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A comparative analysis between static and dynamic chip technology organoid culture techniques

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Summary

Abstract

The development of self-organized three-dimensional cells cluster, called organoids brought an important improvement in the drug screening studies because they are able to give models closer to reality. Nowadays, the aim of the research in the organoids culture is to find a procedure that is able to simulate in a realistic way the environment which can be found inside an organ. For this purpose, it is of fundamental importance to fully understand the characteristics of cancer cells, their micro and macro environment and the external factors that characterize their composition and associated metastatic phenomena. More and more complex systems have been devised to effectively simulate the tumour environment, such as the microfluidic technique that will be tested in this thesis.

This technique, called Organ-on-chip, combining micro-engineering and biology, constitutes an enormous progress compared to previous techniques. Through the aid of pumps and regulation systems it gives the opportunity to make the culture medium flow in a chip by simulating the shear stress which in vivo is caused by the capillaries. The synergistic integration of matrices with new culture methods could pave the way for the development of increasingly faithful models providing reliable results capable of bringing benefits to medical research. These results are capable of bringing benefits to research as well as providing a platform for testing personalized therapies for the patient.

Introduction

In drug screening field, the main factor that leads to the failure to obtain a compound that is able to treat the targeted disease is the lack of coherence between the in vitro model and the conditions that can be found inside the human body (Hynds & Giangreco, 2013). The main methods used nowadays during the preclinical test of new compounds are based on 2D cell culture and in vivo experiments. Cell cultures often don't represent the complexity of the organ's environment, for example with 2D cultures the modification due to the passage of the drug through the tissue that surrounded the target can't be simulated. Moreover, 2D culture cells often give rise to altered phenotypes due to the lack of tridimensionality and so lead to erroneous conclusions. Another drawback of 2D culture as study models is that those must be genetically altered to be immortalized so they gain oncogenic potential making it impossible to simulate a true human phenotype (Schutgens & Clevers, 2020). For what concern the in vivo methods, they mimic in a more precise way the real conditions but despite ethic questions these techniques can give not precise results because of the many differences between small mammalian organisms and human being. With the goal to overcome the different problems that affected the in vitro and in vivo models in the last decades organoids cultures have been developed.

Organoids

The development of a more realistic three-dimensional model started several decades before the first organoid creation. Spheroids and rosettes were the first 3D tissue-derived models and are the conjunction ring between in vitro immortalized cell cultures and in vivo grafted tumours. (Corrò, et al., 2020) Unlike organoids, spheroids don't require a matrix, they are free floating.
Spheroids can be divided in three distinct categories (Białkowska, et al., 2020):

- **Multicellular tumour spheroids** → derived from bi dimensional culture of cancer cells

- **Tumourospheres →** derived from cancer stem cells
- **Organotypic spheroids →** derived mechanically from in vivo tumour

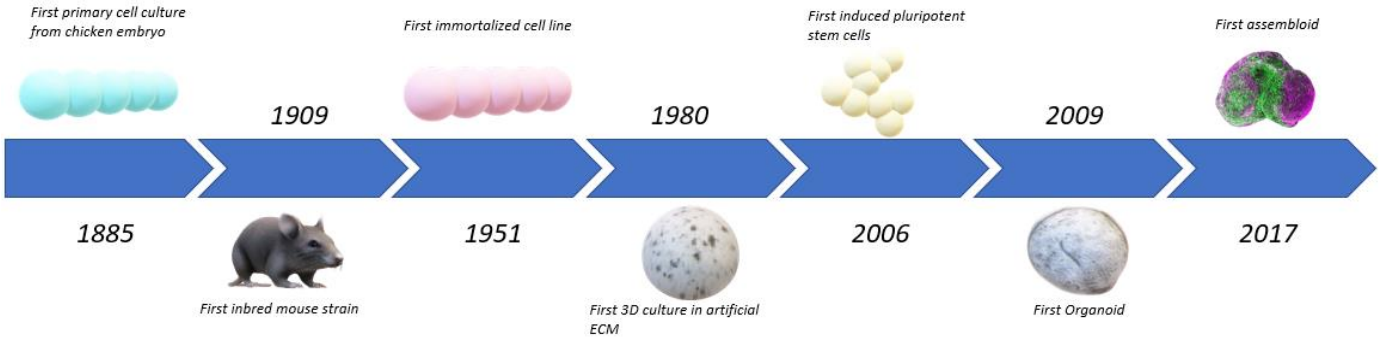


Figure 1 *A brief history of Organoids*

A milestone in the development of the first organoid was the study conducted in 2006 by S. Yamanaka and K. Takahashi which were able to transform mouse skin cells in embryonic stem or induced pluripotent stem cells (iPSCs). This important achievement was reached through the introduction and expression of a group of genes using viral transfecting vector. iPSCs are still today one of the main tools in the research related to medical field. An interesting aspect of this kind of cells is that they retain the genotype of the organism from which they were taken and so allow a personalized medicine study where a drug is tested against an iPSCs cell culture derived from the patient being treated (Takahashi & Yamanaka, 2006). In 2016 Fatehullah and the other members of the research group, give a definition of the key features of an organoid: Organoids are in vitro self-organizing and self-renewal 3D culture systems derived from primary tissue, ES cells or iPSCs and have to resemble the organ of origin (Fatehullah, et al., 2016). Organoids, as previously reported can be originated from ES cells, iPSCs and also adult stem cells (ASCs). These organoids derived from different cell's type exhibit also different characteristics and then different purposes. For example, in the drug screening studies and in the cancer therapy research organoids derived from iPSCs cells are preferred because give a better level of complexity respect to ASCs- derived ones, thanks to the genetic properties of the iPSCs (Huch & Koo, 2015). The first organ simulated with organoids was the intestine when Sato et al in 2009 were able to derive small intestine organoids from murine intestine (Sato, et al., 2009), since then many organs have been simulated from the "simple" salivary gland (Maimets, et al.,

2016) to the complex brain generated from human and mouse pluripotent stem cells in 2017 (Watanabe, et al., 2017)

ASC derived organoids

One of the first study to obtain a method for culture ASC-derived organoids was done by Sato et al. in 2009 achieving the creation of intestinal organoids picked up form isolated staminal cells from intestinal crypts identified by Lgr5 (Sato, et al., 2009). The creation of this kind of organoids was facilitated by a peculiar characteristic of the intestinal stem cells that, in vivo conditions maintain a high self-renewal capacity that allow them to replace often the entire intestinal epithelium. ASCs retain their self-renewal and the capacity of differentiate into the organ's specific cells also when cultured giving a good tool in medical research. Not all tissue have ASCs with an high regenerative capacity like small intestinal tissue and then the ASCs derived present a difficult expansion in vivo. To overcome this problem during the last years, the better culture conditions to mimic the specific conditions present in the staminal niches were studied. It was demonstrated that the main mechanism, which regulates the self-renewal of the ASCs is a class of proteins called Wnt signalling (Clevers, et al., 2014). Hunch et al. ,in 2013, performs a study where a toxic compound (corn oil or CCl_4) was injected in the mice causing them liver damage. After the biopsy was found the presence of $Lgr5^+$ cells in their livers indicating the activation of the Wnt pathway for the healing of the damage. These cells were extracted and when cultured in vitro in presence of a culture media containing RSPO1 formed organoids (Huch, et al., 2013).

ASCs derived organoids present several advantages (Hofer & Lutolf, 2021):

- By taking cells from an adult tissue, they will display an adult phenotype giving a realistic model of the studied organ
- Culture methods are easier respect to the PSC ones because ASCs are taken from a specific tissue and so the cultured organoids will have its specific characteristic
- They can be maintained for long periods of time through maintenance procedures

- Unique model for non-genetic, cancer pathology studies and cellular drug screening giving the possibility of personalized therapy

The two main disadvantages are:

- A biopsy is required to obtain ASCs thus limiting their availability
- By conserving an adult phenotype ASCs organoids are not suite for early stage organoids development studies

PSCs derived organoids

One of the main differences between ASCs derived organoids and PSCs is that the former represent an homeostatic state of the studied organ while the latter recapitulate the different development states of the studied tissue. PSCs, due to their pluripotency, are able to form more complex organoids and with them is possible to obtain tissues which are very difficult to obtain without harming the patient life like for example brain tissues (McCauley & Heather, 2017). An important example of the use of iPSCs in the creation of brain organoids is given by the research of Lancaster et al, they started with a culture of free floating embryoid bodies that subsequently were incorporate in Matrigel and cultured using a spinning bioreactor. An Histological analysis confirms the iPSCs's capacity of self-organize and to form specific brain regions such retina, meninges, choroid plexus and also cerebral cortex (Lancaster & Knoblich, 2014). This kind of procedure was then applied to other disease related to human brain like microcephaly, Zika virus infection and autism spectrum disorders (Watanabe, et al., 2017). As can be seen from the examples cited above, most of the case of studies concern developments in neural development, due to the intrinsic characteristic of PSCs / iPSCs of representing an organ in the early stages of development and not a phenotypically adult organs as in the case of ASCs.

Main advantages of PSCs derived organoids are:

- Obtaining the cells to generate organoids is easier than obtaining ASCs
- It's possible to obtain patient specific iPSCs and so genotypic personalized organoids able to offer a precise model
- Without the needing of biopsies and with the ability to obtain multiple specific

organoids from one culture changing the composition of the culture it's possible to conduct experiments otherwise impossible due to ethical problems or difficult obtaining of the samples

Despite the previously described advantages of PSCs derived organoids present also a more complex protocol that often require multiple passages in order to simulate the natural development process that occur in vivo (Kim, et al., 2020).

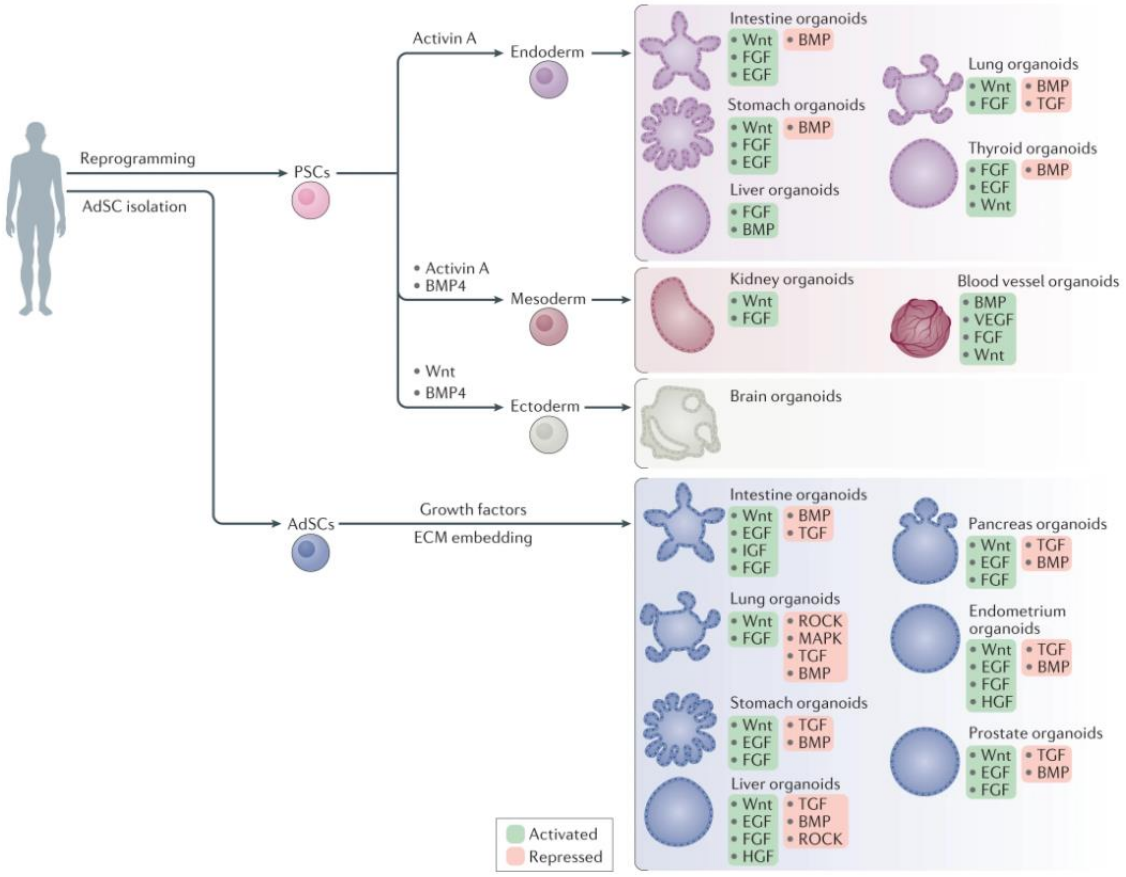


Figure 2 *Guided differentiation of organoids using different growth factors* (Kim, et al., 2020)

