed in Table 2.7 below.

Table 2.7 *Characteristics of the objectives available for Operetta™. (Operetta Technical Manual)*

Objective	Numerical Aperture (NA)	Working Distance (WD) [mm]	Field of view [µm²]	Depth of focus [µm]	Optical resolution xy [µm]	Correction collar	Possible values [mm]
2x long WD	0.08	6.2	6754 × 5086	145.5	3.75	No	-
10x long WD	0.3	10	1351 × 1017	10.3	1.00	No	-
10x high NA	0.4	3.1	1351 × 1017	5.8	0.75	No	-
20x long WD	0.45	6.6-7.8	675 × 509	4.6	0.67	Yes	0-2
20x high NA	0.75	0.6	675 × 509	1.7	0.4	No	-
40x long WD	0.6	2.7-4	338 × 254	2.6	0.5	Yes	0-2
40x high NA	0.95	0.18	338 × 254	1.0	0.32	Yes	0.11-0.23
60x long WD	0.7	1.5-2.2	225 × 170	1.9	0.43	Yes	0.1-1.3
60x high NA	0.9	0.2	225 × 170	1.1	0.33	Yes	0.11-0.23
100x longWD	0.85	1.2-1.9	135 × 102	1.3	0.35	Yes	0-0.7

The setting of the correction collar in the objectives is required to correct the optical aberrance due to the plate material thickness in the kind of objectives that inherently do not have this correction. It has to be done manually before to put the plate in the instrument and it is showed in Figure 2.11. The value of the correction collar to set is displayed by the software when the plate type and the objective are selected.

Figure 2.11 *Correction collar manual settings on the objectives that include this feature (Operetta Technical Manual)*

The objective is composed by a fix part with a set point and a grid mounted in a wheel. With the help of a mirror if the grid is not visible it is possible to spin the wheel until the value in the grid matches the set point. The objectives with high numerical aperture (NA) cannot be used for both the microfluidic platforms and the 96-well plate because the height of the plate bottom

of the support and the multi-well is higher than the limit value set by the instrument (300µm) to avoid the collision between the rid of the bottom frame. A 20X objective long working distance (WD) is selected to provide good statistical results for the fibroblast cells analyzed. The value of the correction collar set for this objective is the same of the plate bottom thickness of the bottom plate and respectively is set equal to 0.17mm for the 96-well plate (Celvis) and 1mm for the support for the microfluidic platforms.

2.3.1.4 Channels selections

Using a sequence of different channels it is possible to perform a measurements in the same position using different optical settings, e.g. emission and excitation filters, exposure times and focus height. The channels in the database can be loaded and each default ones includes an excitation filter and an emission filter suitable for the respective dye/wavelength. The spectrum of excitation and emission of dyes used in the immunofluorescence assay, Hoechst 33342, Calcein AM and Ethidium homodimer are showed in Figure 2.12 below.

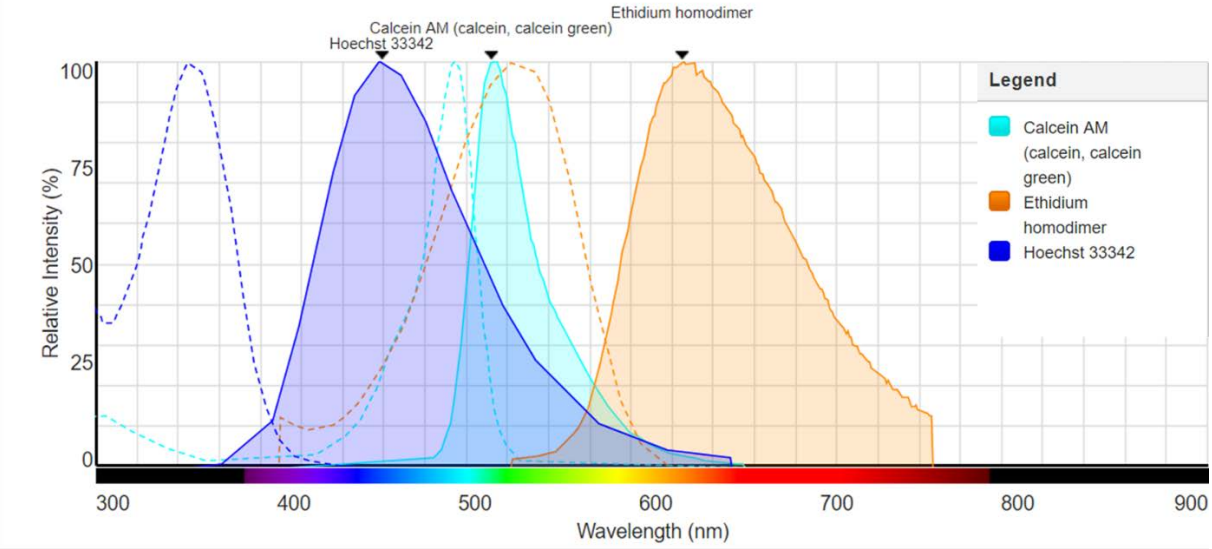


Figure 2.12. *Excitation and emission spectrum view of the fluorophores used in the immunofluorescence assay. The outlined curves represent the excitation spectrum instead the continuous curves are the emitted spectrum of the dyes.*

The emission spectrum of the the Hoechst 33342 overlaps a bit the one of the Calcein AM (Figure 2.12). To avoid the crosstalk of the two fluorophores a narrow emission filter (500-550 nm) is selected for the Calcein AM in order to collect the emitted photons of the dye at the maximum intensity and reducing the interference of the emitted light of the Hoechst 33342. In Table 2.8 the excitation and emission filters are reported for the dyes used in the assays.

Table 2.8 *Selected Channels for imaging each fluorescent probe*

Object fluorescently labeled	Fluorophore	Channel Name	Excitation filter [nm]	Emission filter [nm]
Nuclei	Hoechst 33342	HOECHST 33342	360-400	410-480
Dead Cells	Ethidium Homodimer	Ethidium Homodimer	520-550	580-650
Live Cells	Calcein AM	Alexa 488	460-490	500-550

To avoid the photobleaching of the samples the excitation lamp power is kept around 50% and the exposure times for the channels selected around 200ms.

2.3.1.5 Wells and field selection

The number of the wells and the field inside can be selected for the final measurements. This corresponds to the regions where the objectives are moved and the positions where the images of the samples will be acquired. The software does not allow a custom selection of the fields for every well, so for every well analyzed the images will be collected in the same positions. When the microfluidic platforms are placed in the instrument the aim is to identify the channels inside the wells selecting the correspondent fields.

2.3.1.6 Focusing of the samples

Once objectives and the plate type have been selected, it is possible to interface the samples with the instrument. To avoid focus failures especially when the microfluidic platforms are measured, the bottom surface that face the objective have to be dry and clean. It is important to define the optimal focus height for every light channel selected. An object when it is focused appears to be sharp and bright. Not all the objects in a cell are stained at the same plane and for this reason the focal plane for each channel have to be defined independently. Two kinds of approach it is possible to follow. The first one uses the z-stack (Figure 2.13), a tool where it is required to provide an initial plane of acquisition, the number of planes and the distance between the planes where the instrument collects the images for each light channel.

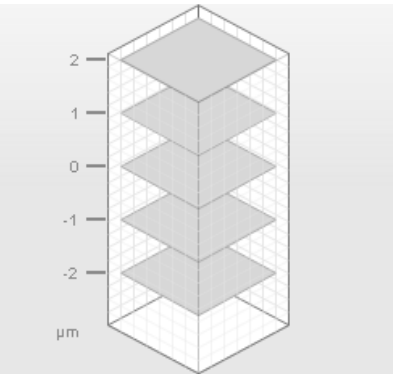


Figure 2.30 *Example of a z-stack of a field inside the well. Distances are in [µm]. (Operetta Operation manual, Perkin Elmer)*

Only one field inside the well is selected for the z-stack. The software calculates the focus height with an offset, that depends on the type of objective and on the optical plate material, giving the possibility to provide as initial plane of acquisition a negative value. When the microfluidic platform has been measured as starting plane it was given -20 µm, with 20 planes far 2 µm from each other. Once the optimal focal plane has been identified for every light channel, the position of the plane is reported on the focus height box in the light channels settings.

A second approach is to evaluate manually the optimal focal plane inserting the height of the planes for every channel with a trial and error procedure. A value of height is inserted in the appropriate box and is taken a snapshot of one field inside the well: if the objects are not well focused another value is inserted and it is taken another picture. The process continue until the object of interest is well focused and the height correspond to the optical focal plane. This procedure is done for every light channel.

When the optimal focal planes for every channel has been determined, it is possible to start the analysis.

2.3.1.7 Temperature and CO_2 controls

Temperature and CO_2 concentration have been controlled during the measurements thanks to a live chamber present in the instrument that allow to keep the temperature and CO_2 concentration of 37°C within a range of ±1°C and a CO_2 concentration of 3 – 8.5 within a range of 0.5%. The live chamber stable temperature is reached after 45-90 min depending on the environmental temperature. At 22°C it will take approximately 1h, instead a value of 5% of CO_2 concentration is reached after 5 min. To perform the experiments the instrument has been set up in advance and the analysis has started when the constant temperature and concentration have been reached.

2.3.2 Images analysis

In this step has been used the imaging analysis software integrated in Operetta™ adapted to the samples that have been analysed. The building blocks that give the image analysis get as input the segmented image where it is possible to select the different light channel based on the type of properties that have to be calculated and they give as output the desired properties. Starting from an input image, the following building blocks have been selected:

- *Find Nuclei*. The nuclei stained by Hoechst are detected.
- *Find Cytoplasm*. The software recognizes the cytoplasm stained by the Calcein AM around the Nuclei and identify a cell region (Nuclei + Cytoplasm).
- *Calculate Intensity properties*. The fluorescence intensity of Calcein AM, Hoechst 33342 and Ethidium Homodimer are calculated.
- *Select Population*. The cells that appear completely in the picture are kept to the further steps, the ones in the edge that are cut in the pictures are discarded.
- *Calculate Morphology properties*. The software calculates area, roundness, width, length, width over length ratio of the cell region selected in step "Select Population".
- *Select dead cells*. From the cells selected on step "Select Population" the dead cells are detected with the following criterion: Intensity of Ethidium Homodimer > background Intensity of Ethidium Homodimer in the negative control (0% DMSO concentration sample).
- *Define results*. The software allows to calculate new properties from the ones determined in the previous steps. For example, to describe the effect of the toxic agent DMSO the percentage of dead cells (D%) has been defined by the equation (2.6):

$$D\% = \frac{Number\ of\ Dead\ Cells}{Total\ Number\ of\ Cells} \cdot 100 \qquad (2.6)$$

  Where dead cells are the ones stained by Ethidium homodimer, detected in step "Select dead cells", and the total number of cells has been detected in step "Select Population". In addition, it has been evaluated how the DMSO change the morphological properties of the cells like area, roundness, width, length. The following outputs, calculated in the previous steps were selected to be exported from the software: the total number of cells selected, number of dead cells, percentage of dead cells, cytoplasm intensity (mean and standard deviation), cell roundness (mean and standard deviation), cell area (mean and standard deviation), cell length (mean and standard deviation), cell width (mean and standard deviation), cell width over length ratio (mean and standard deviation).

The evaluation step allows to calculate the properties selected with the building blocks in the image analysis for all the selected fields and wells selected in an assay. The wells where errors

on focusing of the images or where the images did not appear clear are discarded from the evaluation. The software calculates the properties selected in the building block for all the field and it makes a mean for every well. The results are expressed in a table where are reported the values of every properties calculated for the wells. In addition, it is possible to select graphs, bar chart, scatter plots where it is possible to combine the different properties calculated to express the results. The data have been exported in .txt file and processed with Matlab™ and Excel™. Furthermore, in this step the images acquired of the chambers were saved in .png file.

Chapter 3

Results and discussion

This chapter shows the progressive steps and the results obtained along the development of a microfluidic culture platform compatible with a high-throughput imaging instrument for screening, namely Operetta®.

3.1 Standard procedure for high-throughput screening imaging

First, we describe the conventional method to perform high-throughput imaging with Operetta when using conventional multi-well plates for cell culture. In order to have a clear and robust readout throughout the development of the microfluidic platform towards the interfacing with the high-throughput instrument we set up a practical screening case study based on a toxicity. For these reasons human skin cells (fibroblast, HFF-1) have been chosen to prepare the assay because they are a kind of cells easy to culture and to expand both in multi-well plate and microfluidic platform. The dimethyl sulfoxide (DMSO) was used as toxic agent due to its tolerance for cell viability at low concentration where has been reported in literature that for concentrations below 1% (volume/volume in Dulbecco's Modified Eagle's medium (DMEM) the agent is not toxic generally for most mammalian cell type (Singh M. et al, 2017), instead it results toxic causing cell mortality at a concentration(volume/volume in DMEM, of 10%. Furthermore, the DMSO have the advantage to generate toxic effects on cells in a short time (24 hours), changing the morphology of the cells (area, length, roundness) at first and cell death later. The concentrations of the assays have been chosen in a range between 1.25% and 10% of DMSO, respectively 1.25%, 2.5%, 3.5%, 5%, 10% in order to evaluate the effects of the toxicity where at low concentration it is expected only a low percentage of dead cells and overall a low effect of the toxic agent on the morphology of the cells, while increasing the concentrations it is expected that the percentage of dead cells start to rise and the morphology of the cells starts changing too. At a concentration of 10% of DMSO it is expected an elevated mortality of the cells and a huge modification on the morphology of them.