Assembloids

The possibility of combining different cultures characterized by different types of organoids has been discovered a few years ago, these combinations are called assembloids. Since with assembloids it is possible to create cultures that simulate several interconnected tissues at the same time, it is therefore possible to represent entire organs such as the human brain. A representative study of this concept is the one conducted in 2017 by Birey et al. who simulated the structures of neural circuits using assembloids. Using this technique, they were able to simulate much more complex neural circuits than those represented in single organoids, this result is also obtained thanks to the creation of a microenvironment similar to the real one which in fact is characterized by a wide variety of cell types (Birey, et al., 2017). Assembloids are of particular interest for studies concerning development processes in the biological field. In these studies, it is required that the organoids have a long life to simulate the different steps of the development process. The assembloids, simulating more faithfully the natural environment, last longer than conventional organoids and therefore better meet the requirements of the research. (Marton & Pașca, 2020)(Sloan and others 2018)

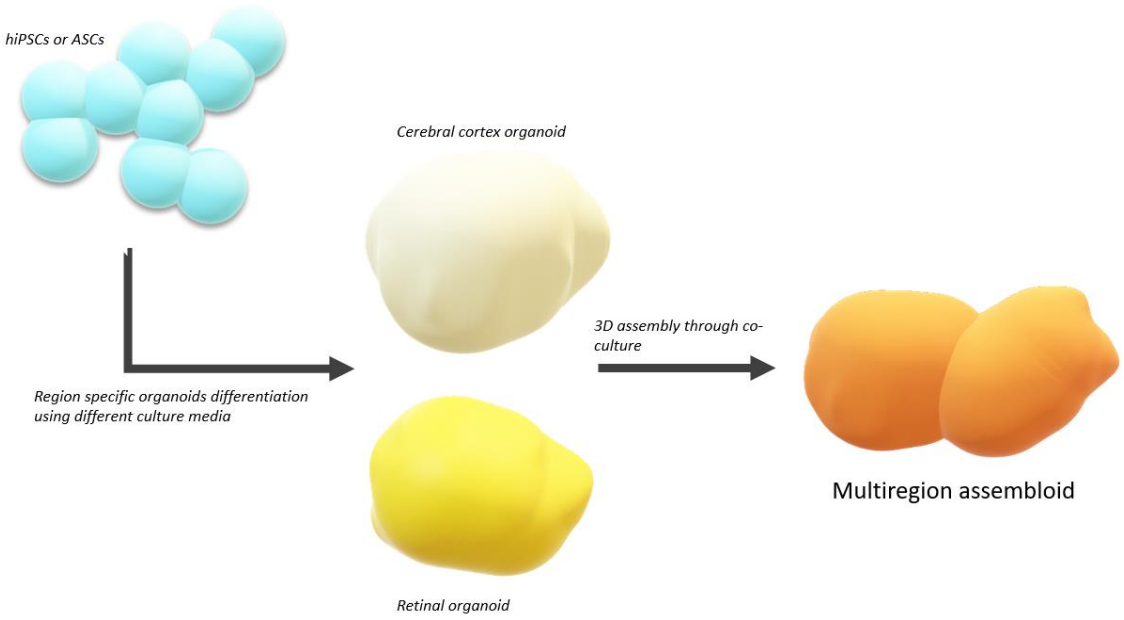


Figure 3 *A schematic representation of an assembloid production*

Ascites

Several research aimed at counteracting the drug resistance of cancer cells and investigating the processes that lead to their proliferation make the use of organoids from ascites. In medicine, ascites are defined as an accumulation of fluid in the peritoneal cavity following liver problems or metastases resulting from neoplasms of the gastric tract or reproductive system. In fact, this abnormal accumulation of fluid causes a lowering of the patient's quality of life due to the numerous associated symptoms such as nausea, abdominal pain, anorexia and difficulty in walking (Ford, et al., 2020). The problem of this pathology is that it is often a symptom of ovarian, liver, colorectal and pancreatic cancers. It is also usually related to a low life expectancy and a failure of anticancer therapies. Since ascites derive from tumour metastatic phenomena and therefore contain cells deriving from the primary tumour, they provide an accessible and very abundant source of samples of the specific tumour type of the patient. Ascites, through the study of their components, are therefore very important for studies aimed at understanding the phenomena of tumour spread and provide a tool for the development of therapies capable of counteracting tumour formation and growth. The fluid of ascites is copied from both tumour cells and non-tumour cells such as adipocytes, fibroblasts and mesothelial, endothelial and inflammatory cells (Li, et al., 2019). Two of the main causes that lead to the formation of ascites is the overexpression of the vascular endothelial growth factor (VEGF), which results in an abnormal composition of the blood vessel walls that allow the release of liquids containing cells that obstruct the lymphatic drainage (Herr, et al., 2012).

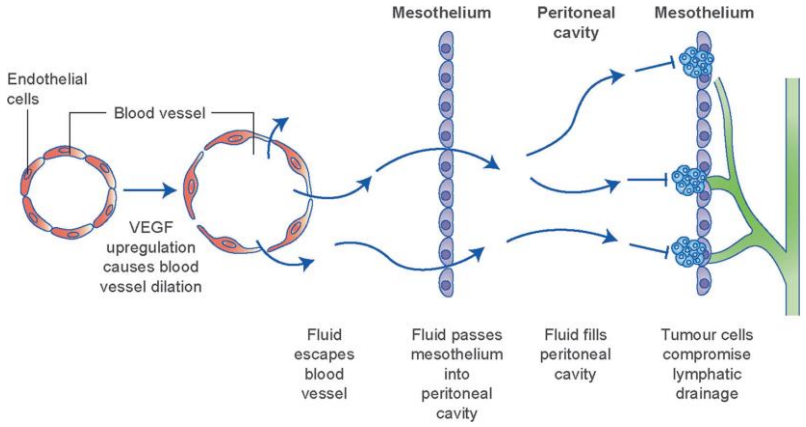


Figure 4 *Role of VEGF in the formation of ascites* (Ford, et al., 2020)

Ascites fluids in the presence of malignant tumours have among their components tumour-promoting cytokines, chemokines, growth factors and proteinases (Kim, et al., 2016). This heterogeneous composition makes ascites an important representation of the tumour microenvironment for the study of metastasis and associated chemoresistance phenomena. To demonstrate that ascites are associated with the promotion of metastatic phenomena, several studies have been carried out and have shown that by extracting cell-free liquid from malignant ascites, this promoted the appearance of metastases through the weakening of the strength of the tight-junctions (Ford, et al., 2020). There is also evidence that ascites promote epithelial to mesenchymal transition by transforming cancer cells into cancer stem cells. This transformation causes an aggravation of the prognosis as it implies that cancer cells can self-differentiate, self-renew, aggregate into spheroids and thus increase their resistance to chemotherapy drugs (Pakuła, et al., 2019). Ascites usually occur in large volumes and even if drained they recur several times during the disease, for this reason they become an important source of samples capable of representing the patient's tumour. Through the collection of ascites, it is possible to avoid the problems related to obtaining tumour material through biopsy as it is sufficient to practice the procedure of paracentesis and obtain large quantities of sample (Ford, et al., 2020). In recent years, protocols have been developed to grow organoids from single tumour cells taken from ascites in the presence of ovarian cancer. It has also been shown that these organoids reflect the molecular diversity of the primary tumour (Velletri, et al., 2018). Organoids derived from ascites, providing a model that faithfully reflects the patient's tumour and being readily available and in large volume, represent a precious opportunity for the creation of models that can be used in studies of resistance to chemotherapeutic drugs, individuation of markers and the formulation of personalized cancer therapy (Patch, et al., 2015)

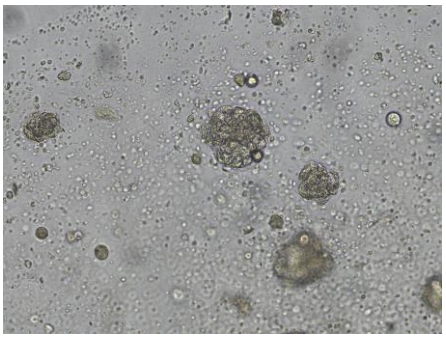


Figure 5 *Organoids derived from ascites obtained during the development of the thesis*

Main Organoids culture techniques

To the present day, there are mainly three different techniques to obtain organoids in vitro. The simplest one and also the most replicated is based on the cultivation of Organoids supported by ECM scaffolds, these scaffolds can be natural such as collagen or Matrigel or synthetic (Kaushik, et al., 2018). Synthetic scaffolds result very interesting because knowing that all the batches have the exact chemical composition, it's possible to obtain reliable and repeatable results. These results can be misrepresented using Matrigel due to it's not full known composition. Matrigel is derived from a natural source that is the basement membrane of the mouse sarcoma ECM and so its composition is affected by a batch-to-batch variability. The main components of Matrigel are laminin, collagen, entactin and several growth factors that mimic an environment very similar to in vivo ECM (Aisenbrey & Murphy, 2020). A pioneering study regarding the above citated method was done by Sato T. et al. in 2009. They were able to generate intestinal organoids starting from $Lgr5^{+}$ cells taken from the intestinal crypt. In order to obtain self-regulating organoids without a niche, they used Matrigel in combination with a culture media supplemented with growth factors (Sato, et al., 2009). Organoids cultured with this method exhibited characteristics of intestinal crypts. A valid alternative to Matrigel is the collagen gel. It, as in the case of synthetic matrices, presents a chemical well known structure and so also the majority of its possible interactions with the cells and tested compounds can be predicted. Collagen gel used in organoid's culture is often composed by collagen type I supplemented with Ham's F12 nutrient medium and sodium bicarbonate (Jee, et al., 2019). Sachs et al. were able to replicate simple epithelium structure using organoids embedded in collagen matrix, thus demonstrating its validity as a culture matrix (Sachs, et al., 2017).

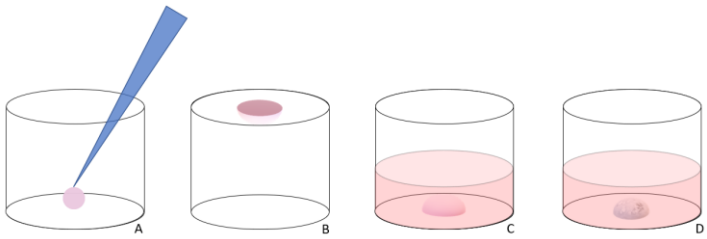


Figure 6 *Schematic of the procedure for obtaining a submerged organoid culture: in step A a drop of matrix is gently placed in the centre of the well. Subsequently, as illustrated in step B, the plate is inverted and incubated for 30 minutes at 37 ° C. Subsequently in step C, the culture medium is added starting from the edges. The first organoids should appear after 24 h (figure D)*

Another culture approach generates organoids starting from PSC or embryonic body (EB), the cells are agitated in a rotating bioreactor that is used to simulate the dynamic conditions gave by blood flux. The conditions that occurred inside the bioreactor also facilitate the diffusion of the nutrients. This technique is particular suitable for culture of brain organoids (Lancaster & Knoblich, 2014).

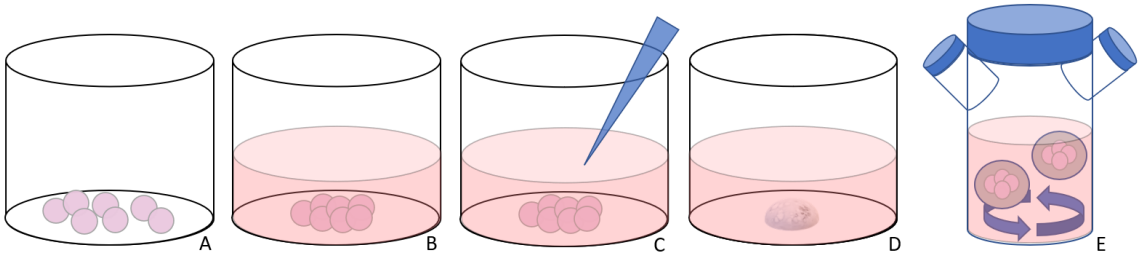


Figure 7 *Protocol for obtaining a culture of free suspended organoids in a rotating bio reactor: (A) Plating of Human pluripotent steam cells on a low attachment surface (B) Embryoid body (EB) formation (C) Add differentiation medium to EB (D) Epithelium formation inside Matrigel scaffold (E) Matrigel droplet transferred in rotating bioreactor to enhance nutrient absorption*

Last of the three methods it's the so called air-liquid interface method (ALI-method), it was used the first time by Li et al. in the creation of long-lasting gastrointestinal organoids using collagen without supplement of growth factors that are instead required in the other methods. This method consists in the creation of two layers of collagen, one immersed in the culture media and the other that contains the organoid exposed to the air. Between the two collagen layers, there is the creation of an air-liquid interface. This technique allows the culture of organoids for over 60 days (Li, et al., 2016).

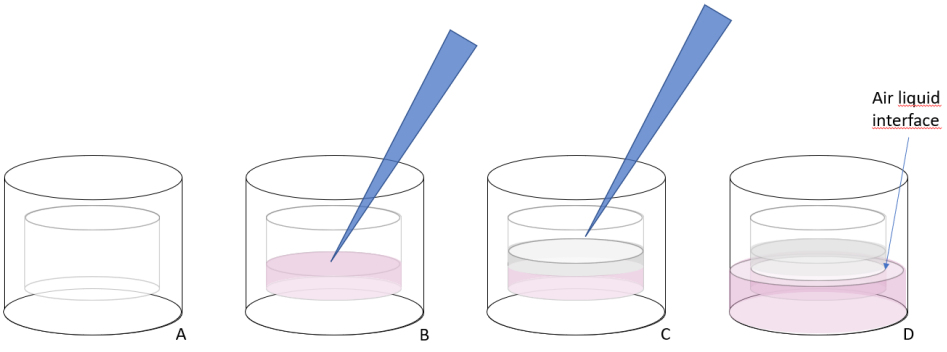


Figure *8 Schematic diagram of the Air liquid interface protocol: an insert is placed in a well (figure A) subsequently a layer of collagen is inserted into the well (figure B) once polymerized following incubation at 37 ° C for 30 minutes, another layer of organ organoid-containing collagen is added on top of the previous one (figure C). In figure D it is possible to see the formation of the air-liquid interface following the addition of the culture medium*

Organs on chip

Although modern organoid cultures provide tissue-like patterns in vivo, these, due to their static nature, are unable to provide a realistic representation of nutrient diffusion. In fact, in the tissues in vivo, the blood flow spreads the nutrients uniformly and also exerts a force on the cells. The diffusion of the culture medium is also inhomogeneous since the inner part of the drop of the matrix, where the organoids are included, with the passage of time will be increasingly depleted of nutrients and oxygen. This condition leads to cell death and compromise of the experimental results (Grebenyuk & Ranga, 2019). For the same reason, any clinical tests concerning compounds could also give equivocal results in the long term experiments. The new studies aimed at better understanding the new organs-on-chip technology are therefore of great interest. This technique combines microfluidic technology with the previously mentioned plating protocols that use natural scaffolds such as Matrigel. Organ-on-chip are define as microfluidic devices lined with living cells for drug development, disease modelling and personalized medicine. This technology is a biomimetic system that try to better resemble all the functions of an organ in vivo (Sonntag, et al., 2010).

Generally, the different parts that compose the system are:

- The core of the system that is a polymeric 3D chip composed by microchannels where the growth media pass. In the chip are present the organoids that simulate the three-dimensional structure of the organ. It's possible to co culture different types of organoids to better simulate the heterogeneity of the in vivo tissues
- A pump system that gives a stimulation using precise mechanical forces and so resembles the sheer stress which can be found inside an organ

The greatest advantage of the organ-on-chip technology is that, unlike the other procedures previously mentioned, it allows to accurately and in real time regulate various important parameters such as: speed, quantity of nutrients, drug tested and the shape and size of the ECM (Duzagac, et al., 2021). The possibility of integrating different types of organoids in

the same chip or in parallel chips provides a useful tool to simulate the passage of pharmaceutical compounds through different tissues and therefore also allows to evaluate the effect due to any metabolites. It is therefore of vital importance to know the essential characteristics that must be taken into consideration in order to obtain a system that is as close as possible to reality. The first example of organ-on chip technology was developed in 2010 by Sonntag et al. who used a chip with six multi-micro-organoids bioreactors in order to maintain human liver, brain, cerebral cortex and bone marrow organoids by customizing the physiological conditions of maintenance for each culture. The ability to simultaneously use different culture conditions for different organoid co-cultures provides a useful tool for studying the organ-organ interface. The precise modulation of the experimental parameters provides repeatable results that are less affected by errors due to the frequent manipulations that characterize the classic methods of organoid culture. With current chip manufacturing techniques, it is possible to precisely create the space where the organoids will be grown and it is also possible to integrate mechanical (Nawroth, et al., 2019) and electrical (Eng, et al., 2016) stimulation devices into the chip. These implementations shortening the time in which the organoids will reach maturity. An important example of the integration of electromechanical and biochemical stimuli to organ-on-chip technology is provided by the study by Marsano et al. where a 3D heart model was created for the study of hypertrophy. Marsano and colleagues first created a place inside the chip with a very specific morphology to confine and shape the material where the tissues were simulated. The matrix was placed on a pneumatic actuator system to deliver cyclic stimuli and precise cultured cells. The system was then used to generate micro-engineered cardiac tissues from human and mouse iPSCs. Compared to the control test, the integration of mechanical stimuli has therefore shown to favour a higher differentiation of heart cells and also to promote the appearance of a synchronized heartbeat (Marsano, et al., 2016). In addition to the integration in the chip of particular technologies to stimulate the cells, it is also possible to modulate the microchannels that make up the latter in order to achieve different objectives. For example, in an effort to create an environment that is closer to reality, it is possible to modulate the diameter that characterizes the micro channels to simulate specific types of blood vessels such as capillaries, veins or arteries (Chip, et al., 2016). Simulating specific blood vessels provides the opportunity to create specific micro and macro environments for almost any tissue to be simulated. Another potential goal in

modulating the geometry of microchannels is the creation of a chemical gradient for the generation of a heterogeneous microenvironment. In this case, the goal is achieved by modulating the shape of the channels using a Y or T shape (Atencia, et al., 2009). In the same way, by modulating the size and shape of the microchannels, it is possible to modulate the sheer stress and therefore conduct studies on how the latter influences cell growth and differentiation (Soffe, et al., 2017). Thanks to the possibility of modulating the concentration of nutrients in real time, of being able to adjust the flow rate and being able to simulate a vascularization system, it is possible to mitigate if not solve the problem of necrosis of the organoid nucleus. With this technology, it is therefore possible to obtain organoids of greater dimensions that do not suffer from the lack of oxygen and nutrients in their nucleus and above all to conduct experiments over much longer periods of time.

Chip fabrication

As previously highlighted, the core of the organ-on-chip system is the chip. This chip is characterized by channels with at least one of them of micrometric size and small chambers where to prepare cell cultures. For the first experiments of this technique, the chip was produced using already tested techniques for the manufacture of microchips. Initially, the materials used were silicone and glass shaped with photolithography and surface micro-matching techniques. Nowadays, the production materials used for the chips have changed, in fact polymeric materials are used, these materials provide various advantages such as low cost, biocompatibility, transparency and greater mechanical resistance. Polydimethylsiloxane, due to its transparency and its permeability to gases, is the most used material for biological purposes. However, this material also has several disadvantages such as low resistance to extremely basic and acid pH, tends to deteriorate even with the use of solvents and has a structural fragility that leads to deformation of the channels and consequent hydraulic losses (Nge, et al., 2013). To cope with these problems, polydimethylsiloxane has been replaced with thermoplastic materials which do not present the problems mentioned above. As in the production of microchips, the technique of photolithography is used for the chips used in organ-on-chip technology. A non-polymeric material such as glass or silicone is used as a substrate upon which a photoactive material is

placed. To model the microchannels and chambers to the desired shape, a mask is used that lets the light pass only into the desired spaces. The photoactive material, in the parts exposed to light, undergoes photochemical reactions that lead to different results depending on whether the material is positively or negatively photoresist:

- **Positive** → Areas exposed to light become soluble
- **Negative** → Areas exposed to light become insoluble

While with positively photoresist materials it is possible to directly obtain the creation of the various elements that will characterize the chip, with negatively photoresist materials it is possible to obtain a mould used to obtain chips in soft lithography techniques. The product thus obtained can be used as a mould to obtain an inverse polymeric replica. The process briefly involves pouring the liquid polymer directly onto the mould. Once solidified, the polymer is removed from the mold, which will have formed the required channels and cavities. The chips can also be obtained by ablative processes by means of laser beams and in the case of thermoplastic polymers by means of thermal modelling processes. Whatever the process, the channels of the chip once completed must be sealed and therefore a polymer layer is fixed on top of them (Alrifaiy, et al., 2012).

Applications of organoid technology

Organoids are used in various fields of research such as tests of pharmaceutical compounds, research on hereditary diseases, the study of diseases that characterize the early stages of development, studies on tumours and also research on the relationships between infectious diseases and tissues damages in the human body (Artegiani & Clevers, 2018). The organoids, as previously mentioned, can be derived from human tissues and therefore provide a realistic and better than animal models. Furthermore, the heterogeneity and the possibility of obtaining specific tissues by modulating the growth factors of the culture medium make organoids an excellent model for the study of infectious diseases. An emblematic study for this application is the one conducted on the relationship between

ZIKA virus and embryo malformations. In this study, brain organoids were used to study the early developmental stages of the human foetus. Cultivated organoids have been infected and the teratogenicity of the pathogen and the mechanisms by which it causes malformations have been demonstrated (Cugola, et al., 2016). After uncovering the processes by which the Zika virus causes malformations, the brain organoids were used to test pharmaceutical compounds capable of counteracting the latter (Nowakowski, et al., 2016). Other types of organoids such as gastric organoids have also been used in studies aimed at investigating the pathogen-epithelium interactions and therefore the mechanisms of infection (Bartfeld, et al., 2015). Organoids used in the study of human infectious diseases are generated from a biopsy of the patient's tissue under examination or through the genetic modification of organoids of the type needed. The genetic modification of organoids has been made faster and easier by the advent of technology clustered regularly interspaced short palindromic repeats (CRISPR). Through this technology, it is possible to target specific genomic locus in a simple and effective way through specific RNA sequences. As for organoids derived from biopsies, an important example concerns human intestinal organoids originating from the tissues of patients suffering from cystic fibrosis. The organoids, like the cells taken, had the CFTR AF508 mutation and are used for the response to various pharmaceutical compounds. The results showed that organoids are an excellent platform for personalized medicine (Matano, et al., 2015). Organoids derived from hiPSCs have also been used in genetic disease studies, for example hiPSC-derived kidney organoids have been treated with CRISPR technology to induce knockout of the PODXL and PKD genes (Cruz, et al., 2017). These mutations have given rise in organoids, as occurs in vivo, to nephrotic syndrome and polycystic kidney disease, providing useful information on the two diseases and their correlation with genetic expressions (Freedman, et al., 2015).