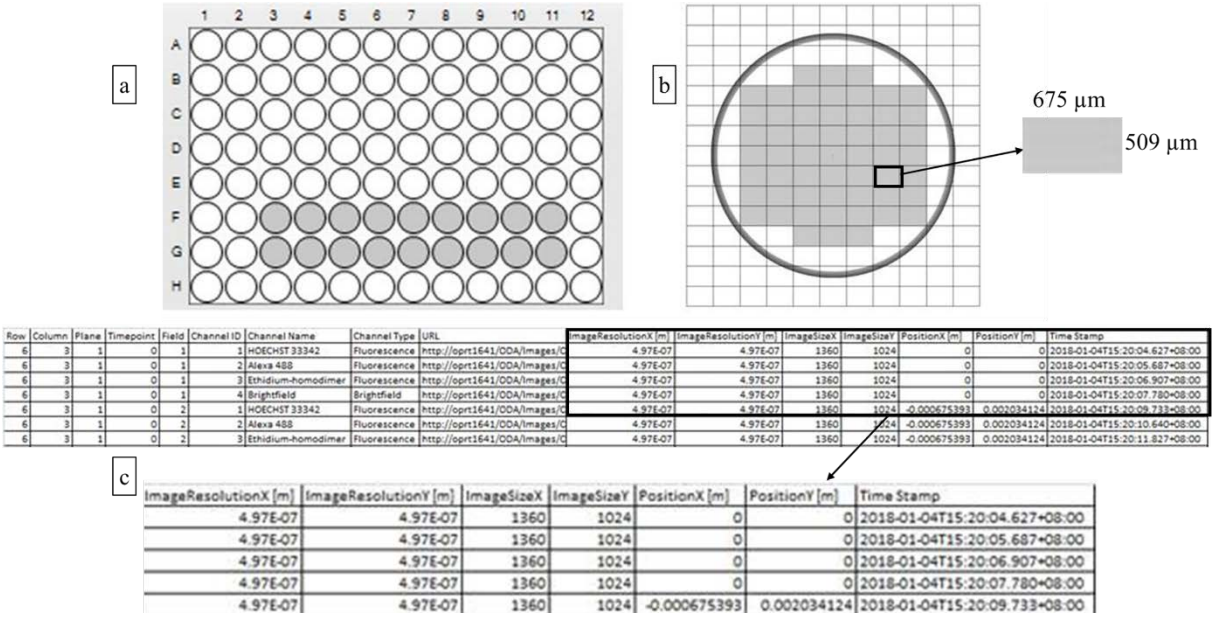
First of all, the high-throughput screening instrument has been set up with the optimal characteristic for the acquisitions of the test multi-well plate in terms of plate type dimension, type of objective, number and type of light channels based on the type of dyes used in the assays, field and well selections, time of acquisition as described in section 2.3.1. In parallel a toxic assay has been developed starting from the cells culturing step in a conventional 96-well

glass bottom plate, then adding toxic solutions to the samples made by DMEM and DMSO and at the end preparing the immunofluorescence staining to label the live and dead cells after the treatment as reported in section 2.2. This kind of multi-well plate guarantees high performance imaging due to the good optimal properties and low thickness(0.17mm) of the bottom glass compared to the common polystyrene bottom plates. Also, the black colour of the lateral walls of the wells guarantees no interference of signal between wells and minimize the reflection of light when it hits the sample.

The measurements have been performed at constant temperature of 37°C and constant concentration of CO_2 equal to 5% in air (volume/volume) for pH control.

3.1.2 High-throughput screening acquisition

The pictures of the immunofluorescence assay done in a 96-well plate are acquired with a 20X long working distance (WD) objective using the high-throughput screening instrument(Operetta®). The selection of the wells and the fields inside them where to acquire the pictures of the samples is reported in Figure 3.1.



Row	Column	Plane	Timepoint	Field	Channel ID	Channel Name	Channel Type	URL	ImageResolutionX [m]	ImageResolutionY [m]	ImageSizeX	ImageSizeY	PositionX [m]	PositionY [m]	Time Stamp
6	3	1	0	1	1	HOECHST 33342	Fluorescence	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:04.627+08:00
6	3	1	0	1	2	Alexa 488	Fluorescence	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:05.687+08:00
6	3	1	0	1	3	Ethidium-homodimer	Fluorescence	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:06.907+08:00
6	3	1	0	1	4	Brightfield	Brightfield	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:07.780+08:00
6	3	1	0	2	1	HOECHST 33342	Fluorescence	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	-0.000675393	0.002034124	2018-01-04T15:20:09.733+08:00
6	3	1	0	2	2	Alexa 488	Fluorescence	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	-0.000675393	0.002034124	2018-01-04T15:20:10.640+08:00
6	3	1	0	2	3	Ethidium-homodimer	Fluorescence	http://oprt1641/ODA/Images/C	4.97E-07	4.97E-07	1360	1024	-0.000675393	0.002034124	2018-01-04T15:20:11.827+08:00

ImageResolutionX [m]	ImageResolutionY [m]	ImageSizeX	ImageSizeY	PositionX [m]	PositionY [m]	Time Stamp
4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:04.627+08:00
4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:05.687+08:00
4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:06.907+08:00
4.97E-07	4.97E-07	1360	1024	0	0	2018-01-04T15:20:07.780+08:00
4.97E-07	4.97E-07	1360	1024	-0.000675393	0.002034124	2018-01-04T15:20:09.733+08:00

Figure 3.1 *a) Wells selection in the navigation panel of Harmony® software; b) fields selection inside the well in the navigation panel of Harmony® for an assay with acquired with a 20x objective (field of view 675×509 µm); c) tables generated by the software where each picture acquisition conditions are detailed*

The fields inside the well (figure 3.1b) were selected at the centre and close to the edge to have a better statistical result in the further evaluations and to consider the different distribution of the cells in every single well.

The samples are stained with Hoechst 33342 to identify the nuclei, calcein acetoxymethyl (AM) to identify the live cells and ethidium homodimer to identify the dead cells as reported in section 2.2.3 and the pictures of the samples taken after the treatment with DMSO for 24 hours are reported in Figure 3.2. Some technical difficulties were encountered during the staining of the control samples (untreated with DMSO). For this reason, the control sample are not shown as it was not possible to build up an accurate image analysis.

A first aspect that is possible to notice in the pictures stained by Calcein AM showed in Figure 3.2 is that a concentration of 1.25% of DMSO does not affect the shape of the cells because they keep the classic elongated shape that characterizes fibroblast cells. In the pictures of the staining with Ethidium homodimer, the dye marking the dead cells, the orange points start rising while the DMSO concentration increase from 1.25% to 10% shows the expected growth of mortality due to the effect of the toxic agent in the samples. Another aspect that is possible to notice is the change of the morphology of the cells from low to high concentration of the toxic agent, in particular between 5% and 10% where the DMSO act by changing the shape of the cells and its effect on the morphology appears to be more evident. The images acquired at 10% of DMSO appears a bit unfocused because the samples are acquired in a fixed focal plane and when the cells are dead they detach from the surface and it is not possible to focus them during the measurement. As a consequence, the final evaluation of the area, length and roundness that give an indication of the morphology of the cells at this concentration of DMSO was difficult because it was complicate by software to recognize the edge of the single cells and to make the calculation.

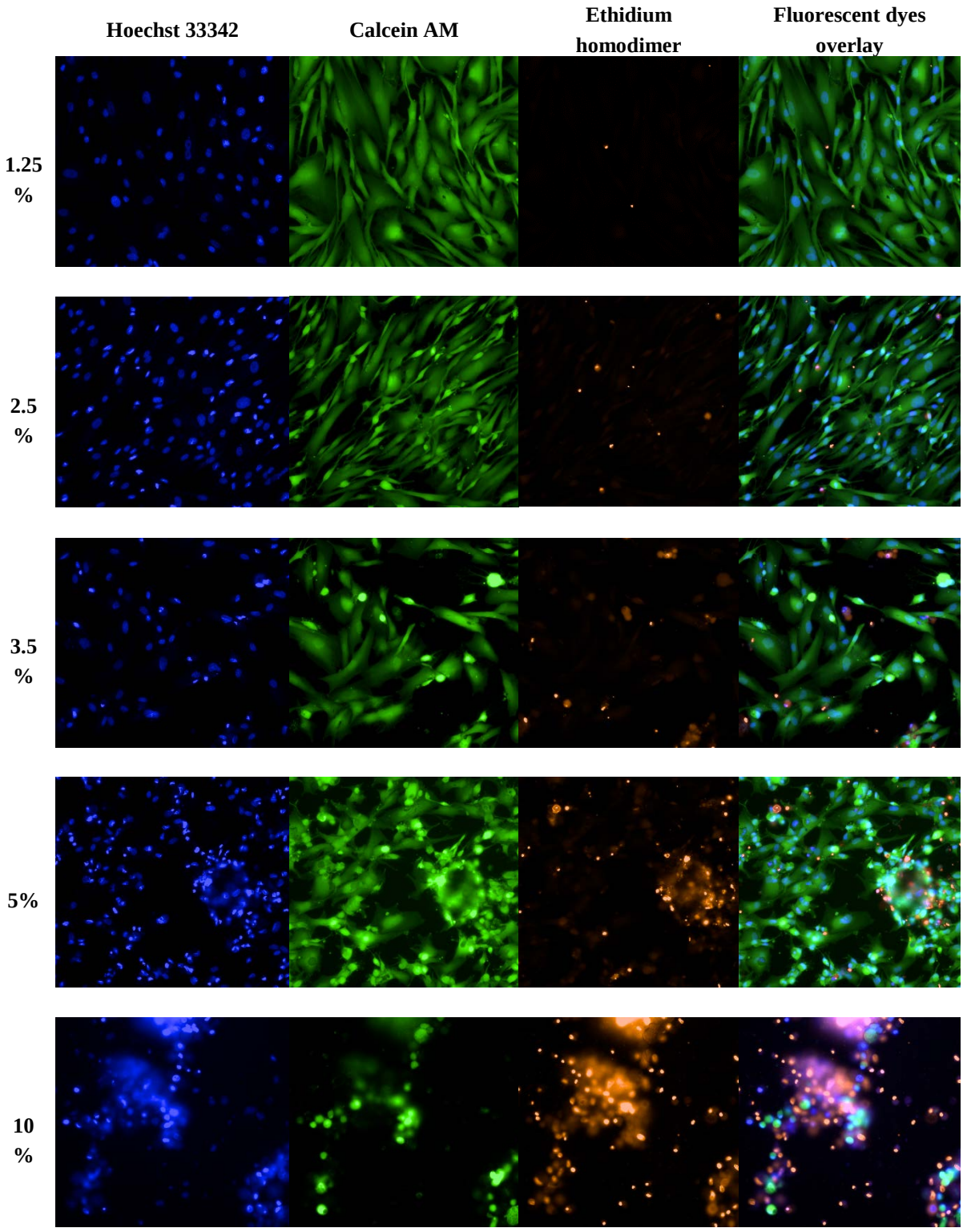


Figure 3.2 *Immunofluorescence assay of a 96-well plate after treating with DMSO at 1.25%,2.5%,3.5%,5% and 10% (volume/volume) for 24h acquired at 20x of magnification using Operetta®. Nuclei are marked in blue with Hoechst 33342, live cells are marked in green with Calcein AM and the dead cells are marked in orange with Ethidium homodimer. In the last columns is reported the fluorescent dyes overlay.*

3.1.2 Image analysis sequence

After the acquisitions of the assay an image analysis procedure has been set up to evaluate the assay faced in the high-throughput screening instrument. The protocol to build up the image analysis sequence in Harmony® software is described in Appendix A.3.2 while the detailed explanation of how the images are segmented and analyzed is reported in section 2.3.2. Below in Figure 3.3 it is showed an example of how the image analysis of a sample has been implemented for a sample treated with 3.5% of DMSO. Dead cells are selected between the ones that show an intensity of ethidium homodimer higher than 500, value determined analyzing the samples with a low concentration of DMSO equal to 1.25% where the toxicity effect of the agent on the cells was low to cause cell death and the average intensity of the ethidium homodimer that have been measured represented the background signal of the dye.

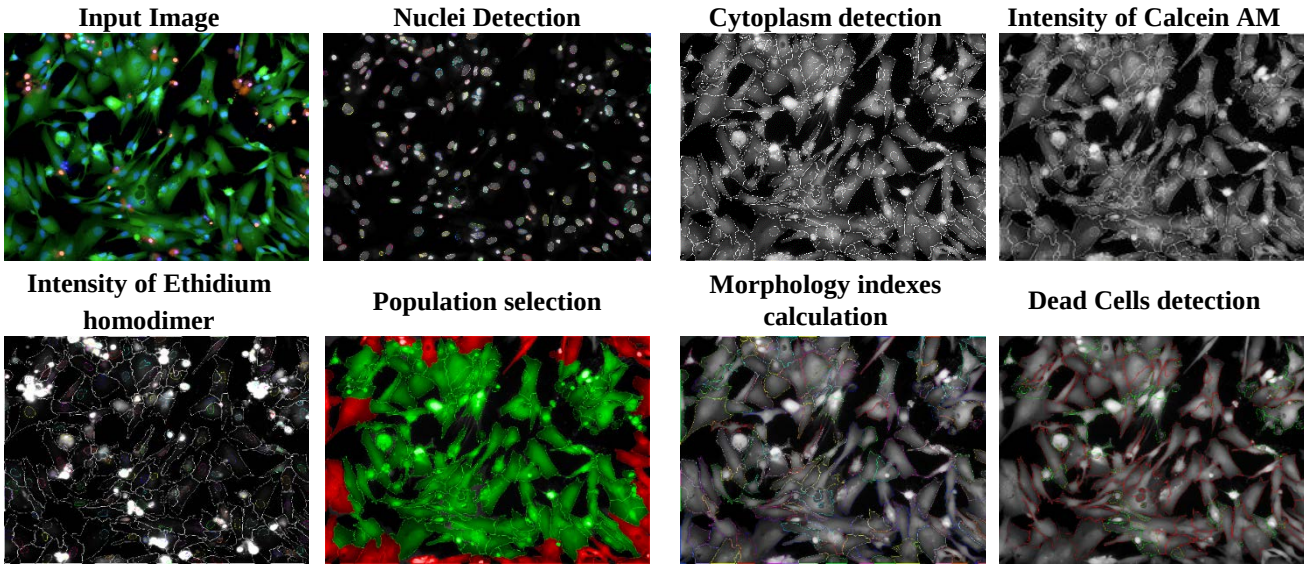


Figure 3.3. *Image analysis sequence done in a sample at 3.5% of DMSO. From top-left the order: Input Image, Nuclei Detection, Cytoplasm detection, Intensity of Calcein AM calculation, Intensity of Ethidium homodimer calculation, Population selection, Morphology indexes calculation, Dead Cells detection.*

3.1.3 High-throughput screening evaluations

When the software detects the cells in a field of the well, it calculates the properties selected in the image analysis step for every single cell detected. Before to run the evaluation for the entire assay based on the image analysis showed in Figure 3.3, it has been considered as example a field of a well treated with DMSO at concentration equal to 3.5% to show how the properties of the single cells are distributed. In particular the probability density function of the intensity of calcein AM, the cell area and the cell roundness have been plotted in Figure 3.4a for the field selected. It is possible to see that in this particular case roughly 14 cells have an area equal to 1000 μm^2 and a roundness about 0.4. These values for the properties selected gives an idea of

how the effect of the DMSO at 3.5% of concentration on cell morphology. Doing this first evaluation for different fields in a well it is possible to have statistical significance of the final result for the well. Then a comparison between the same properties related to single cells detected in samples treated at concentration of DMSO from 1.25% to 10% is done calculating the cumulative distribution function it is showed in Figure 3.4b. This comparison between different condition it is useful to understand whether the parameters to calculate the cell properties have been selected properly or if it is necessary to tune them to improve the final results for the wells.