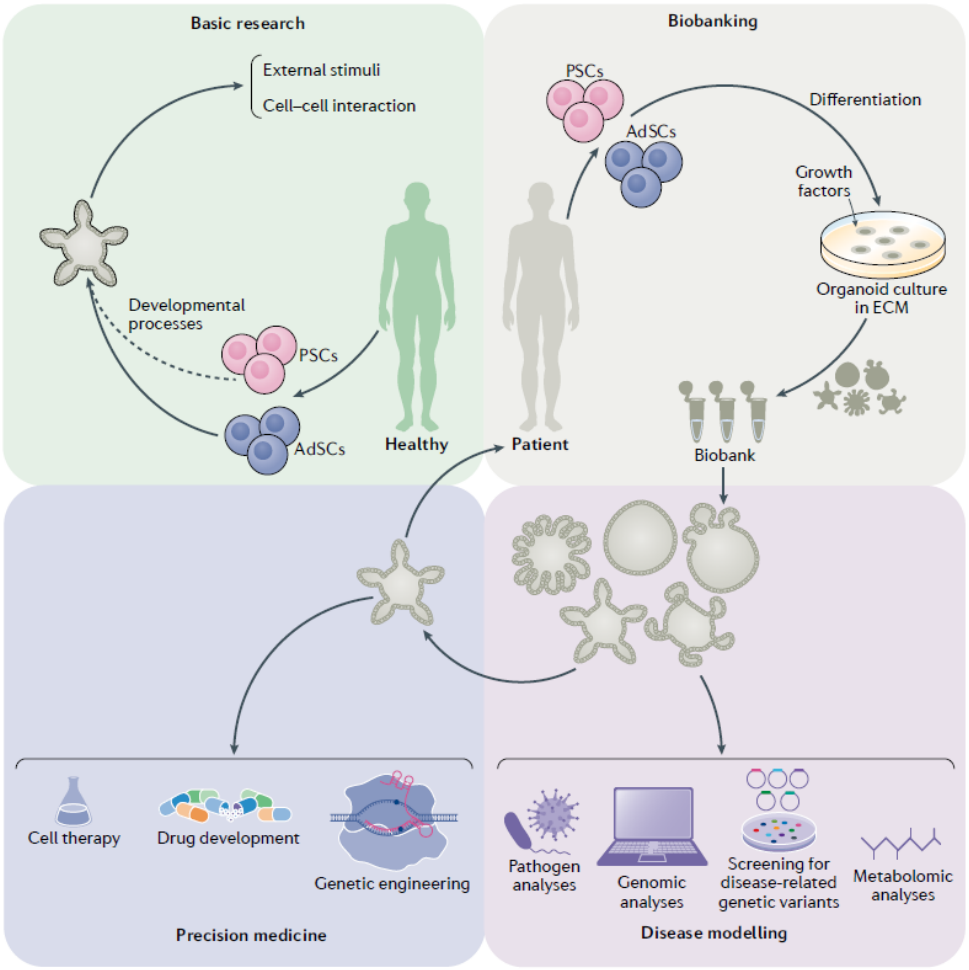


Figure 9 *Potential applications of the organoids in multiple research fields* (Kim, et al., 2020)

Even in the field of cancer research, organoids play a role of fundamental importance. In this field, they are generated starting from cells taken from patient biopsies and are called tumoroids (Lee, et al., 2018). To date, different types of tumoroids have been produced starting from biopsies of primary tumours and also from the collection of metastases. One of the main advantages of tumoroids is the ability to be passed and propagated multiple and potentially infinite times in in vitro cultures. Due to the aforementioned characteristics, tumoroids are suitable for building biobanks representing different types of tumours taken from different patients and which can be indefinitely propagated in order to carry out different tests (Broutier, et al., 2017). The tumoroids contained in this biobank retain the genotype and mutations typical of parental tumour cells and therefore constitute material for experimentation that faithfully represents the tumour tissues and these mutations are

preserved even after several expansions. For the previous characteristics, they are effectively used for the tests of tumour drugs and have the advantage of not diverging from human tumours in vivo as opposed to what happens with tumours transplanted into mice (xenotransplantation). As in the case of the study of genetic diseases, organoids can also be genetically modified through the use of lentiviruses and CRISPR technology to mutate specific associated genes thus making it possible to study even the early stages in tumour development (Tsai, et al., 2018).

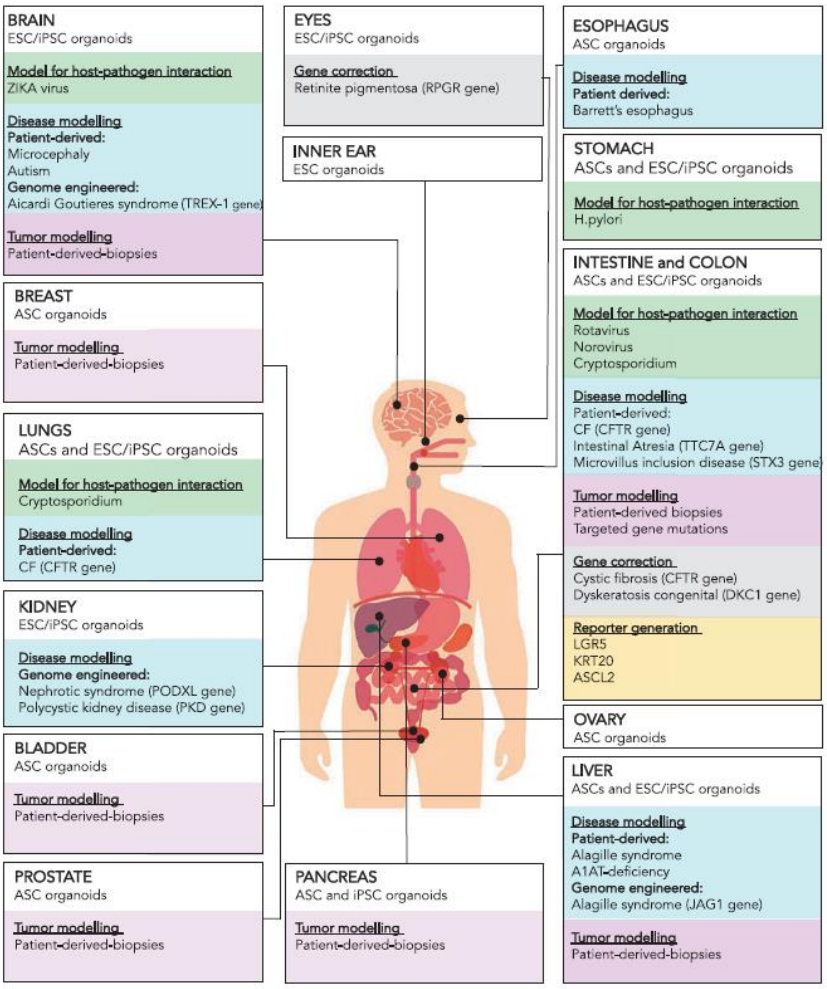


Figure 10 *Representative diagram of the applications of human-derived organoids* (Artegiani & Clevers, 2018)

Aim of the thesis

The aim of the thesis is to analyse how the methods described above influence the growth of organoids and what their potential advantages, defects and uses could be. For this purpose, the already consolidated technique of immersion of the matrix obtained from a reworking of the protocol by Broutier et al. of 2016 was used as a positive control. Several tests were also carried out for the proposed methods. As regards ALI-method, different methodologies have been tested to obtain an efficient gelation of the matrix and also the importance of growth factors in this technique has been tested. In the organ-on-chip technique, different flow rates have been tested in order to obtain a stable environment that allows to obtain a compromise between organoid growth and mechanical resistance of the matrix. Having found the optimal conditions for each proposed technique, a comparative analysis was carried out. Through the analysis of images obtained at different time points and thanks to the use of software for image analysis, advantages and disadvantages for each technique have been highlight. Whit the obtained data it was possible to infer their specific potential use in different research fields.

Materials and methods

Culture of ovarian mouse 3D organoids

Submerged culture using Matrigel® ECM

The protocol used during the experiment is derived from the protocol Broutier et al., 2016. Ovarian organoids were defrosted from frozen culture and then maintained during the period of the experiment. This technique was used as positive control because it's know that assure, in the majority of the cases, the growth of organoids.

Materials used:

- Ovarian mouse medium
- Matrigel® matrix derived from Basement membrane
- Falcon® 24-well Clear Flat Bottom
- Gibco® HBSS (1X) Hanks' Balanced Salt Solution
- Sigma Aldrich® Dulbecco modified eagle's medium/nutrient mixture F-12 Ham (10%FBS 1%P/S)
- Sigma Aldrich® Dulbecco's Phosphate Buffered Saline
- Biological hood
- Incubator 37°C, 5% CO_2
- Falcon® tubes 15/50 mL
- Centrifuge
- FBS: Fetal Bovine serum, Sigma Aldrich®
- DMSO: Dimethyl Sulfoxide, Sigma Aldrich®
- EuroClone® Trypsin 0,05% w/o Calcium, w/o Magnesium w/ Phenol Red Sterile Filtered

Organoids defrosting protocol

Organoids, that were stocked in liquid nitrogen at -180°C, were taken out from the liquid nitrogen container and then defrosted using a hot bath (37°C). The content of the vial is transferred in a 15 mL tube containing 8ml of Dulbecco modified eagle's medium/nutrient mixture F-12 Ham (10%FBS 1%P/S) in order to dilute the Dimetilsulphoxyde contained in the freezing solution. The 15 mL tube is centrifuged at 1500 rpm for 5 minutes to obtain a visible pellet made of cells. The supernatant was removed first using a pipet connected to a vacuum pump and then with a micropipette to avoid the loss of the pellet. The remaining pellet was diluted in a proper amount of Matrigel® (10µl for each well) and then carefully resuspend using a micropipette. Matrigel® was previously transferred from the -20°C fridge where it was stocked to an ice bath to allow its melting. For each well a single 10µl drop was plated and, then, the plate was turned upside down and put into the incubator at 37°C, 5% CO_2 for 30 minutes until the Matrigel® is well polymerized. So, 500µl of medium were added in each well and was substituted every three days.

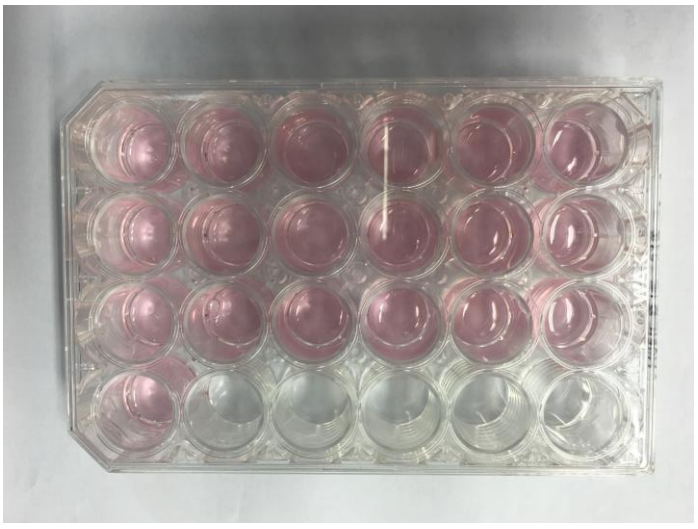


Figure 11 Organoid's plate

Organoids maintenance protocol

Removed the media from the well, organoids are washed with 1 ml of HBSS. 200µl of trypsin 0.05% were added over the mixture of organoids and Matrigel® pipetting energetically in order to break up the organoids into single cells. The resulting mixture was transferred in a 15 ml tube containing Dulbecco modified eagle's medium/nutrient mixture F-12 Ham (10%FBS 1%P/S) used to neutralize the trypsin. The 15 mL tube is left in ice for 15 minutes to allow the liquefaction of the Matrigel® in solution. The sample is then centrifuged at 1500 rpm for 5 minutes and after the elimination of the supernatant the organoids are plated using the procedure described above.

Organoids freezing protocol

Pellet derived from detaching procedure can be stored in liquid nitrogen. Organoids were placed in a cryovial containing 225 µl of media, 225 µl of FBS. At the end 50µl of Dimetilsulfoxide were added as cryoprotectant. The cryovial was freeze in isopropanol at -80°C and then transferred in liquid nitrogen.

ALI methods

The protocol used for these experiments was derived from the one designed by Li et al. (Li, et al., 2016). Different composition for the buffer solutions were tested. Three different solution (A,B,C) are mixed together in a ratio of 8:1:1 and the resulting solution is used to create two distinct layers of reconstituted collagen. The first collagen layer that forms the basal layer with support function and the second one that contains the organoids.

Materials used:

- Sigma-Aldrich® Collagen, type I solution derived from rat tail
- Matrigel® matrix derived from Basement membrane
- Mouse ovarian medium
- Human ovarian medium
- Sigma Aldrich® Nutrient Mixture F-12 Ham
- Milli-q water
- Merck Millipore® Millicell ® Cell Culture inserts 0.4µm, 30 mm diameter
- Sigma Aldrich® Sodium Bicarbonate
- Sigma Aldrich® Sodium Hydroxide
- Falcon® 6-well Clear Flat Bottom
- Falcon® 24-well Clear Flat Bottom
- Litmus paper
- Sigma Aldrich® Dulbecco modified eagle's medium/nutrient mixture F-12 Ham (10%FBS 1%P/S)
- Matrigel® matrix derived from Basement membrane
- Merck Millipore Vented Millex® 0,22 µm filter unit

Solutions preparation

The first part of the protocol is dedicated to the realization of three solutions that during the beginning of the experiments will be mixed together.

Solution	Composition
A (cell matrix type I-A)	*Collagen type I solution derived from rat tail or Matrigel® matrix derived from Basement membrane*
B (10X culture media)	*1 vial of Ham's F12 dissolved in 100ml of sterilized milli-Q water*
C (sterile reconstitution buffer)	*-100 ml sterilized milli-q water* *-0,05 N NaOH* *-200 mM HEPES* *-2,2g $NaHCO_3$*

Table 1 Composition of solutions A, B and C

Solution B preparation:

Ham's F12 powder was taken from its container and transferred to a sterile bottle. 100 ml of sterilized milli-q water were added to the powder inside the bottle and then manually stirred to obtain a 10X medium. The medium was supplemented with 1.176 g/l of sodium bicarbonate. Finally, the reconstituted medium was filtered with a 0,22 µm filter and transferred in two 50 ml falcon tubes.

Solution C preparation:

An amount of milli-q water was sterilized through filtration using vented Millex® 0,22 µm filter unit and subsequently via UV irradiation under biological hood. Sodium hydroxide and then HEPES were added to obtain the specified molarity, finally 2.2g of Sodium bicarbonate were added. The obtained solution was filtered again using Millex® 0,22 µm filter unit. The entire procedure described above was done under biological

hood to ensure proper sterile conditions.

Three different buffer solutions with three different pH were prepared with the goal of finding the proper condition to obtain two stable collagen layers. The three different pH conditions were obtained adding an extra amount of sodium hydroxide 3M solutions.

Buffer solution	**pH**
Buffer solution 1	7.8
Buffer solution 2	11
Buffer solution 3 (*no HEPES*)	11.17

Table 2 pH values for the three buffer variants tested

Plating procedure

Add ice-cold 8 parts of solution B to 1 part of solution A and mix very well pipetting up and down keeping the solution on ice. At this point the solution should appear yellowish due to the interaction of the phenol red with the acid collagen. Add one part of ice-cold solution C to the previously obtained solution, mix very well by pipetting up and down until the colour of the solution turns pink indicating an increase of the pH value. If bubbles are present is possible to eliminate them through a spin using the centrifuge and then mixing again the solution with a micro pipette. The obtained mixture was pipetted in the Millicell ® Cell Culture insert previously inserted in a well of a Falcon® 6-well Clear Flat Bottom. The plate was inserted in the incubator at 37°C, 5% CO_2 to facilitate the polymerization of the first layer of collagen. After approximately 30 minutes the first layer should be polymerized and then is possible to proceed with the deposition of the second layer, containing the organoids, above the first one. A second solution composed by solutions A, B, C was obtained following the same procedure described above, but the volume of solution B, in this case, was previously used to dilute the pellet obtained and then mixed with solution A. The mixture obtained was pipetted over the first layer of reconstituted collagen and incubated at 37°C, 5% CO_2. After 30 minutes also the second layer of reconstituted collagen containing the organoids should be polymerized. If the collagen successfully polymerized, it's possible to add 1 ml of the desired medium at the bottom of the well avoiding touching the reconstituted collagen layers. The

organoids cultured with this methodology can last for more than 30 days.

Changing of media

The medium was changed every three days. Exhausted medium was removed and Organoids were washed twice with 300 µl of PBS, so 500 µl of medium was added to each well.

Detaching of organoids from the cell culture insert

At the end of the experiment the medium was removed using a pipette connected to a vacuum pump. Then 200µl of trypsin 0.05% were pipetted inside the cell culture insert pipetting energetically breaking up the two layers of collagen. The resulting content was transferred into a15 mL tube containing 6 ml of washing medium and so centrifuged at 1500 rpm for 5 minutes. At this point a very dense pellet should be visible at the bottom of the tube and after the elimination of the supernatant 200µl of trypsin 0.05% is added again. 6 ml of Sigma Aldrich® Dulbecco modified eagle's medium/nutrient mixture F-12 Ham (10%FBS 1%P/S) was added to the solution and then the latter was centrifuged at 1500 rpm for 5 minutes. After the elimination of the super natant the pellet containing the organoids was used to make further experiments.

Organ-on-chip

The microfluidic setup is composed by the following components:

- Pressure pump
- Fluigent™ Flow EZ™ EZ LU-FEZ-7000
- Falcon® tubes 15/50 mL
- Fluigent™ Pressure unit EIPS345
- Pipes
- Evos Auto 2 incubator chamber
- Organ-on-a-chip – Complete setup (MS-kit-01)
- Becker

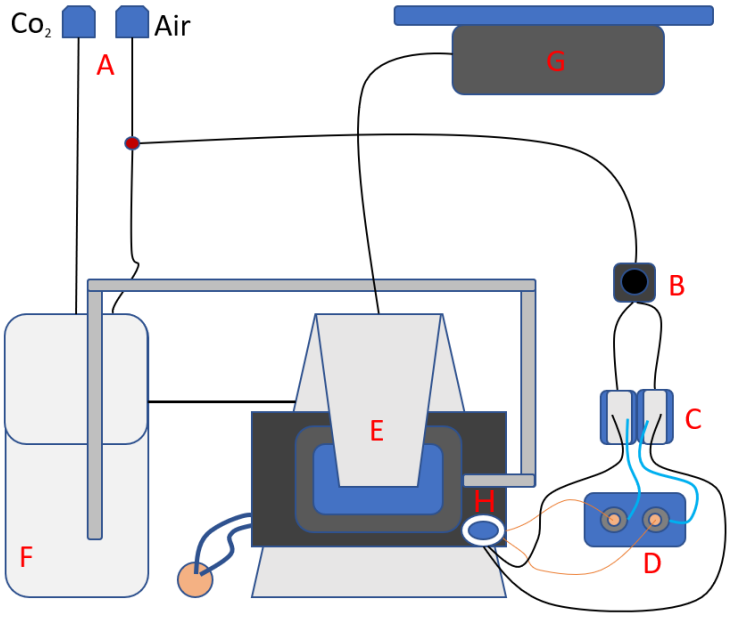


Figure 12 Organ-on-chip system setup used in the thesis. In the image are depicted the different parts that compose the system: **A** CO_2 and air valves used for incubator (CO_2 and air) and for EZ system to give pressure. **B** Valve to regulate the air inflow. **C** Fluigent Flow EZ **D** Falcon tubes containing the media and connected with the system with p-caps **E** Evos Auto 2, incubator chamber containing the chips **F** Incubator system **G** Computer and monitor **H** Pressure unit

The organoids were resuspended in a 30 µl Matrigel drop and then plated on the circular cavity located in inferior part of the chip MS-kit-01. Using the four magnet at the edge of

the chip, the latter was closed and placed in Evos Auto 2 incubator chamber (fig 12 E). The main characteristic of this system is the continuous flow of growth medium through the chip where the organoids are plated. In order to provide a regular influx of media, a system of different devices was connected to the chip. A pump provided a pressure of 2 bar to the Flow Ez (fig 12 C). This device regulates the pressure and the flow rate of the entire system. The desired pressure, regulated by Flow Ez was then applied to the medium contained in a 50 ml tube which is connected to the system with a P-cap (fig 12 D). Due to the pressure the medium flows through the pipes to the chip. Before reaching the chip, the flux encounters the flow unit (fig 12 H) that, measuring the change in temperature gives information to Flow EZ for regulating the pressure. This system allows of obtaining the desired flow rate (1µl/min). After passing the drop of Matrigel the medium is discharged into a Becker. Auto 2 software was used to set a photos time lapse and to regulate the incubation conditions, 37°C, 5% CO_2.

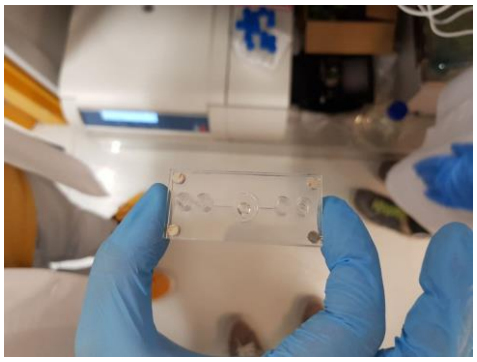


Figure 13 Drop of Matrigel inside the Chip

Figure 14 P-caps and pressure units

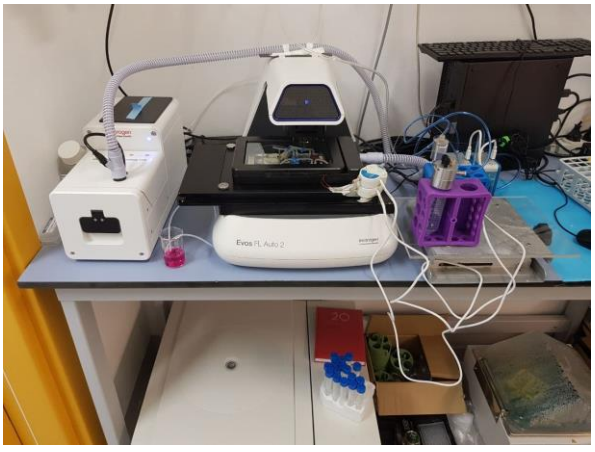


Figure 15 Final setup for two chips

Methods of analysis

To evaluate the difference in the organoid's growth cultured in the different methodologies previously described, several photos were taken at regular interval of time. Two different microscopes (Evos FL Auto 2 and Nikon eclipse TS2R) were used to take the photos of the experiments.

Nikon eclipse TS2R

This optical microscope was used to take photos of the plates in which organoids were cultured. Nikon eclipse TS2R was mainly used when the Evos FL Auto 2 was unusable because occupied by the chip of the microfluidic technique. The photos were taken with a zoom of 2x,4x,10x.

Figure 16 Nikon eclipse TS2R (Nikon, s.d.)

Invitrogen EVOS™ FL Auto 2 Imaging System

This instrument is a high-performance, fully-automated, multi-channel fluorescence microscopy. This microscope has an incubator chamber where the microfluidic experiments were performed.

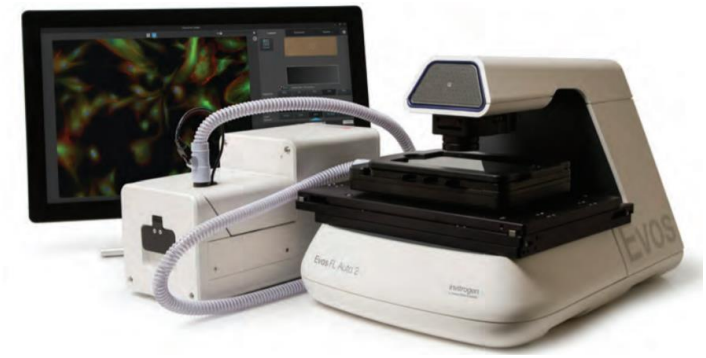


Figure 17 Invitrogen EVOS™ FL Auto 2 Imaging System with incubator system **(Scientific™, s.d.)**

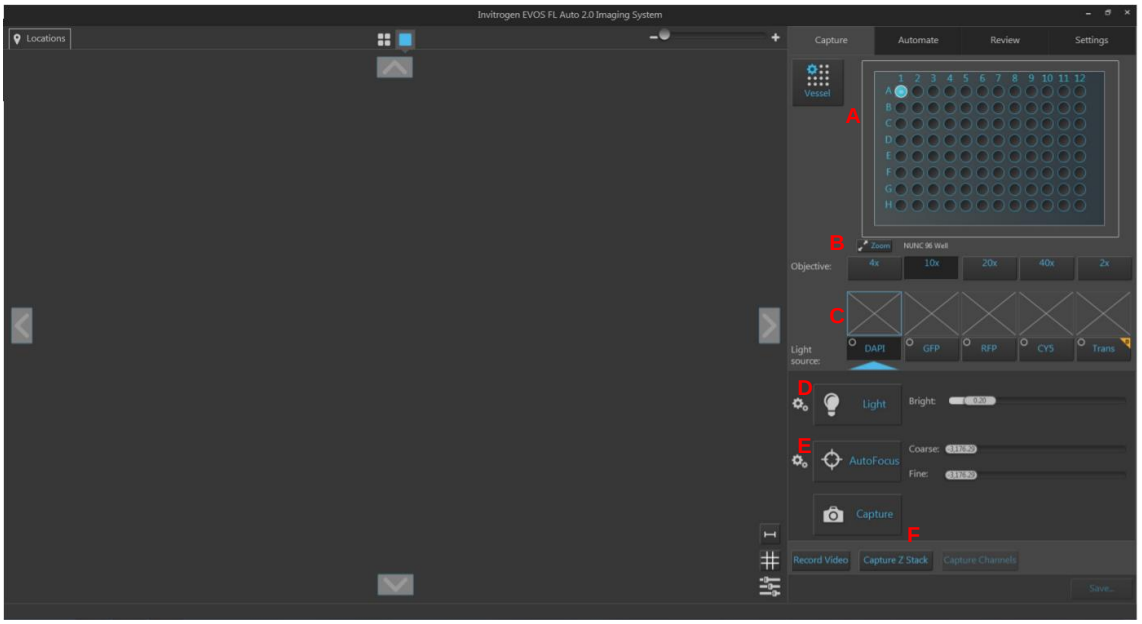


Figure 18 Image capture screen **A** Selection of the type of plate **B** Zoom selection **C** Selection of type of light source **D** Light source intensity selection **E** Focus settings **F** Commands for image acquisition

Inclusion in paraffin

The exhausted medium was eliminated from the plate and the organoids were washed with 200 µl of PBS. So, 20 µl of PBS were added to each well and were used to help to detach the organoids drop with a 0,5 cm large spatula. The Organoids were placed in a Tissue-Tek Cryomold filled with 0,8 mL of liquid BioAgar maintained at 50° C. When the BioAgar was solidified it was transferred inside the two wraps previously wetted with milli-q water and then into the cassette. The cassette is maintained in formalin 10% and then included in paraffin. After the inclusion in paraffin the sample was cut in slice wide 2.5 um using a microtome to perform Immunohistochemical analysis.