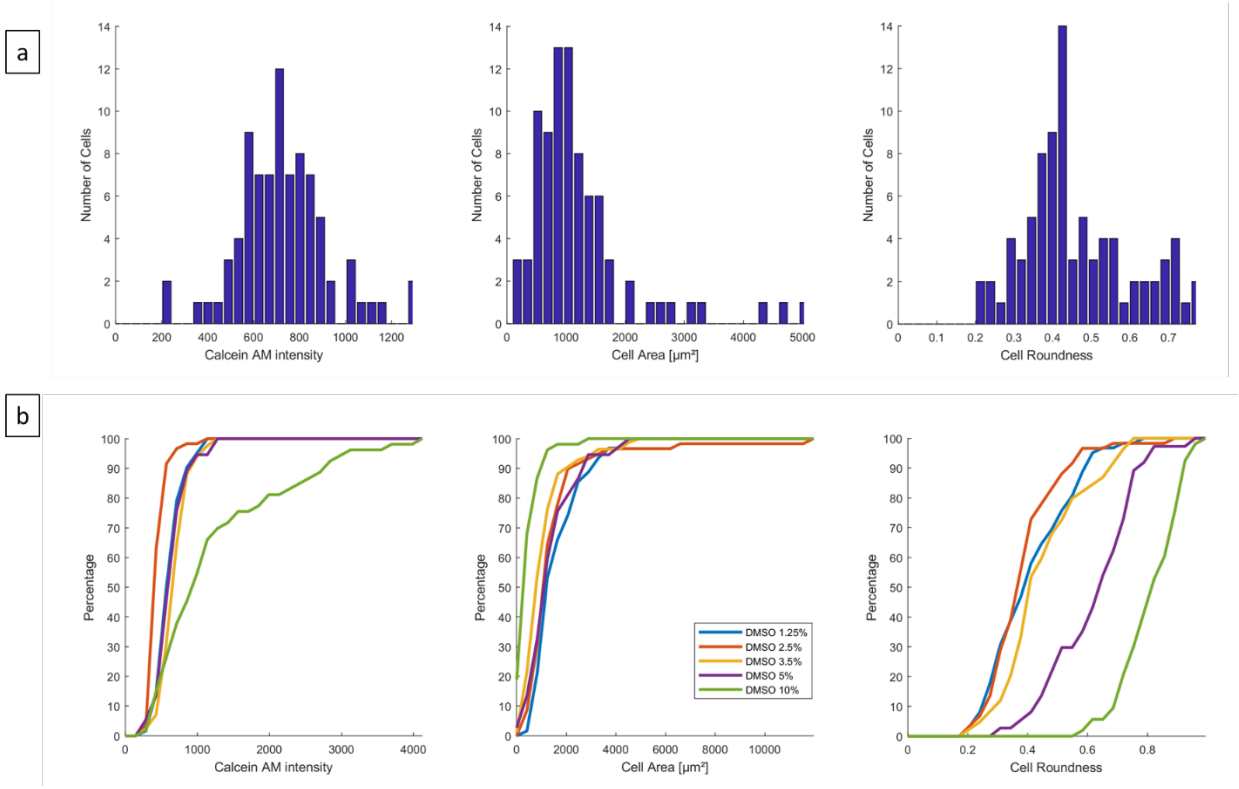


Figure 3.4 *a) probability density functions of the Calcein AM intensity, cell area and cell roundness for a field in a sample treated with DMSO at 3.5% of concentration; b) cumulative density function of Calcein AM intensity, cell area and cell Roundness at different DMSO concentration: in blue is reported the condition at 1.25%, in orange the one at 2.5%, in yellow the one at 3.5%, in purple the one at 5% and in green the one at 10%.*

The quantification of the effect of the DMSO on cell viability and morphology indexes (area, length, and roundness) of the entire assay is reported in Figure 3.5. For every cell properties measured a one-way analysis of variances (ANOVA) have been performed to show significant differences in each properties in the sample as function of different concentration of DMSO.

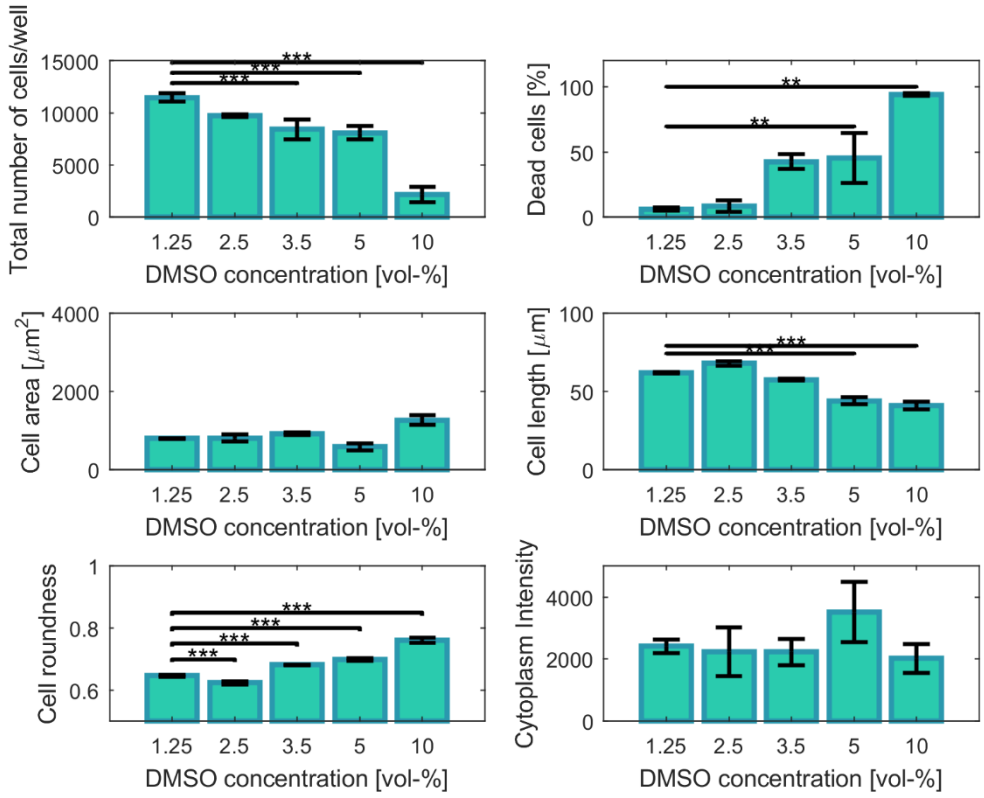


Figure 3.5. *Data evaluation related to an immunofluorescence assay of fibroblast cells performed in a 96-well plate (Cellvis). From the top left are displayed in order the bar plots of total number of cells per well, percentage of dead cells, cell area, cell length, cell roundness and cytoplasm intensity in function of the DMSO concentration [volume/volume]; number of replicates n=2; The symbols (∗∗) means a p-value<0.01 and (∗∗∗) means a p-value<0.001.*

As reported in Figure 3.5, total number of cells per well decrease when the concentration of the toxic agent is increased because of the toxic action of the DMSO. In particular over 10000 cells per well are present in the sample treated at 1.25% of DMSO and the number is decreased below 5000 at 10% of DMSO concentration. Furthermore, the percentage of dead cells increase with the concentration of DMSO. This result shows the low toxicity of the DMSO at low concentration and the effect is relevant when the concentration is increased, reaching a percentage of cell death close to 100%. Regarding the area of the cells it does not change a lot with the concentration of DMSO with a value below 2000 μm^2 for all the conditions. Instead the length of the cells overall decreases when the concentration of DMSO is increased: this behavior proves what is showed in Figure 3.2 in the pictures related to the Calcein AM where the length of the cells is reduced when the DMSO concentration increase, especially in the range of concentration between a 3.5% to 10%. Cell roundness that is a morphological index that indicate a variation of the cell shape. If it is reached a value of 1 a perfect circle shape is reached. As it possible to see the result of cell roundness in Figure 3.5, for all the samples the roundness is higher than 0.5 for all the toxic agent concentration and this means that the cells in the multi-

well environment tends to have a round shape instead of expected extended shape. Furthermore, the increase of DMSO concentration shape the cells rounder. Overall these results show that the toxicity assay conditions were set properly to be meaningful when used as a readout during the microfluidic platform development.

3.2 Main constraints of the imaging platform

Microplates suitable for high-throughput screening measurements are commonly made of transparent polystyrene. The bottom of the plate could be made of polystyrene treated for tissue-culture or glass with good optical properties that ensure an optimal acquisition of the images in the platform in terms of compatibility with the objectives, the plateholder and the capacity to focus the samples.

A first constrain that the plates have to respect is their external dimensions compatible with the ones indicated by the American National Standards Institute (ANSI) reported in Table 3.1, the organism that has standardized the microplate external dimensions to make them compatible with different kind of high-throughput screening instruments. This constrain allow to have a standard frame that ensure a reading of the imaging platform with the advantage to work with a configuration where the external dimensions are fix and they are already implemented in the software and instrument settings.

Table 3.1. *External standard dimensions of the microplate according to American National Standards Institute (ANSI). (ANSI SLAS 2-2004).*

External Dimension	Value
Length	127.8 mm ± 0.5 mm
Width	85.5 mm ± 0.5 mm
Height	14.35 mm ± 0.25 mm

However, these dimensions are not the only limitations when plates are interfaced with Operetta®, others will be discussed next: high thickness of the bottom plate, possible autofocus failure when two different material are overlapped on top of each other and the possible hit of some type of objectives with the plate bottom.

3.2.1 Thickness of the bottom plate

The plate bottom should be as thin as possible to take into account the additional thickness of the glass of the microfluidic platform. The bottom plate thickness affects the choice of objective and the planarity of the bottom, in combination with the material that is it made the plate. For example, the thickness of the glass that make the microfluidic platform is 1 mm and the thickness of a common polystyrene plate is 1 mm thus the total thickness of the bottom plate

when the microfluidic platform is inserted in a polystyrene plate becomes 2 mm. This thickness does not allow using objectives with high numerical aperture (NA), which however provide images with better resolution, due to their low working distance. Bottom thickness of plates tolerated to use an objective high NA are below 200µm and since the total bottom thickness of the system is 2mm only objectives with long working distances are compatible.

3.2.2 Autofocus failure

When a polystyrene plate overlapped with a glass plate are interfaced together to an objective the laser based autofocus unit cannot detect the samples even though the bottom thickness of the plate made by the microfluidic platform glass thickness and the plastic bottom of the plate have been provided correctly in the software. The reason of this problems comes from the working principle of the laser based autofocus system implemented in Operetta®: it detects the different reflection of the laser beam at surfaces due to the different refractive index of the material (Figure 3.6a). A first peak is detected in the interface between the air and the plate bottom and a second peak is detected in the interface between the glass and the buffer. The second peak is the essential reference point for focusing of the samples in order to identify the cells in every location in the well at exactly the same focus height independently on the possible changes on the height of the bottom plate. The focus height defined by the user is calculated from this second peak detected. In Figure 3.6b it is showed the case when two different material, particularly glass and polystyrene are interfaced to an objective. In this case the second peak of the laser beam reflection corresponds to the interface between the glass and the plastic and not the interface between the buffer and the glass causing failures in the focusing of the samples. The algorithm implemented in the autofocus unit works knowing the plate bottom height and thickness and even setting up in the plate definition only the thickness of the plastic, disregarding the thickness of the glass, the software displayed errors focusing the samples. For the reasons explained above it is not possible to couple a plastic plate where is placed in the bottom surface a glass plate because the different materials in which the system is made cause errors in the autofocusing of the samples.