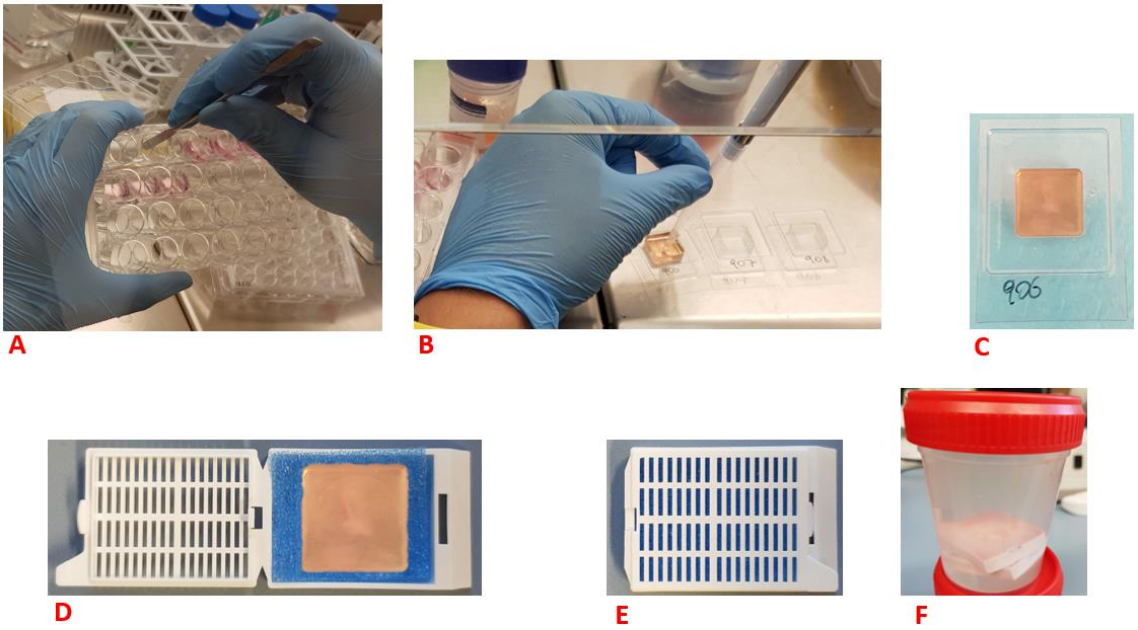


Figure 19 Procedure for embedding the sample in paraffin **A** Removal of the sample from the well **B** Deposition of the sample in the Bio Agar **C** Solidification of the Bio Agar **D** Deposition of the solidified Bio Agar in the wet sponges **E** Closing the cassette **F** Immersion of the cassette in paraffin

ImageJ

To better evaluate the growth of organoids, ImageJ graphics software it's been used. This software it's been used to measure the area of each organoid trough the "measurement tool". Once the areas of all the organoids of the image were defined, the "summarize command" was used to obtain an average size. The average area was then normalized to average area taken at T0.

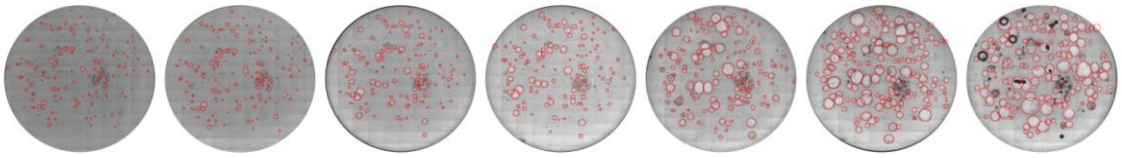


Figure 20 Example of image processing to find the average area

Results and discussions

In the following tests, the advantages and disadvantages of the three techniques for the culture of organoids mentioned above were analysed. For alternative, such as Ali methods and microfluidic, various conditions and variations to the original protocol were tested in order to obtain the optimal conditions for the comparative test. For the different tests, the images obtained by EVOS™ FL Auto 2 Imaging System and Nikon eclipse TS2R optical microscope will be proposed. For each experiment, the growth curves, sizes, shapes and number of organoids grown in the culture protocol were also analysed.

Buffer solutions selection for the ALI-method technique

The protocol for the ALI method technique was obtained following the procedures described in the paper "An Air – Liquid Interface Culture System for 3D Organoid Culture of Diverse Primary Gastrointestinal Tissues "by Xingnan Li, Akifumi Ootani, and Calvin Kuo". In this paper, as explained in the materials and methods section, three distinct solutions are used (A corresponding to the scaffold, B consisting of 10X medium and C buffer solution) with a ratio of 8:1:1. In the native paper, it is suggested to increase the pH of the C solution to reach a value of 11 (when Sigma-Aldrich® Collagen, type I solution derived from rat tail, is used). For this reason, it was tested different buffer compositions in order to understand which one gives the best results in collagen formation. Three different buffer compositions were then tested.

For this test, organoids were plated with both ovarian mouse medium which contains growth factors and HAM's F-12 medium without growth factors. In the original protocol, the ALI-methods is presented as it is able to generate organoids even without growth factors. Below, in the following table, photos of five different time points for different cultures conditions, obtained using the EVOS™ FL Auto 2 Imaging System, were shown.

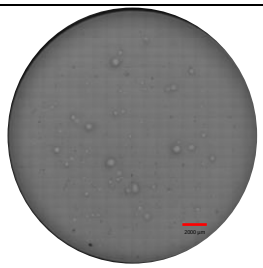
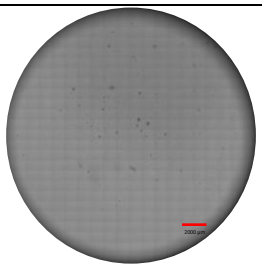
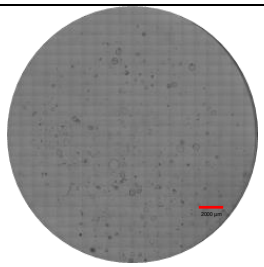
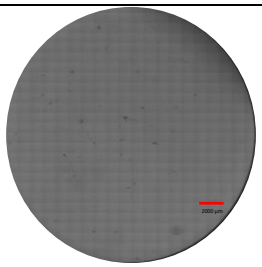
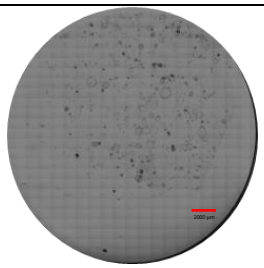
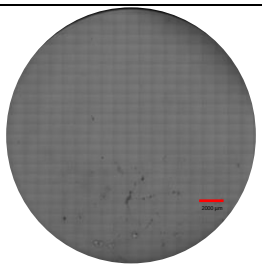
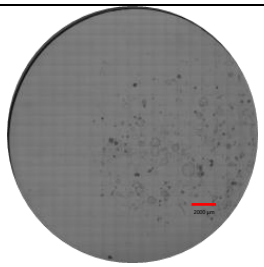
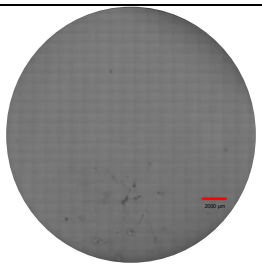
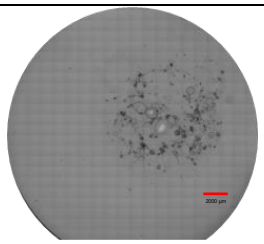
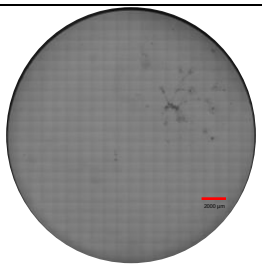
Buffer 1 with Ovarian mouse media (10X zoom)	*Time(h)*	*Buffer 1 with HAM's F12 media (10X zoom)*
	***24***	
	***48***	
	***72***	
	***96***	
	***120***	

Table 3 Time points of the wells containing murine liver organoids. Comparison of culture medium

From the previous images (Table 3) it can be seen that already at the time T0 (24h) it is

possible to observe organoids formations. The growth of organoids is increased by using culture medium specific for mouse ovarian organoids, which is characterized by containing specific growth factors, small molecules and other components that effectively simulate the conditions of stem's niche, as it is in vivo condition. On the contrary, using HAM's F12 medium devoid of growth factors, at T0 no formation of 3D clusters of cells is noted. On the contrary, probably suffering cells are noted forming 2D aggregates. This aspect becomes more evident at time T5 (120h) where these aggregates become more extensive and form connections between each other (fig 21).

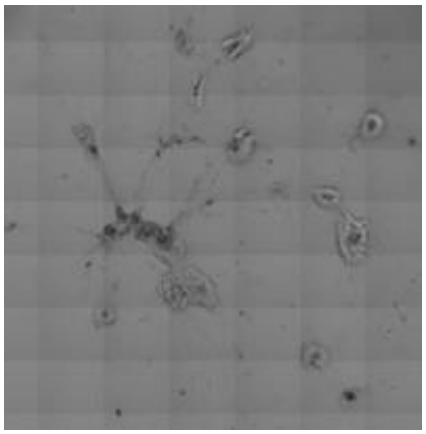


Figure 21 Formation of bidimensional clusters of cells

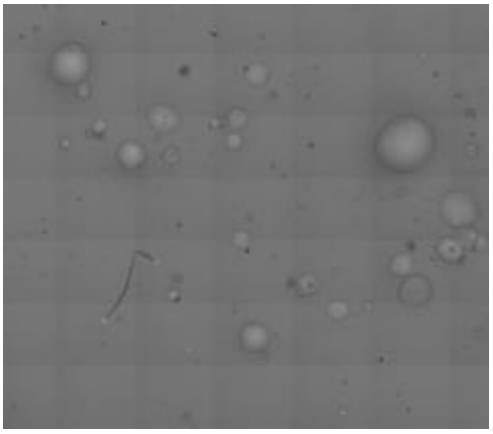


Figure 22 Formation of three-dimensional ovarian mouse organoids

Although there is a formation of organoids with specific and well-defined shape (fig 22) already from the time T2 (72h), there is a progressive weakening of the upper layer of collagen which leads to a progressive liquefaction of the latter. This phenomena leads to a consequent thickening of the organoids and therefore to their death (fig 23). This phenomenon occurs in both types of growth media. It was hypothesize that it is due to an incorrect pH condition during the first phases of collagen gellation. The wrong pH (too acidic) could therefore create a weak collagen layer unable to withstand the stress caused by a progressive environmental acidification due to the growth of organoids.

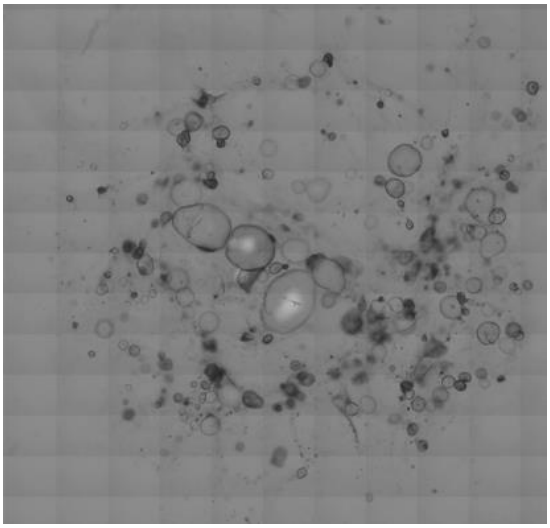


Figure 23 Collapse of the collagen layer and death of the organoids

Indeed, collagen needs a basic environment to polymerize and therefore an acid environment necessarily leads to non-polymerization.