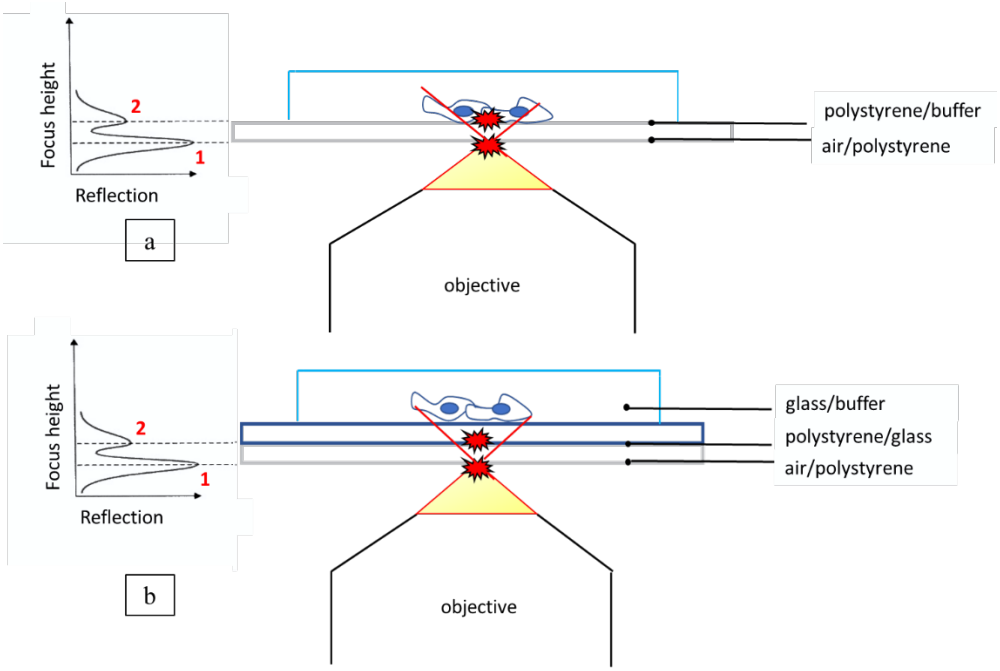


Figure 3.6. *Working principle of the autofocus unit implemented in Operetta®. a) Representation of a plate made by polystyrene interfaced to an objective: the first star corresponds to the first peak of the laser beam reflected at the air/polystyrene interface and the second star corresponds to the second peak of the laser beam reflected at the glass/buffer interface. b) Representation of a polystyrene plate overlapped by a glass plate interfaced to an objective: the first star corresponds to the first peak of the laser beam reflected at the air/polystyrene interface and the second star corresponds to the second peak of the laser beam reflected at the interface between glass and polystyrene. (Operetta High-Content Screening Manual)*

3.2.3 Objective collision

Not all the objectives are compatible with the imaging platform. An important parameter that make feasible or not the selection of an objective for a specific measurement of a platform is the bottom height of the plate. High bottom plate over 300 µm have a high probability of measurement area limitations when objectives with high NA are used, caused by the short working distance of the objectives that can hit the scan table or the plate's ribs (Figure 3.7).

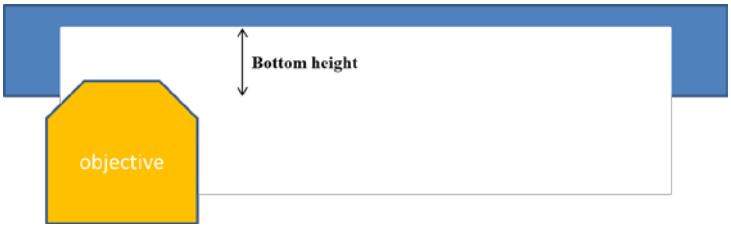


Figure 3.7 *Example that represent the collision of the objective with the plate rib.*

3.2.4 Frame for high-throughput screening of microfluidic platforms

To interface the microfluidic platforms in the high-throughput screening instrument and taking into account all the limitations in the applications of the imaging platform encountered and explained from section 3.2.1 to section 3.2.4, a frame has been designed to analyze optimally the microfluidic platforms in high-throughput way when they are faced in Operetta®.
As explained in section 2.1.5 from a commercial one-well the bottom has been cut and the geometry of the final frame is showed in Figure 3.8.

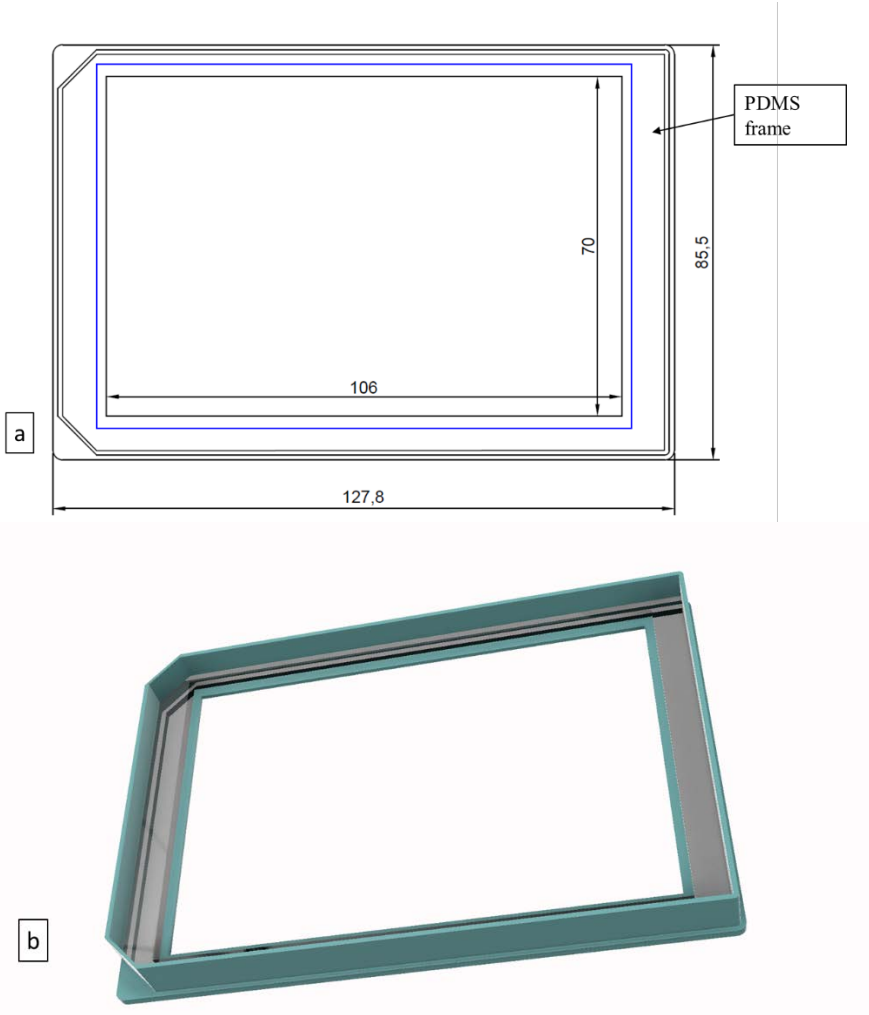


Figure 3.8. *Frame from one-well plate: a) top view. Dimensions are reported in [mm]; b) prospective view. In gray is colored the PDMS frame and in cyan the polystyrene frame.*

A PDMS frame has been added to the one-well frame in order to keep the microfluidic platform in a fix position during the measurement. The bottom height and bottom thickness have been measured with a calibre. In Table 3.2 are reported the main dimensions of the frame.

Table 3.2 *Dimensions of the designed frame for high-throughput screening of microfluidic platforms*

Dimension	Unit of measurement	Value
Length	mm	127.8
Width	mm	85.5
Height	mm	14.4
Bottom height	mm	1.68
Frame bottom thickness	mm	1
Detectable surface	mm^2	106×70

The frame respects the standard external dimension according to ANSI as reported in Table 3.1 in terms of length, width and plate height, guaranteeing an easy readout of the microfluidic platforms faced in the high-throughput screening instrument where the user has only to provide the correct dimension of the culture chambers of the microfluidic platform and distances of them from the sides of the frame. To avoid the focus failure explained in section 3.2.2 the one-well plate bottom has been cut creating a free detectable surface where the objective interface only with the glass of the microfluidic platform. In this manner the bottom plate becomes the glass of the platform and the autofocus unit is able to detect the cells attached in the surface without errors. The plastic frame left at the bottom can support the microfluidic platform glass in both the configurations described below (76×26 mm and 110×75 mm). However, the limitation of the objective collision was not overcome due to the geometrical characteristics intrinsic to the one-well plate. As a consequence, due to the high bottom plate of the frame (higher than 0.2 mm) it is not possible to work with high numerical aperture objectives that can hit the rib of the bottom plate and only long working distance objectives can be selected for the analysis in the detectable surface.

Furthermore, it is not possible to do live-cells imaging because the frame cannot guarantee a sterile environment: the microfluidic platforms have to be coupled manually with the frame moving the platform from the sterile vessels to the platform and in this passage, sterility is compromised. The frame cannot be sterilized in autoclave because the temperature reached by the process(120°C) is higher than the glass transition temperature of the polystyrene (around 100°C) and the frame can be deformed. In Table 3.3 the main features and limitations of the frame are summarized.

Table3.3 *Features and limitations of the one-well frame that hold the microfluidic platforms*

Features	Limitations
Standard external dimension according to ANSI	High numerical aperture objectives cannot be used
Compatibility with autofocus	Autoclave sterilization not compatible.
Compatibility with microfluidic platform having different dimensions	Sterility maintenance not guaranteed.
Compatibility with long working distance objectives	

3.3 Microfluidic platforms and image acquisition: software constraints

The development of a microfluidic platform to be interfaced with the imaging instrument started with a prototype that past studies done in Shanghai Institute of Immunochemical Studies(SIAIS) laboratory have found that from a biological point of view has the necessary proportion to create a confined cell culture environment where it is possible to study relevant processes of cellular self-regulation. In particular the context of pluripotent stem cells will regard the final subject of the optimized high-throughput screening (HTS) developed during this thesis work.

Harmony® is the software integrated in Operetta®. It is automatized, and it has in its database the settings of many commercial plates. Thus, we first started adapting the microfluidic platform to have geometrical dimensions compatible with those already registered in the software. Fitting the culture chambers of the microfluidic platform in a well simplify the acquisition of the samples thanks to the software that gives the possibility to select specifically different regions of interest (ROI) inside a well in order to identify the culture chambers or part of them. For these reasons a 6-well plate has been taken as reference and modifying in the software the dimensions of the bottom height and thickness with the ones of the one-well frame reported in Table 3.2. In order to maximize the number of detectable culture chambers in a microfluidic platform to provide more replicant for the analysis to improve the accuracy of the final results, five chambers have been fit in each well for a total number of chambers equal to thirty in the platform. A glass plate 110×75 mm has been used to assembly the microfluidic platform in order to have a fix position of the culture chambers once they are faced to the instrument. The configurations designed is showed in Figure 3.9

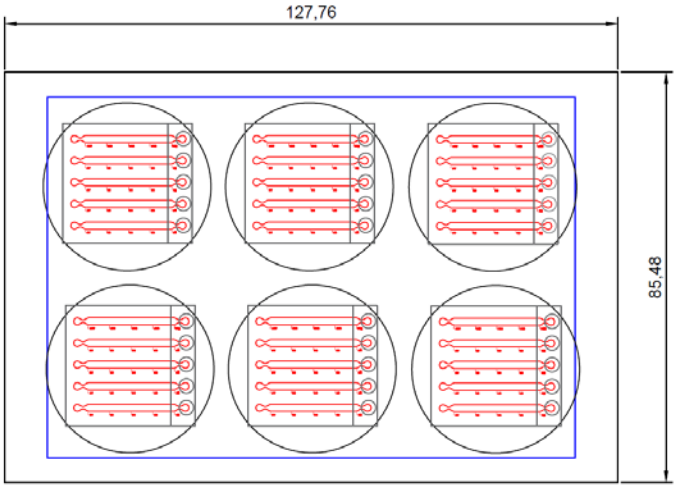


Figure 3.9. Configuration of a microfluidic platform adapted to a 6-well plate. In blue the glass plate of the microfluidic platform is marked, in red the culture chambers are labeled and in black the 6-well reference plate is highlighted. Dimensions are reported in [mm].

In this assay a toxicity test at different DMSO concentration has been developed followed by an immunofluorescence test where the samples were stained as described in section 2.2.2 in order to acquire the images of the culture chambers and analyze them using the same image analysis sequence used in the case study of section 3.1. Experiment time to analyze the entire length of all the culture chambers of the platform was very long. To speed up the acquisition of the pictures only the central part of the culture chamber almost 6 mm long has been analyzed and evaluated. This configuration gives problem on the evaluation of the single culture chambers of the platform: Harmony® software is designed to evaluate a specific property defined in the image analysis sequence of each image acquired inside the well and then making the average of the property for the entire well. The user has to select the ROI inside the well and the wells of the platform where to make the evaluation. The position of the ROI selected is the same for all the well of the platform and the software does not allow to customize this selection for every of the platform. Inside a single well five culture chambers are fitted and they are represented by the ROI selected. When the evaluation is performed in this configuration the software gives as output the mean values of the properties related to the wells. In this case the results of each single culture chamber inside the wells are taken into account in the mean calculation of the property of the well, so the value that come out from this evaluation is meaningless.

Since one aim of this work was to develop a microfluidic platform suitable for high-throughput screening completely legible and evaluated using the tools of Harmony® software, this microfluidic platform does not allow an easy evaluation of the culture chambers. However, there are two ways to overcome this limitation of the software to obtain meaningful results:

1. Evaluating the desire property for every single object detected by the software in every single image that compose the culture chambers. Higher is the number of properties selected and higher is the computational effort in terms of time of evaluation. Then all the data have to be exported and rearranged with a third part software (Matlab®, Excel, etc);
2. Evaluating the desire properties selecting a single region of interest that corresponds to a culture chambers in one well; then selecting the other five wells this ROI selection is kept at the same position. In this case the mean value of the properties that the software gives as output is related on the culture chambers at the same position in the wells. Repeating this procedure selecting one by one the ROI that correspond to the five culture chambers inside the well is possible to have a meaningful readout of the assay. Data can be exported and manage with a third part software (Matlab®, Excel, etc);

Following the second approach it was possible to obtain an evaluation of area, length, roundness, percentage of dead cells, number of cells per chamber and cytoplasm intensity of an immunofluorescence assay performed in this platform (Figure 3.10) as described in section 2.2 to see the effects of the DMSO in the cells. For every cell property measured, an ANOVA test have been performed to show significant differences in the mean property in the sample between different concentration of DMSO respect to the control (0% of DMSO concentration).

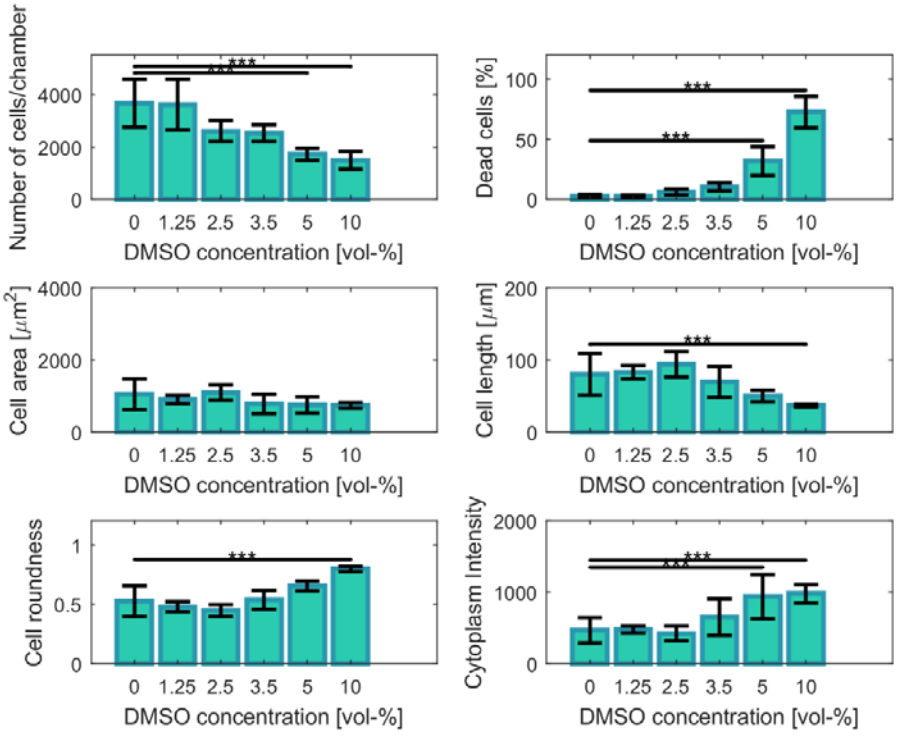


Figure 3.10. *Data evaluation of a microfluidic platform adapted to a 6-well plate. From the top left are displayed in order the bar plots of total number of cells per well, percentage of dead cells, cell area, cell length, cell roundness and cytoplasm intensity in function of the DMSO concentration [volume/volume]; number of replicates n=6; The symbols (∗∗∗) means a p-value<0.001.*

Results of the microfluidic platform adapted to a 6-well plate are different compared to the ones obtained for a 96-well plate (Figure 3.5). As a consequence, the variability of the results is higher (standard deviations are higher). However, the main results remain unchanged: while the total number of cells per chamber decrease with the DMSO concentration, the percentage of dead cells increase with the concentration of DMSO, the cell area does not vary significantly with the concentration of the toxic agent, the cell length decreases and cell roundness overall increases when the concentration of DMSO is increased. Cytoplasm intensity is also not affected by DMSO concentration as long as cell membrane is integer (at 10% DMSO the high cytoplasmic intensity is due to a general cell membrane damage that let the dye enter the cell)

3.4 Image analysis software constraints

The way how it works Harmony® software to evaluate the image analysis sequence over all platform explained in the previous section represents a limitation on the evaluation of the culture chamber: the approaches used to overcome the problem are not comfortable in the management of the data and the idea is to use the potentiality of the software to obtain meaningful results for every single culture chamber evaluated. For this reason, a configuration of a microfluidic platform ten chamber geometry was built taking as reference a 96-well plate where every culture chamber fit a single well as reported in Figure 3.11. A glass plate 110×75 mm has been used to assembly the microfluidic platform in order to have a fix position of the culture chambers once they are faced to the instrument. Each culture chamber of the microfluidic platform is treated like a single well of a multi-well plate and the evaluation of the image analysis sequence for the complete assay gives as output the mean value of the properties related to a single culture chamber.

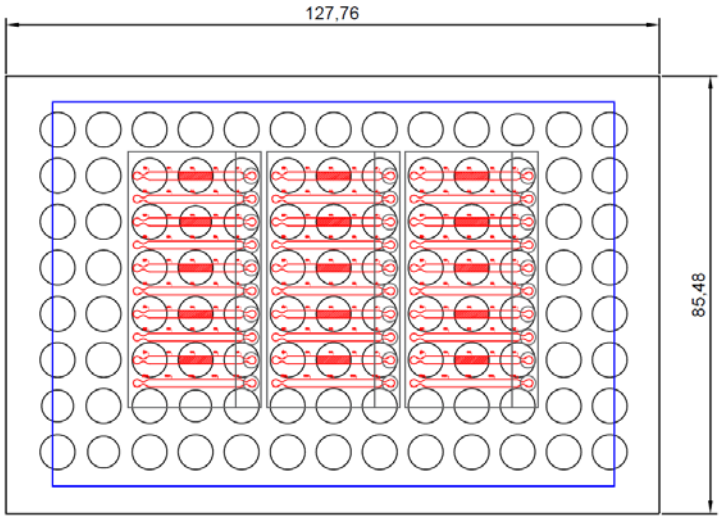


Figure 3.11. *Configuration of a microfluidic platform adapted to a 96-well plate. In blue the glass plate of the microfluidic platform is marked, in red the culture chambers are labeled and in black the 96-well reference plate is highlighted. Dimensions are reported in [mm].*

In this platform an assay has been developed for high-throughput screening where a toxicity test using DMSO at different concentration and an immunofluorescence working solution have been injected in the culture chamber as reported in section 2.2 to take the pictures to see the toxic effects of DMSO on cells.

Below in Figure 3.12 are illustrated the results of the evaluation done in this assay. An ANOVA test have been performed to show significant differences in the mean property in the sample between different concentration of DMSO respect to the control (0% of DMSO concentration).

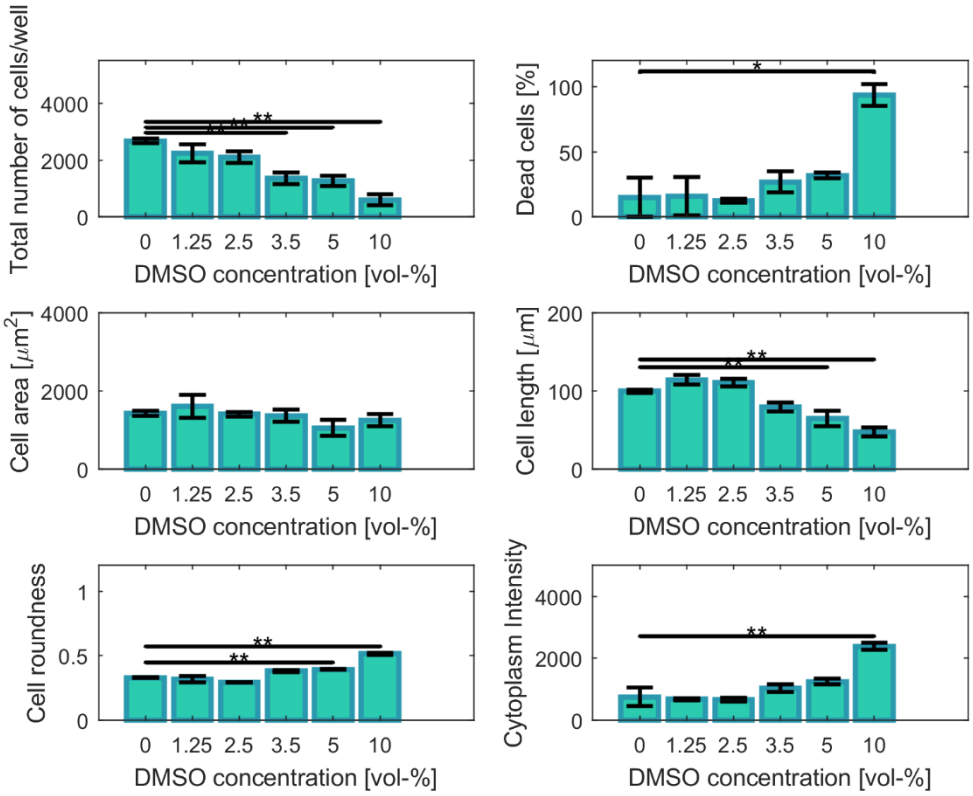


Figure 3.12 *Data evaluation of a microfluidic platform adapted to a 96-well plate. From the top left are displayed in order the bar plots of total number of cells per well, percentage of dead cells, cell area, cell length, cell roundness and cytoplasm intensity in function of the DMSO concentration [volume/volume]; number of replicates n=2; symbols (∗∗) means a p-value<0.0 and symbols (∗∗∗) means a p-value<0.001.*

Results obtained from this configuration are less accurate due to the low number of replicates that impose this configuration. Overall the mortality of the cells increases with the DMSO concentration until a percentage closer to 100%. The effect of the DMSO on the morphology of the cells are described by the results obtained by the morphology indexes: cell length decrease when the concentration of the toxic agent is increased with a marked effect between 2.5% and 10% of DMSO concentration where the length of the cells is halved; Cell roundness increase a bit with the concentration of the toxic agent but it the effect of the DMSO on the shape of the cells is lower compared to the result obtained in a microfluidic platform adapted to a 6-well plate.

Although this configuration was designed to overcome the limitation on evaluation of the microfluidic platform, as it is showed in Figure 3.11 there are some geometrical constrain that cannot be avoided:

- The length of a single chamber is too big to fit in a single well.
- It is possible to acquire the pictures only in a section of the chambers and it was decided to take the pictures in the central part of the chambers where the cells are not affected by the inlet and by the reservoir. A section of the chamber ~7mm long is acquired, although the chamber is 17-mm long
- Not all the ten chambers of the platform fit a single well due to the geometry of the ten chambers wafer used for the PDMS replica molding. The distance between culture chambers in the platform does not correspond to the distance between wells in the 96-well plate. For this reason, only 15 of the total 30 culture chambers are available to be detected and this consist in a big limitation on the number of replicates that is possible to perform for an experiment that brings in an inaccurate final evaluation of the result.

3.5 Optimal configuration

The idea to adapt the configuration of the microfluidic platform to a 6-well plate and to a 96-well plate shows some limitations of the platform in high-throughput screening application in Operetta®. Thanks to the previous tests done in the adapted platform it was possible to design a configuration suitable for high-throughput screening that was able to overcome the problem on the acquisition where only section of the culture chamber were available to be screened and regarding the evaluation where it was difficult to get meaningful result using the tools of Harmony® software. This platform is completely automatized from the acquisition phase of the pictures to the evaluation of the image analysis sequence using Harmony®. The design of the optimal configuration is reported in Figure 3.13

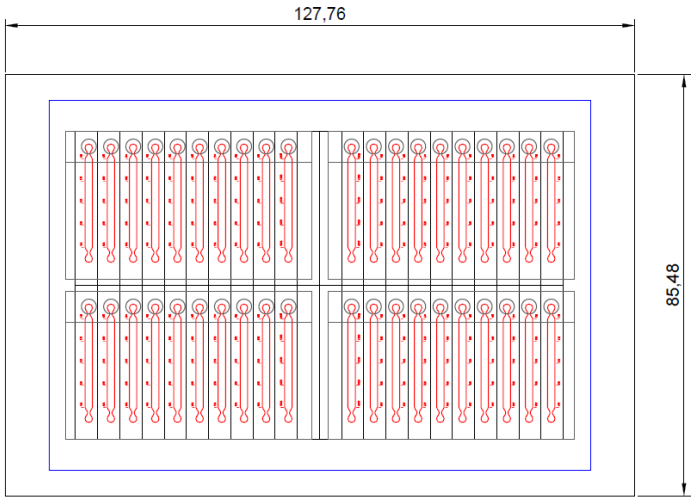


Figure 3.13. *Custom design of a microfluidic platform suitable for high-throughput screening. In blue the glass plate of the microfluidic platform is marked, in red the culture chambers are labeled and in black the custom grid is marked. Dimensions are reported in [mm].*

This microfluidic platform joins the advantages of the microfluidics platform adapted to a 6-well plate and to a 96-well plate. Furthermore, it has been maximized the number of culture chambers that is possible to acquire over a surface in a glass plate(110×75mm) up to 40 chambers. Moreover, the grid designed for the platform, labeled in black in Figure 3.13, is suitable to contain in each section a single culture chamber. This grid was designed to simulate in the software a multi-well configuration where each well correspond to a culture chambers in order to exploit the functionality of the software that is implemented by default to handle with commercial plates. The wells in this case are represented by the rectangles of the grid around the culture chambers.

The grid is composed by 2 rows and 22 columns and it is able to contain the 40 culture chambers of which the custom configuration of the microfluidic platform is made. Each single rectangle is 4.5mm long and 31.25mm wide and it is able to contain a single culture chamber. The main feature of this microfluidic platform is reported below (Figure 3.13):

- It is compatible with the high-throughput screening instrument when it is placed in the one-well frame and it is completely automatize.
- The position of the culture chambers ensures a clear acquisition avoiding that the objectives hit the rim of the one-well frame
- The assembly of the platform with the glass plate(110×75mm) allow to have the culture chambers always in the same position avoiding errors on the placement of the platform in the one-well plate and on the matching of the culture chambers using the software.

- A number of culture chambers equal to 40 ensure an adequate number of replicates in order to have statistically good result.
- The platform resembles a multi-well plate when it is interfaced in Operetta simplifying the acquisition of the culture chamber and the evaluation of the pictures taken.
- It is possible to select different section of the culture chamber or the entire length of the channel for the analysis.
- From a biological point of view the microfluidic platform assembled guarantee cells viability inside the chambers allowing the possibility to perform any kind of assay, especially the ones for high-throughput screening.

In the same way of the other two microfluidic platforms configuration the DMSO at different concentration have been injected in the culture chambers and then an immunofluorescence assay has been performed to quantify with Operetta® the toxic effect of the compound, as described in section 2.2

Below in Figure 3.14 are illustrated the results of the evaluation of the immunofluorescence assay done in this configuration. An ANOVA test have been performed to show significant differences in the mean properties in the sample between different concentration of DMSO respect to the control (0% of DMSO concentration).