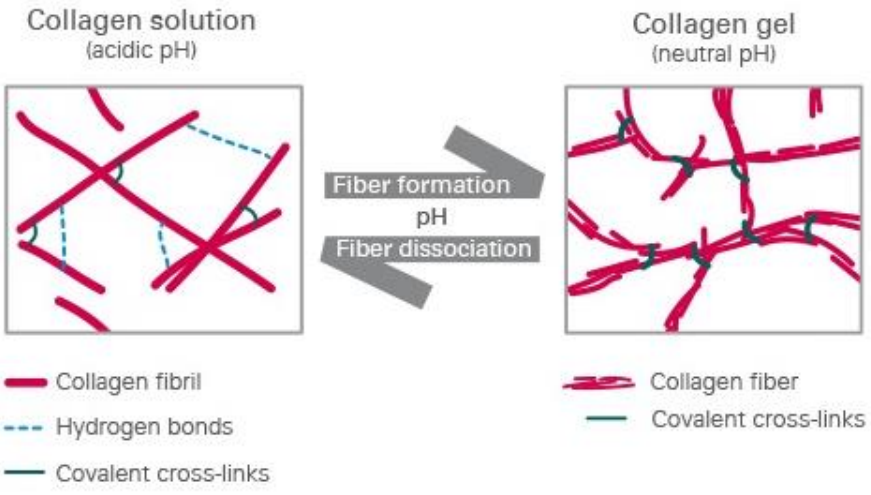


Figure 24 Collagen gelation process

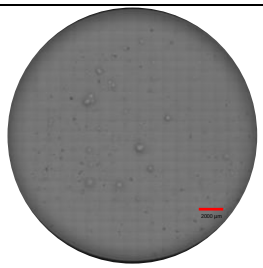
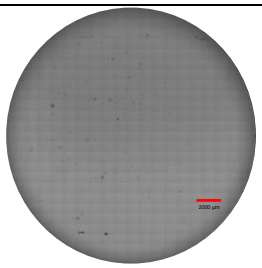
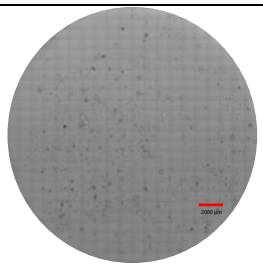
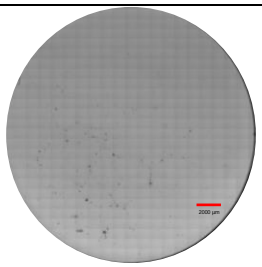
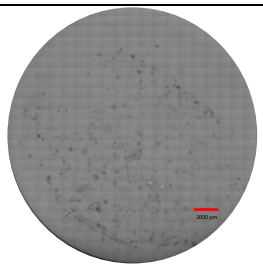
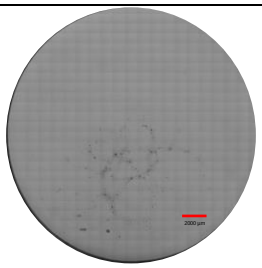
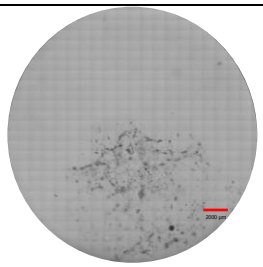
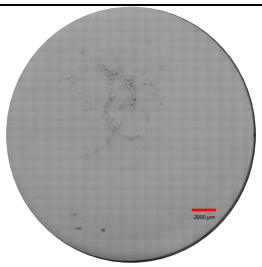
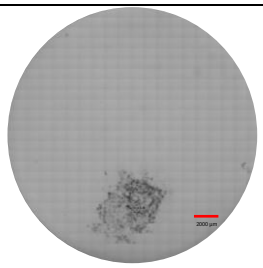
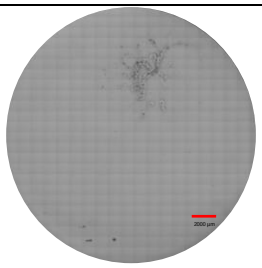
Buffer 2 with Ovarian mouse media (10X Zoom)	*Time(h)*	*Buffer 2 with HAM's F12 media (10X Zoom)*
	***24***	
	***48***	
	***72***	
	***96***	
	***120***	

Table 4 Time points of the wells containing murine liver organoids. Comparison of culture medium

The results obtained using buffer 2 (table 4) are similar to those obtained with buffer 1 but the organoids grown with the ovarian mouse medium already appear to be in distress

at time T1(48 h) (fig 26). At the same time point the collagen begins to yield following a similar course to the previous one but slightly faster (fig 26)

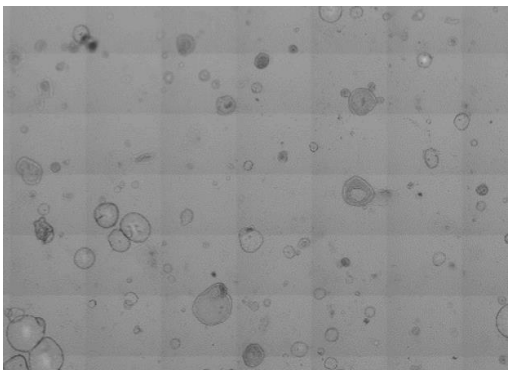


Figure 26 Organoids in distress at time point T1(48h)

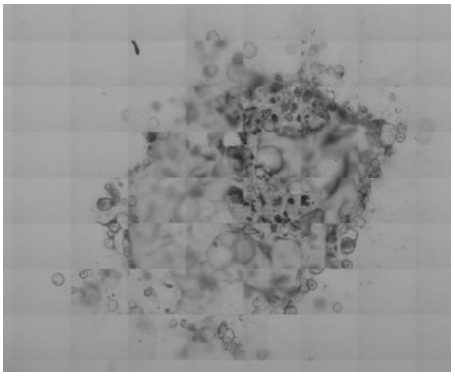


Figure 25 Collapse of the collagen layer and death of the organoids

As regards the use of the HAM's F12 medium, an initial formation of 3D clusters is noted (fig 27) which, however, already at the time T1 (48h) begin to differentiate and form clusters of 2D cells as in the previous case (fig 28).

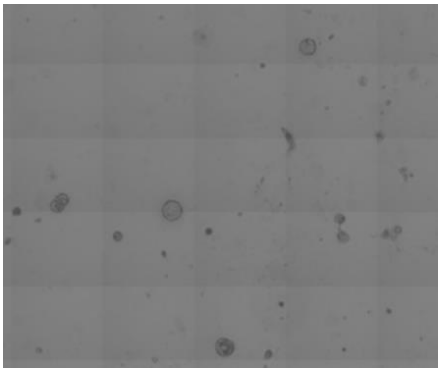


Figure 27 Formation of organoids in the absence of growth factors

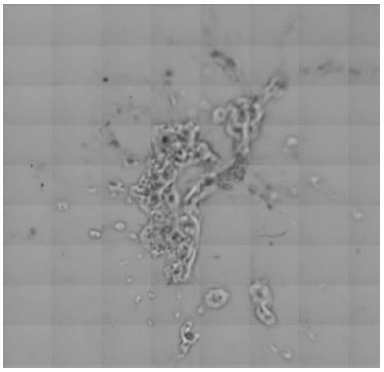


Figure 28 Formation of bi-dimensional cluster of cells

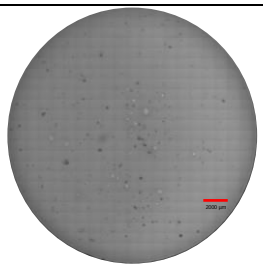
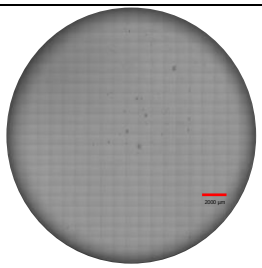
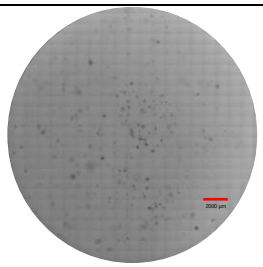
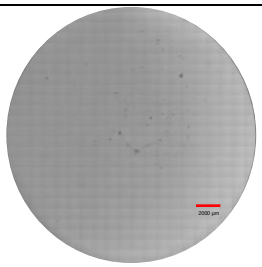
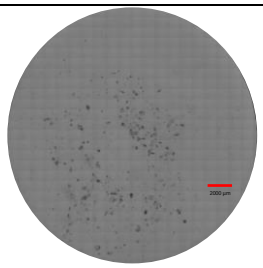
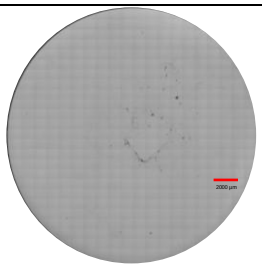
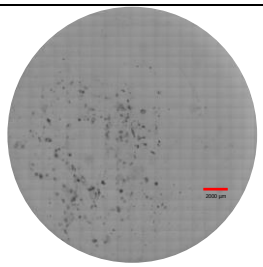
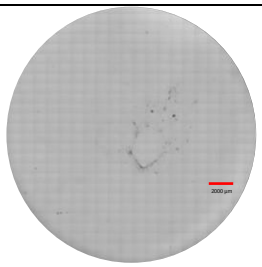
Buffer 3 with Ovarian mouse media (10X Zoom)	Time(h)	*Buffer 3 with HAM 12 media (10X Zoom)*
	***24***	
	***48***	
	***72***	
	***96***	

Table 5 Time points of the wells containing murine liver organoids. Comparison of culture medium

As regarding the buffer 3, the cell development is similar to the previous tests but in this case the collagen layer showed sealing problems already at the time T0 (24h) and was unable to withstand until the time T4 (96h) with consequent suspension of the experiment. Therefore, albeit with the limitation of the settlement at time T2, buffers 1 and 2 appear more promising, respect to the buffer 3. The images obtained by the Evos Auto 2, were then analysed with the imageJ program using the method described previously in the materials and methods section. In such way, it is possible to obtain a

growth curve that allows to evaluate the growth in terms of organoid size.

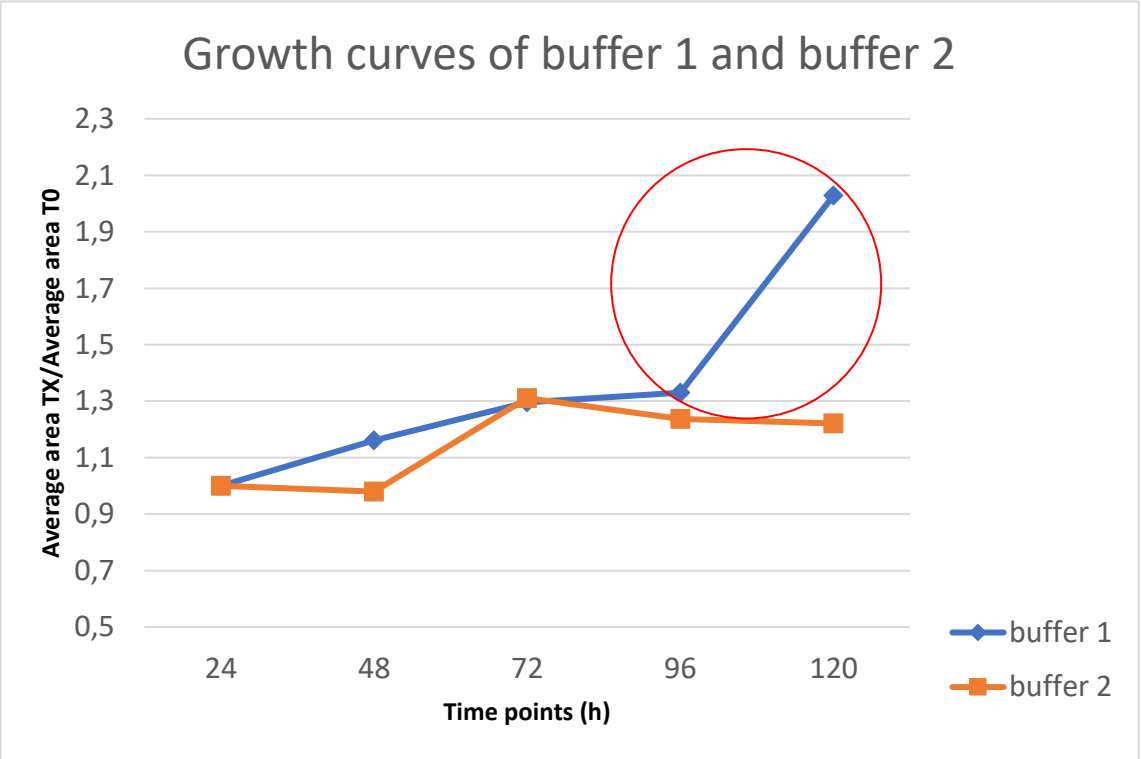
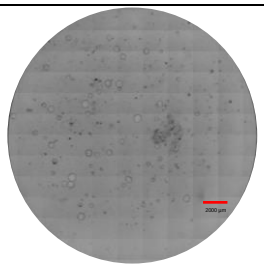
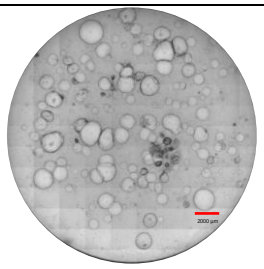
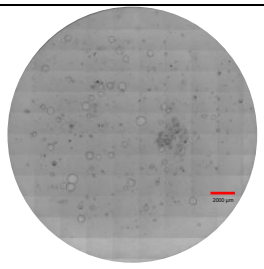
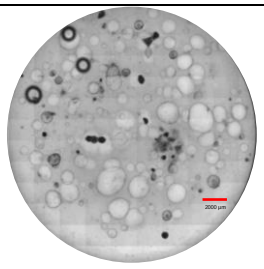
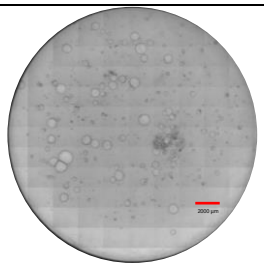