Overall the behavior of the cells properties when the DMSO concentration increase is the same found in the microfluidic platforms adapted to a 6-well plate and to a 96-well plate. The percentage of dead cells increase with the DMSO concentration and the cell area and length decrease when DMSO concentration rise. Cell roundness increase due to the change of morphology of cells when the DMSO concentration increases and this means that the morphology of the cells is changed. In this configuration the DMSO decrease the area of the cells when the concentration of DMSO is increased, as it is possible to see in Figure 3.14 where the cell area is halved. Also, cell length change when the concentration of DMSO is increased, in particular between 5% and 10% of DMSO concentration. Overall, the results suggest the suitability of this microfluidic system for screening purposes.

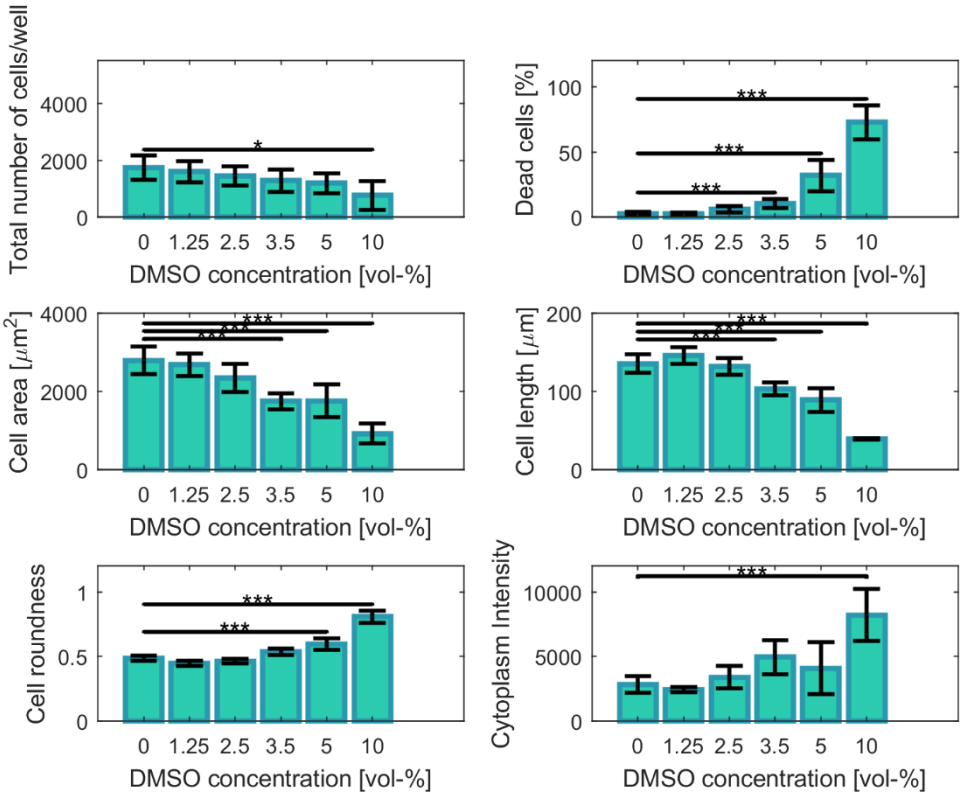


Figure 3.14 *Data evaluation of a microfluidic platform with custom configuration. From the top left are displayed in order the bar plots of total number of cells per well, percentage of dead cells, cell area, cell length, cell roundness and cytoplasm intensity in function of the DMSO concentration [volume/volume]; number of replicates n=6; symbols (∗∗) means a p-value<0.0 and symbols (∗∗∗) means a p-value<0.001.*

3.6 Decision-making suggestions

The main advantage that the optimal configuration of the microfluidic platform brings for high-throughput screening purposes is that allow to analyse an assay in Operetta® in the same way of a commercial multi-well plate using the features of Harmony® software depending on the type of experiment that have to be performed. In order to perform correctly and efficiently a high-throughput screening with this optima configuration some aspect need to be consider. The first one is the time of experiment. For example, in some assays long experiment times can lead to a degradation of a fluorophore that do not allow the detection with the instrument of the particular target molecule stained by the marker compromising the analysis. The main factors of which time of experiment depends are listed below:

1. type of objective selected for an acquisition
2. number of fluorescent channels selected for the experiment
3. number of plane to acquire for a single field
4. total number of field and wells selected

Regarding the type of objective selected, it depends by the type of component that one wants to investigate but generally higher is the magnification and higher is the resolution of the image but lower is the field of view: to take pictures of a surface of a sample the time of imaging is higher if it is selected an objective with low field of view. The features of the objectives compatible with Operetta is reported in Table 2.7 in section 2.3.1.3. The number of light channels selected depends on the type of analysis performed in the high-throughput screening instrument. Generally, at least two fluorescent channels are required to detect the nuclei of the cells and the cytoplasm to identify a cell region. Increasing the time of acquisition for every single channel increase the time of experiment: it should be maintained as low as possible to capture the cells in similar conditions from beginning to end of acquisition. The instrument allows to acquire many pictures of a single field in z-coordinate thanks to the z-stack tool. Even though the z-stack is a useful tool to obtain 3D information from a cell sample and it increases the dept of focus, it increases the experiment time by a factor proportional to the number of planes selected. For this reason, it could be used in cases when only few images of samples are required. The total number of wells and field selected for the images acquisition depends on the type of platform that is used. Table 3.4 shows the time required to acquire a surface of a 96-well plate using a 10X ling working distance objective and a 20X long working distance objective for different fluorescent channel. To cover a surface of well equal to 32mm2 a number of fields equal to 24 are necessary using a 10X objective, instead using a 20X objective 94 fields are necessary to cover the entire well surface

Table 3.4 *Time requirements for imaging the surface of one well of a 96-well plate at the default acquisition time of 200ms for every channel (32 mm^2 total surface,24 fields at 10X long working distance(WD) objective, 94 fields at 20X long working distance(WD) objective)*

		Type of objective	
		10x long WD	**20X long WD**
Time for imaging (min)	**1 channel**	3	5
	2 channels	6	7
	3 channels	7	9

Usually the field are selected at the centre of the well and possibly far from the areas that are affected by liquid handling and it is possible to reduce the field selected to reduce the time of experiment.

Regarding the acquisition of the microfluidic platform, even though the surface is lower to cover compared to the one of a 96-well plate ($26mm^2$ against $32mm^2$) it is necessary to take into account some extra-fields that are needed to detect the chamber due to errors during the manual assembly of the microfluidic platform that create a mismatch between the field. Table 3.5 shows the time required to acquire a surface of a microfluidic chamber using a 10x long

working distance objective and a 20x long working distance objective for different fluorescent channel. To cover the surface of a culture chamber equal to 26mm2 a number of fields equal to 23 are necessary using a 10X objective, instead using a 20X objective 149 fields are necessary to cover the entire well surface.

Table 3.5 *Time requirements for imaging the surface of one culture chamber of a microfluidic platform at default acquisition time of 200ms for every channel (total surface 26 mm², 23 fields at 10X long working distance(WD) objective, 149 fields at 20X long working distance(WD) objective)*

		Type of objective	
		10X long WD	**20X long WD**
Time for imaging (min)	**1 channel**	3	7
	2 channels	6	10
	3 channels	7	13

Times for imaging related at 10X long working distance objective of a culture chamber in a microfluidic platform are comparable with the ones in a well due to the similar number of fields required to cover the surface of the well and of the culture chamber. Moreover, at this magnification the number extra fields required to ensure the acquisition of the culture chamber is lower due to the larger field of view of the 10X long working distance objective (1351 × 1017 µm). In order to save time avoiding the addition of extra fields that increase the experiment time, it is necessary to pay attention on the assembly of the microfluidic platform.

3.7 Real case study

The final goal of the robust methodology developed to perform high-throughput imaging of the microfluidic platform in Operetta® is it used for biological studies. In particular the developed microfluidic platform will be used for the study of human pluripotent stem cells. Cells with different genetic modifications will be used within the platform to study how these perturbations affect pluripotent stem cell colony production and growth. Preliminarily, the platform has been tested for its ability to image pluripotent stem cells without genetic modifications during culture. This work was performed in collaboration with a master student in biology at SIAIS, due to the level of expertise required to handle this type of cells. The cells were stained with Hoechst 33342 for nuclei detection and with a fluorescent probe for detection of TRA-1-60, a membrane marker expressed by pluripotent stem cells. The pictures of pluripotent stem cell colonies at different time points from cell seeding were taken with a 20X long working distance objective and they are shown in Figure 3.15. Further image analysis in progress to determine the area occupied by the colonies by measuring the area of the yellow

marker respect the background and to quantify the pluripotent stem cells growth in the culture chamber.

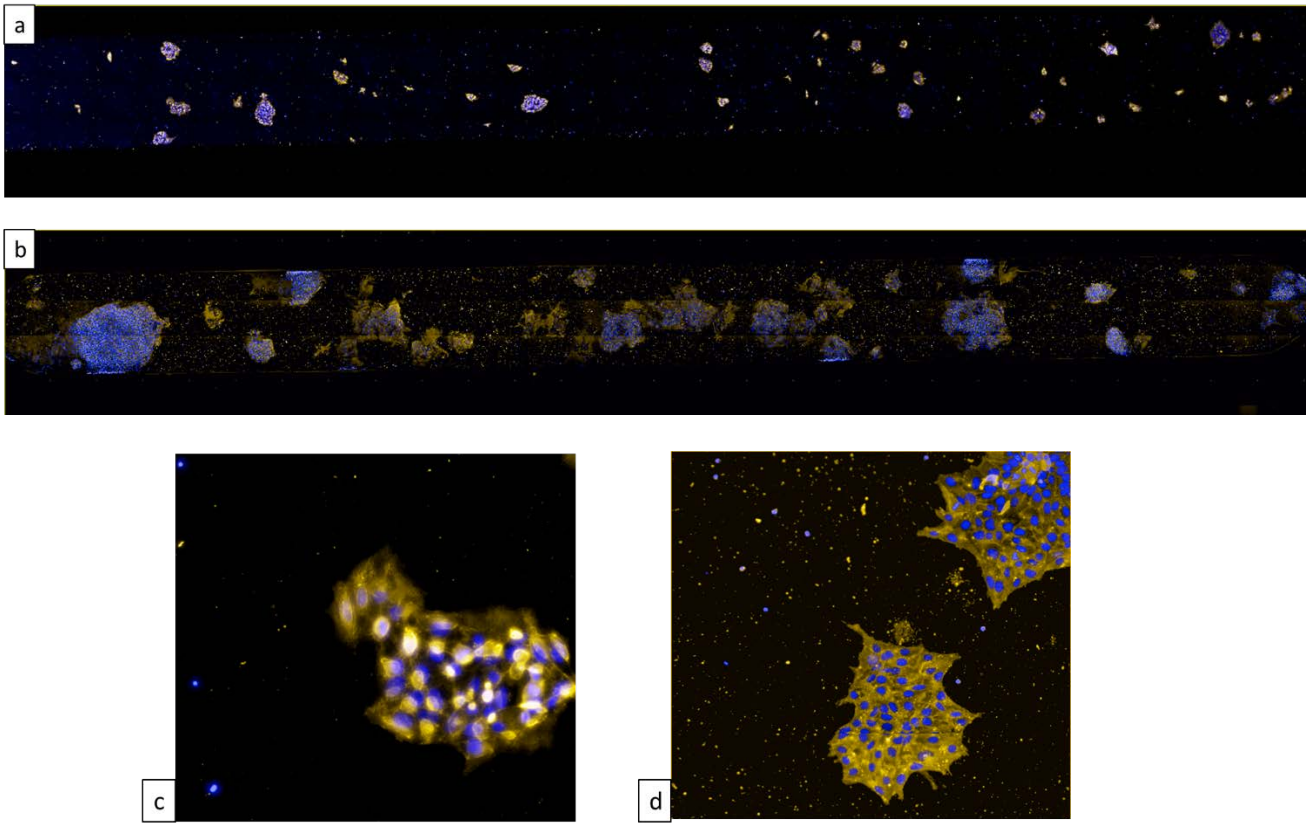


Figure 3.15 *Pictures of the immunofluorescence assay taken in Operetta® at 20x, Nuclei are stained in blue by the Hoechst 33342 and TRA-1-60 is stained in yellow; a) culture chamber at two days after seeding; b) culture chamber at five days after seeding; c) enlargement of a cell colony at two days after seeding; d) enlargement of a cell colony at five days after seeding;*

FUTURE PERSPECTIVES

The principles followed in this work during the development of the microfluidic platforms suitable to be interfaced with a high-throughput imaging instrument can be taken as a reference to develop new custom configurations, according to specific requirements of the culture setup and the specific phenotype to be detected and quantified. For example, a microfluidic platform with a new culture chamber geometry has been designed in BioEra lab: this chamber has a serpentine at the inlet and one at the outlet that are long and narrow, with a volume of few nanoliters. This geometry strongly reduces the longitudinal concentration gradient in the chamber due to the presence of inlet and outlet. These microfluidic platforms can be automatized and the possibility to interface them in the high-throughput screening instrument using the methodologies developed in this work would represent a powerful tool to perform high efficiency phenotypic screening. Following the optimal custom configuration approach for designing an optimal microfluidic platform suitable for high-throughput screening as described in section 3.5, it is possible to develop a new geometry microfluidic platform configuration that exploits the features of the instrument in terms of compatibility and ease of analysis with the software. An example of the new microfluidic platform potential design is showed in Figure F1.

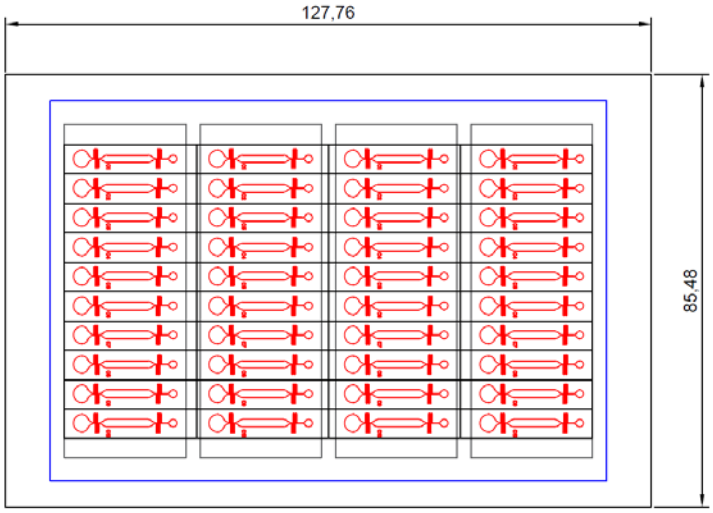


Figure F1. *Custom design of a microfluidic platform with new culture chamber geometry suitable for high-throughput screening at reduced longitudinal concentration gradient. In blue the glass plate of the microfluidic platform is marked, in red the culture chambers are labeled and in black the custom grid for the software is marked. Dimensions are reported in [mm].*

Although the one-well frame used to interface the microfluidic platform with the imaging instrument provided robust results of the assay, one limitation is its incompatibility with

autoclave sterilization due to the low resistance of the material to high temperatures. Designing a new one-well frame in aluminum or polycarbonate, materials compatible with autoclave sterilization, would make possible to perform live-cell imaging, as it would provide a sterile environment that prevents cell culture contamination. The external geometry of the new frame can be maintained as the one-well frame used in this work, but it can also be improved. In particular reducing the bottom height of the frame would make possible the use of high numerical aperture objectives, reaching higher imaging resolutions.

CONCLUSIONS

In this work, a microfluidic platform was integrated with an automated high-throughput imaging system, optimizing the number of parallel conditions detected, the achievable resolution of detection, the size of the field of view, and the automation of the image analysis. The platform already proved suitable for a test case where a toxicity study was performed stimulating fibroblast cells with dimethyl sulfoxide (DMSO), and with a real study for detecting pluripotent stem cell growth and morphology. In particular, the results obtained from the cytotoxicity assay using different DMSO concentrations performed in a 96-well plate provided firstly a methodology to perform an automatized high-throughput image acquisition using the instrument integrated software and secondly a methodology to set up an image analysis pipeline to quantify different cell properties as cell mortality and morphological changes. As for the first aspect, the main criticalities found in the imaging platform where the thickness of the bottom plate, the autofocus failures and the objective collision with the culture plate. They have all been addressed in the design of a one-well frame as a support of the microfluidic platform compatible with the screening instrument. Moreover, a methodology for an automated robust readout was also implemented. Thanks to the available microfluidic technology, it was possible to build a configuration adapted to a standard 6-well plate and a configuration adapted to a standard 96-well plate. However, these configurations showed limitations in high-throughput screening as for the limited number of parallel conditions observable and the reduced size of the culture chamber portion detectable, and the difficulty of data management. Finally, a microfluidic platform with a custom configuration has been designed to overcome these limitations, which provides robust solutions to perform high-throughput screening exploiting the tools of the instrument for analyzing a large number of culture chambers and providing an easy acquisition and analysis of the pictures taken. The quantitative results collected show robust statistical significance. The time of imaging is a critical parameter affecting operational decisions during the design of the experiments. Thus, tables for instructing the decision-making process have been provided for a well of a standard 96-well and a culture chamber of a microfluidic platform, imaged with different possible objectives, specifically a 10X and a 20X long working distance objectives. These indications can help the trade-off between the time required for the measurement and efficiency of the phenotypes screening.

Finally, the application of the developed phenotype screening methodology to a real case study related to the investigation of human pluripotent stem cell biology was presented. Overall the developed integration between microfluidic and imaging technologies proved robust and effective to be adopted in real situations.

Appendix A

Microfluidic platform production protocols

In this appendix are described the protocols adopted and developed to build the microfluidic platforms, It particular the step from the PDMS replica moulding to the microfluidic platform assembly.

A.1 PDMS replica moulding

The followings steps describe in details how to obtain a mould suitable to be coupled in the glass to create a microfluidic platform for the standard geometry wafers and for the new geometry wafers

1. Place the wafer to use in a hermetic vessel with some paper towel and add some drop of chlorotrimethylsilane. Close the lid of the vessel and leave there for 60 minutes.
2. Clean the wafer with compressed air and remove manually eventually pieces of solid PDMS on the wafer being careful to not damage the channels geometry inside the wafer.
3. Fill the PDMS and the curing agent in a cup and weight them over a balance. The reagents have to be in a ratio 10:1. Mix them energetically under the hood with a wood wand until the mixture turn white.
4. Cover the cup with the aluminium foil and place it in a vacuum vessel for the first degassing step. Turn on the vacuum pump until the liquid PDMS reach the level of the aluminium foil, then turn off the pump and leave there for 20 minutes.
5. Place the wafer in the balance and fill them with 5.5g for each mould (11g of PDMS in total for the wafer).
6. Put the wafer in a vacuum vessel for the second degassing step. Turn on the vacuum pump for some minutes until the first air bubbles appear on the mould surface, then switch off the pump and leave there for 45 minutes.
7. Place the wafers in a hot plate previously preheated at 75°C for 75 minutes.
8. When the wafer is cooled and reach the room temperature cut the mould with a scapel.
9. Punch the inlets with the 1mm puncher for the standard geometry and eventually the reservoirs with the 3mm puncher if the mould has the new geometry
10. Define the shape of the edge using a cutter with the help of a slide glass. The final dimensions of the mould should be roughly 50×26 mm for the standard geometry and roughly 66×26 mm for the new geometry

A.2 PDMS reservoirs production

This protocol is developed to create a reservoir required to be attached on the inlets of the 10-chamber first chip configuration (see section 2.1.3). The passages are similar to the one described on A.1 and they are the following.

1. Clean a 100×100 mm plate with compressed air and remove manually the piece of PDMS that eventually deposited on the edge of it.
2. Fill the PDMS and the curing agent in a cup and weight them on a scale. The reagents have to be in a ratio 10:1. Mix them energetically under the hood with a wood wand until the mixture turn white.
3. Cover the cup with the aluminium foil and place it in a vacuum vessel for the first degassing step. Turn on the vacuum pump until the liquid PDMS reach the level of the aluminium foil, then turn off the pump and leave there for 20 minutes.
4. Fill the plate over a balance with 30g of liquid PDMS.
5. Put the plate over a preheated hot plate at 75°C for 75 minutes.
6. When the plate is cooled at room temperature it is possible to detach the PDMS from the plate
7. Following the sign of the holes drew in a carpet with a distance that correspond to the mould inlets punch the piece of PDMS with the 3mm puncher
8. Shape the reservoirs using a cutter. The final dimensions should be roughly 50mm long and roughly 6.5mm large.

A.1.3 Glass cleaning

Before to attach the mould to the glass slide or glass plate, a cleaning of the glass surfaces have to be done. It is described below the main procedure. All the steps have to be done wearing gloves.

1. Wash with distilled water the surfaces of the glass.
2. Put some drop of Cleaning Solution (2% of Micro 90 Sigma -in distilled water) in the surfaces of the glass.
3. Scrub with the fingers for a few seconds the surfaces and rinse the surfaces with distilled water
4. Place the cleaned glasses to dry temporally over a paper towel and then dry accurately with the compressed air

A.1.4 Microfluidic platform assembly

The surfaces of the silicon mould and the glass are coupled using a plasma treatment building a microfluidic platform. The protocol is described below:

1. 1.Use white tape to clea surface of a glass slide or plate and in the patterned PDMS surface.
2. In the case of the glass slide (76×26 mm), remove the tape from the glass and from the PDMS mould and place it in the centre of the plasma chamber around the cylinder with the surfaces to treat pointed up
3. In case of the glass plate (110×75 mm) remove only half tape from the surface and place it in the centre of the plasma chamber together with the patterned PDMS mould with the surfaces to treat pointed up. Then when the silicon moulds are attached in one side, remove the tape from the other side and repeat the step
4. Close the lid of the plasma chamber and switch on the vacuum pump until to reach a pressure of $9.6*10^{-1}$ mbar.
5. Open the air valve until to reach a pressure of 1.2 mbar.
6. Switch on the plasma power for 15 seconds
7. Switch off the plasma power and open the air valve until to reach the atmospheric pressure.
8. Take out the glass from the chamber and place it in the carpet where a design of the configuration is printed and stuck over there.
9. Attach the silicon mould to the glass following the position of the channels in the printing as much as possible pushing gently the silicon to the glass.
10. Put the platform in a preheat hot plate at 100°C for at least 15 minutes to promote the adhesion of the surfaces. Taking as reference the most high-throughput configuration the placement of the glass plate in the one-well frame at the end of the assembly is showed in Figure A.1

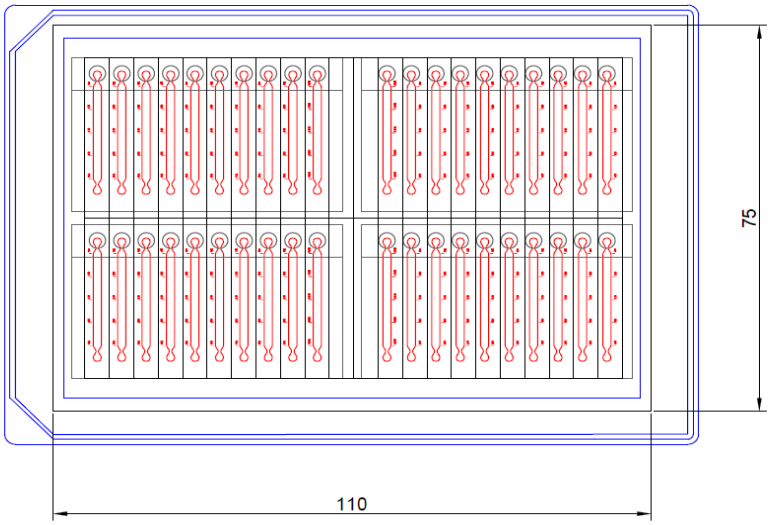


Figure A.1. *Disposition of the forty-chambers microfluidic platforms in the one-well frame. In blue the one-well frame is represented, in red the culture chambers are represented and in grey the PDMS moulds and reservoirs are represented. Dimensions of the glass plate are reported in [mm]. The grid represented in black lines is a virtual well disposition that is needed to set up the parameter to face the microfluidic platform in the instrument.*

A.1.5 Microfluidic platform cleaning and sterilization

The microfluidic platforms have to be cleaned before to be used in the cell cultures. Under chemical hood these following steps have to be done:

1. all the channels of the platforms have to be rinsed with distilled water, isopronanol and distilled water again for two times.
2. The platforms are placed in a hot plate preheated at 100°C until the channels are completely dried.
3. Put the microfluidic platform in the bag suitable for autoclave, close and transfer them in the autoclave at 121°C at 2.2 bar for 27 minutes.

Appendix B

Biological Protocols

B.1 Cell culture

In this section are reported the protocols to perform the cell culture in dish, in the multi-well plate and in the microfluidic platform with the first version of the geometry of the channels (see section 2.1.4.1).

B.1.1 Cells thawing

The cryovials in the -80°C freezer or in the liquid nitrogen vessels are labelled with type of cells, number of cells, number of passage, date of freezing, operator name. The operations to thaw the cells are listed below:

1. Prepare under the hood: a 15-mL tube, at least 10 mL of warm medium, p1000 set at 1000
2. Take the vial containing the cells from freezer (-80°C) or liquid nitrogen tank
3. Put it in the thermostatic bath as soon as possible, shaking a little to speed up the process and progressively closing the cap.
4. When only a little piece of ice is left inside, bring it under the hood after spraying it with ethanol.
5. Transfer all the vial content in a 15-mL tube using p1000.
6. Add 10 mL of warm medium drop-wise, keeping on shaking the tube
7. Centrifuge at 1100 rpm for 8 min.
8. Aspirate medium, leaving the cell pellet, ~50µL of medium are left in the pellet.
9. Add 950 µL of medium and resuspend cells (to make 1 mL of total cell suspension).
10. Count cells and seed them at desired density, passage number is the same as in the cryovial.

B.1.2 Cells Freezing

The procedure to freeze the cells are explained in the following steps:

1. Prepare freezing solution: 400 µL FBS + 100 µL DMSO.
2. Prepare the cryovial labelled with: type of cells, number of cells, number of passage (+1 respect to previous dish), date of freezing, operator name.

3. Suspend cells to be frozen in 0.5 mL of medium in 15-mL tube.Add dropwise 0.5 mL of freezing solution, keeping on shaking the tube
4. Mix cells twice and transfer them in cryovial.
5. Put the cryovial in cryostep in -80°C freezer very quickly.
6. After 24 h, cryovial can be removed from cryostep and left in -80°C freezer for up to 6 months, then it needs to be transferred into liquid nitrogen.

B.1.3 Medium changing

The method to change the medium in the microfluidic platform is reported below:

7. The medium have to be frequently changed in the cell cultivation to guarantee the viability of the cells. For the fibroblast cells the following procedure has been followed:
8. 1.Preheat the tubes of medium solution and the PBS-/- 1x in a thermal bat at 37°C.
9. 2.Take the cells from incubator (closing the flask cap, if not vented).
10. 3.Aspirate exhausted medium with the vacuum pump.
11. 4.Wash with warm PBS-/- 1x to remove dead cells and debris and aspirate.
12. 5.Put fresh warm medium.
13. 6.Place the flask back into the incubator.
14. The medium have to be changed more or less every 3 days.
15. For the microfluidic platforms, the procedure is different and because the medium have to be changed 24 hours after the seeding and the next days every 12 hours.
16. Preheat the tubes of medium solution in a thermal bat at 37°C.
17. 2.Take the microfluidic platforms from the incubator.
18. 3.Fill 12µL of medium solution with a p20 pipette in the seeded channels.
19. 4.Aspire with a high volume pipette some microliter in the reservoirs to avoid a backflow of the medium
20. 5.Add distilled water if the dish of the microfluidic platform is dry and place the platform back in the incubator.

B.1.4 Cell Passaging

The following procedure has been adopted to passage the cells:

1. Take out the cell from incubator and preheat the PBS-/- 1x and the medium solution in a thermal bat at 37°C.
2. Aspire the buffer with a vacuum pump and wash the cells with PBS-/- 1x to remove the dead ones.
3. Add the trypsin EDTA 0.05% to detach the cells from the bottom and the dish is put in the incubator at 37°C for 5 minutes

4. After checking in the microscope if a great part of the cells detached from the bottom add an equal amount of medium solution to stop the effect of trypsin EDTA.
5. Collect the suspension in a 15mL tube and it is centrifugate at 1200 rpm for 5 minutes.
6. Aspire with the vacuum pump the supernatant avoiding touching the cells deposited to the bottom.
7. Resuspend the cells in an appropriate amount of media solution (usually 1 mL) to count them or to seed them directly in a new dish.
8. Put the cells in incubator.

B.1.4 Cell seeding in microfluidic platforms

The following procedure is the same for both microfluidic platforms built in a 76x26mm glass slide and 110x75mm glass plate.

1. Under biological hood, using sterile disposable tweezers remove the chips from the autovlaved bag and place the one built in a glass slide in 100 mm Petri dish. The ones mounted in a big glass plate are placed in a 500 mm diameter Petri dish.
2. Take out from the -20°C freezer the fibronectin and let defrost completely.
3. Fill the channels using a p20 with 12µL of fibronectin and left them to coat for at least 30 minutes at room temperature. Add inside ultrapurified water plus Pen Strep (Penicillin Streptomycin, Thermofisher, diluited 1/100 in ultrapurified water).
4. After 30 minutes rinse the channels with fresh medium solution to remove the exhausted fibronectin.
5. Resuspended the cells and count them with an automatic cell counter. To count the cells, a small volume of 10µL is withdraw from the cells suspension and it is put in a vial where it is added 10 µL of Trypan Blue (Sigma-Aldrich). Then fill with 10 µL of solution (cells suspension + Trypan Blue) each chamber of a counter plate and insert it in an automated cells counter.
6. Seed the channels with 12 µL of cell suspension at the desire density.
7. Put the microfluidic platform in the incubator.

B.1.5 Cell seeding in multiwell plate

This procedure has been tested in a 96 Well Glass Bottom Plate(Cellvis) and is explained below:

1. Take out from the -20°C freezer the fibronectin and let defrost completely.
2. Under biological hood Coat the bottom the wells before to seed the cells with fibronectin diluted in a volume ratio 1:5 in PBS.
3. Leave the plate at room temperature for 30 minutes to let the and in the wells not coated around are filled with 200 µL of PBS to avoid the evaporation.
4. Resuspended the cells and count them with an automatic cell counter. To count the cells, a small volume of 10µL is withdraw from the cells suspension and it is put in a vial

where it is added 10 µL of Trypan Blue (Sigma-Aldrich). Then fill with 10 µL of solution (cells suspension + Trypan Blue) each chamber of a counter plate and insert it in an automated cells counter.

5. Seed the wells with 200 µL of cell suspension at the desire density.
6. Put the multiwell plate in the incubator.

B.2 Toxicity assay

This procedure has been performed to develop a toxicity assay with the DMSO (dimethylsulfoxide) of HFF-1(ATCC® SCRC1041™) cell cultures both in microfluidic platforms and in multiwell.

1. Preheat the tubes of medium solution in a thermal bat at 37°C.
2. Under biological hood, prepare 6 vials and fill them with the DMSO and the medium at this concentration volume/volume: 0%,1.25%, 2.5%, 3.5%, 5%,10%.
3. Take out the multiwell plate or the microfluidic platform from the incubator.
4. Fill each channels of the chip with 12 µL of toxic solutions at different concentration disposed in random positions. In a multiwell, fill each well with 200 µL of toxic solution disposed in random positions.
5. Put the device in the incubator.

B.3 Immunofluorescence assay

The staining procedure is valid both in microfluidic platforms and in a 96 well plate. Before to start the assay, all the vials and tube that contains the fluorophores have to be protected from light covering them with aluminium foil. The staining procedure is described below.

1. Take out from the -20°C freezer the Viability/Cytotoxicity Kit (Thermofisher) that contain Calcein AM and Ethidium homodimer and take out the Hoechst 33342 and let defrost the two vials.
2. Prepare the working solution by diluting the fluorophore in PBS-/- as following:
   - *Hoechst 33342* in a volume ratio of 1:5000.
   - *Calcein AM* in a volume ratio 1:5000 (2µM).
   - *Ethidium homodimer* in a volume ratio of 1:400 (4µM)

   Shake with the vortex the working solution and cover it with aluminium foil.
3. Take out the microfluidic platforms or the multiwell from incubator and wash the sample one time with PBS-/- 1x for 5 minutes.
4. Add the working solution to the channels of the microfluidic chips or to the wells and put in the incubator for 40 minutes.
5. Interface the devices in Operetta™

Appendix C

Imaging Protocols

In this section the protocols are reported to interface the microfluidic platforms with the one-well plate and the Operetta® instrument, to set the parameters for a correct acquisition of the images and to perform the final image analysis.

A.3.1 Image acquisition

Before to perform any experiments with the instrument it is required to set up all the parameters regarding the support combined with the different microfluidics platform configurations and the parameters of the 96-well glass bottom plate (Cellvis) that were not present in the database.

A.3.1.1 Setup of platform layout

Here, it is described the procedure to define the characteristics of a new user-defined plate, specifically the high-throughput microfluidic configuration shown in Figure A.1
All the measurements of the support and the identifications of the wells and the field that correspond to the microfluidic culture chambers are done with the following procedure:

1. Switch on Operetta™ and the desktop computer.
2. Lunch Harmony software
3. Select “Plate Type Definition Wizard” on the setting menu. This allow to insert the measurement of new the high-throughput configuration. The meaning of each variable reported in Figure A.2 are explained in detail in section 2.3.1.2. Below the dimensions to set (Figure A.2) are described, taking as reference the virtual well disposition showed in Figure A.1:
    - set A equal to the distance [mm] between the center of the top-left virtual well and the left side of the one-well frame;
    - set B equal to the distance [mm] between center of the top-left virtual well and the upper side of the one-well frame;
    - set C and D equal to the length [mm] and the width [mm] of the one-well frame;
    - set E equal to the distance [mm] between the center of the top-right virtual well and the left side of the one-well frame;
    - set F equal to the distance [mm] between the center of the down-left virtual well and the upper side of the one-well frame;
    - set G equal to the height[mm] of the one-well frame;

- set I equal to the thickness[mm] of the bottom glass plate of the microfluidic platform;
- set H equal to the distance[mm] between the bottom of the microfluidic platform and the rid of the one-well plate;
- set L equal to the width[mm] and M equal to the length [mm] of a single virtual well;
- set the value of O and P equal to L and M.

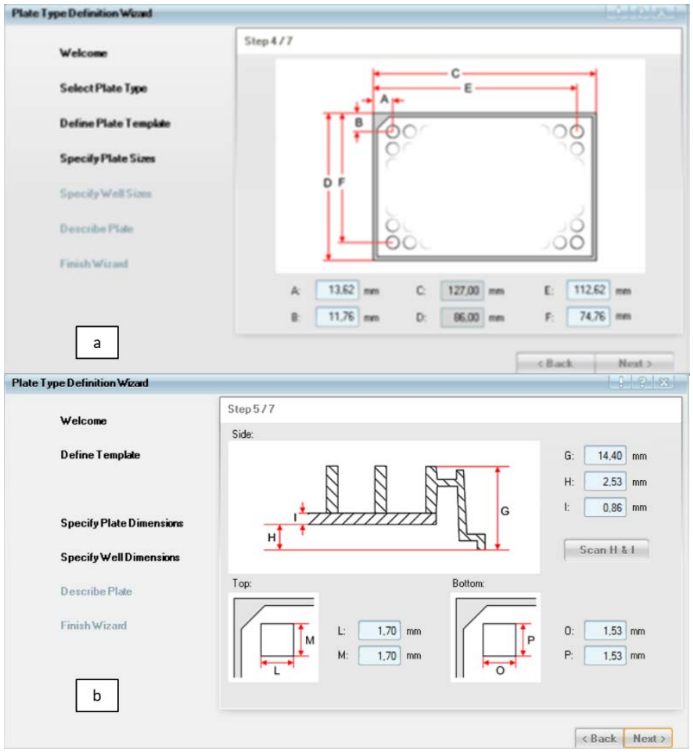


Figure A.3 Screenshot from the "Plate Type Definition Wizard" windows steps for a new plate type; a) specification of the plate dimension[mm]; b) specification of the well and side dimensions[mm] of the plate.

4. Select the plate configured in "Select Plate"
5. Select the type of objective
6. If the objective selected required the correction collar, in the software a message with the value of the correction will appears and it will be possible to open the lid of the instrument and set manually the value of the correction in the objective.
7. Select the Brightfield as light channel and select the wells and the fields that correspond to the edge of the channels or part of it.
8. Save the configurations of the layout selected. Note that even for the same plate, when the objective is changed the layout (wells and fields) have to be selected again

A 3.1.2 Acquisitions of microfluidic platform samples

To acquire the sample performed in the microfluidic platform it is possible to load the layout already set up in the instrument and provide to the software other parameters before to start the analysis. Otherwise it is possible to set up a new configuration. The procedure is listed below:

1. Switch Operetta®, the external lamp and the desktop computer on, and open the Harmony® software.
2. If the acquisition have to be done at constant temperature of 37°C and constant concentration of CO_2 of 5%, activate this feature and close the Live Chamber in the instrument at least one hour before the acquisition.
3. Select the type of the plate suitable for the acquisition.
4. Select the type of objective
5. If the objective selected required the correction collar, in the software a message with the value of the correction will appears and it will be possible to open the lid of the instrument and set manually the value of the correction in the objective.
6. Put the device in the plateholder, close the Live Chamber and the lid of the instrument
7. Select the type of light channels suitable for the analysis, then set the excitation/emission filters, the lamp power, the time of acquisition and the height of focus. All these parameters can be set taking a snapshot in a well selecting a field inside it. This step has to be done for every light channel selected in order to find the best optical configuration.
8. If the layout of the experiment has been loaded, check if the fields selected correspond to the channels of the microfluidic platform. Otherwise find the correspondents fields that match the channels.
9. Run the analysis.

A 3.1.3 Acquisition of multi-well plate samples

The procedure to acquire the samples in multi-well plate is easier than the procedure for the microfluidic platform. Even for the multi-well it is possible to save a suitable layout and load it in the set up phase of the parameter for the acquisition. The procedure is reported below:

1. Switch Operetta®, the external lamp and the desktop computer on, and open the Harmony® software.
2. If the acquisition have to be done at constant temperature of 37°C and constant concentration of CO_2 of 5%, activate this feature and close the Live Chamber in the instrument at least one hour before the acquisition.
3. Select the type of the plate suitable for the acquisition.
4. Select the type of objective
5. If the objective selected required the correction collar, in the software a message with the value of the correction will appears and it will be possible to open the lid of the instrument and set manually the value of the correction in the objective.

6. Put the device in the plateholder, close the Live Chamber and the lid of the instrument.
7. Select the type of light channels suitable for the analysis, then set the excitation/emission filters, the lamp power, the time of acquisition and the height of focus. All these parameters can be set taking a snapshot in a well selecting a field inside it. This step has to be done for every light channel selected in order to find the best optical configuration.
8. Select the wells and fields where the acquisition have to be done. Inside the well select the fields close to the centre of the well.
9. Run the analysis

A.3.2 Image analysis

Once the images have been acquired and stored in the database, it is possible to set up the image analysis. First it is defined a sequence of properties to calculate and then it is done an evaluation for all the assay using the sequence defined.

A.3.2.1 1 Setup of image analysis pipeline

It is built up a sequence of building blocks to evaluate the cytotoxicity of the DMSO in the samples both in the microfluidic platforms and in the 96 well plate.

1. If the analysis has already been done load it from the database in the image analysis step in the software otherwise at the end of the experiment the software conducts there automatically.
2. Select an input image from the wells and in a specific field inside it.
3. Add the building block *Find Nuclei* selecting Hoechst 33342 as light channel.
4. Add the building block Find Cytopasm selecting Alexa 488 as light channel. At this point the cells are detected as a region.
5. Add 2 building blocks *Calculate Intensity properties* of Ethidium Homodimer and Alexa 488.
6. Add the building block *Select Population* in the cell region detected checking the method *Remove Border Objects.*
7. Add the building block *Calculate Morphology properties*. Check the area, roundness, width, length, width over length ratio of the cell region from step 6.
8. Add the building block *Select dead cells*. From the cells selected in step 6 the criteria to discriminate the dead cells is Intensity of Ethidium Homodimer > n where n is the intensity value of the average background intensity of the Ethidium Homodimer in the negative controls.
9. Add the building block *Define results*. Select “Formula Output” and define the Percentage of Dead Cells as a/b*100 where a is the as number of dead cells detected in step *Select dead cells* and b is the number of cells detected in step *Select Population*.

Select “List of Output” and select as output total number of cells selected, number of dead cells, percentage of dead cells, cytoplasm intensity (mean and standard deviation), cell roundness (mean and standard deviation), cell area (mean and standard deviation), cell length (mean and standard deviation), cell width (mean and standard Deviation), cell width over length ratio (mean and standard deviation). Select “none” in single cell results.

A.3.2.2 Run analysis and export results

To evaluate the image analysis in the assay the following procedure have been developed:

1. If the image analysis has just been performed the software switch automatically in this section. Otherwise load a past measurement of an assay to evaluate.
2. Select the wells and the fields inside them to evaluate the image analysis for all the assay. Run the evaluation.
3. Results can be exported in .txt file in section “Export Data” selecting the option “Evaluation Results per Well”.
4. To save a single image of a field of a single light channel or the merge it is possible to save it in 8 bit (.png , .jpg) in the control panel with the option “Save image”. Otherwise it is possible to export the images selecting the option “Export Image” in 16 bits (.tiff) for each light channel used. To take the picture of the entire microfluidic channels select the fields inside the well where the channels is detected and click the option “Overview – Plate and Well realistic”. With this feature it is possible to save the images only in 8 bits.

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