Figure 29 Buffer 1 and 2 growth curves.

The growth trend is similar in the two buffers (buffer 1= blue line and buffer 2= red line). In the case of buffer 1 it is more constant, leaving out the slope (red circle in the graph) of the last time point. This last data has not been considered because the organoids, which are in the sample, have fused together and therefore their apparent size increases. The initial decrease in buffer 2 curve should coincide with the situation of relative suffering that can be seen in the photo of time T1 (48h). Following the analysis of the morphology and number of the organoids, and above all considering the greater resistance of the collagen reconstituted with buffer 1, it was decided to use the latter for the comparative analysis.

Test for the choice of the layer material

In the following experiments, Matrigel and collagen were tested as matrices constituting the two layers that make up the ALI-method system. Collagen is originally used as a matrix in the protocol. Mouse ovarian organoids are first used, and later also human ascites in order to understand if this culture protocol is also suitable for the screening of drugs against human cancers. The mouse ovarian organoids used for the following test were taken from the well where buffer 3 was tested.

Time(h)	*Buffer 1 with Ovarian mouse media and Matrigel (4X Zoom)*	*Time(h)*	*Buffer 1 with Ovarian mouse media and Matrigel (4X Zoom)*
24		***144***	
48		**168**	
72			

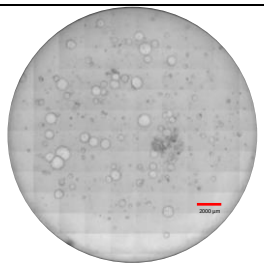
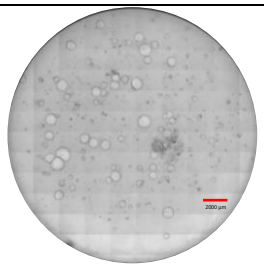
96			
120			

Table 6 Time points of the wells containing murine liver organoids cultured following ALI-method using Matrigel® and buffer 1

As can be seen from the images since the time T0 (24h) there is a growth of large organoids with a proper shape accordingly to the literature (fig 30). The organoids then continued to grow to very large sizes up to over 1 mm at time T6 (144h)(fig 31). The experiment lasted for over a month demonstrating the solidity the endurance of the matrix. By the end of the trial, organoids had reached such a size that all the media added in the morning was consumed by the late afternoon.

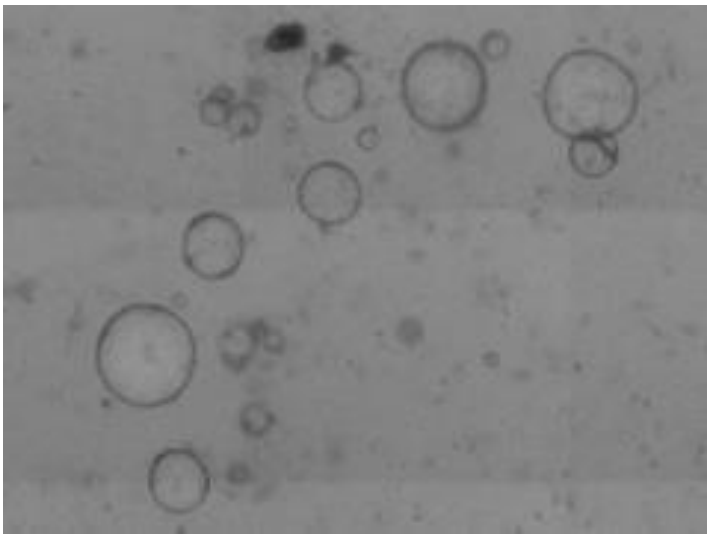


Figure 30 Organoids formation at 24h

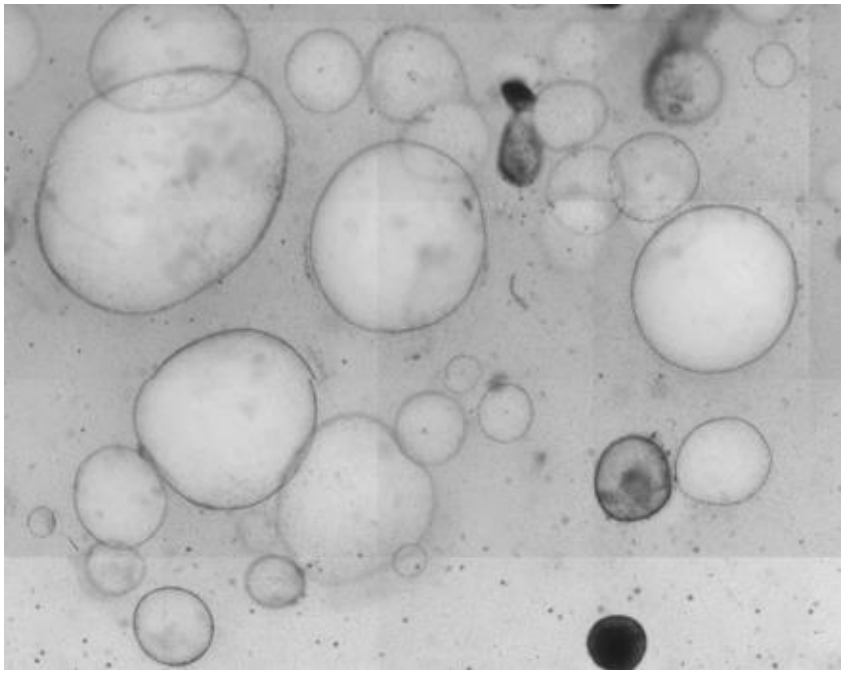


Figure 31 Organoids growth at 144h

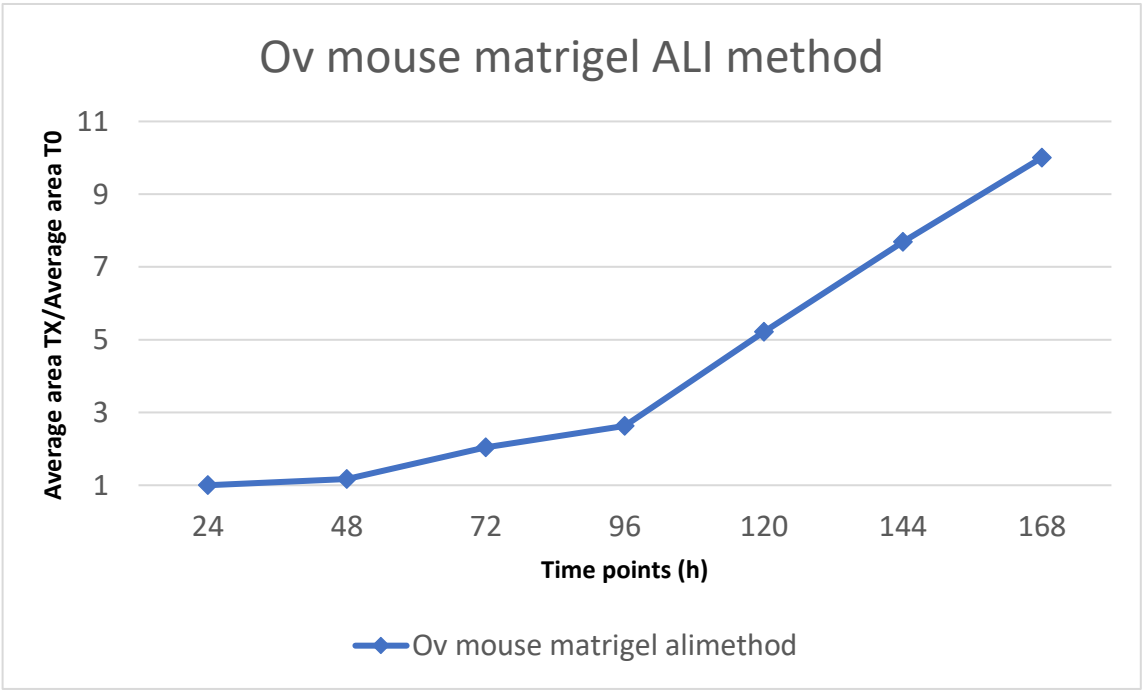
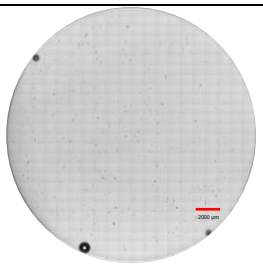
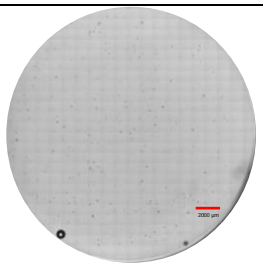


Figure 32 Graph of the growth curve of mouse ovarian organoids in Matrigel following the ALI-method protocol

In the growth curve shown above (fig 32), which describes the growth of the organoids

used in the test just described, a slightly growth can be seen greater than the growth curve obtained using the same buffer (buffer 1) with the collagen. Up to the time T3(72h) the difference is not so marked but from the point T3(72h) onwards it is evident that the Matrigel has provided a better environment for the growth of organoids than collagen. This fact can be explained probably due to its better stability during time. This prolonged growing trend over time demonstrates the ability of the ALI-method to support a constant growth of organoids even for long periods of time. Human ascites (obtained from totally anonymized specimens and, biobank informed consent for research purposes was available to collect the samples at National Cancer Institute of Aviano) were then plated using Matrigel as matrix, solution 1 as buffer and human ovarian medium as culture medium. The test is aimed at testing the survival of this type of organoid which has more "difficult" culture conditions, but which plays a role of great importance in the field of personalized medicine.

Time(h)	*Buffer 1 with Matrigel H.ascites and H.Ovarian media (10X Zoom)*
24	
48	

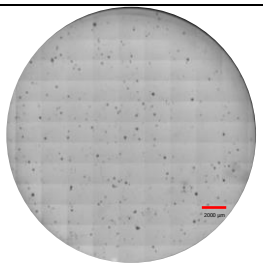
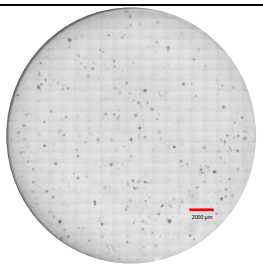
72	
96	

Table 7 Time points of the wells containing H.Ovarian organoids derived from ascites cultured following ALI-method using Matrigel®, buffer 1 and H. ovarian media

A progressive growth can also be noted in the case of this type of organoid (fig 33). To demonstrate that this culture method is also suitable for ascites, a growth curve was then elaborated to compare the results obtained with mouse ovarian organoids.

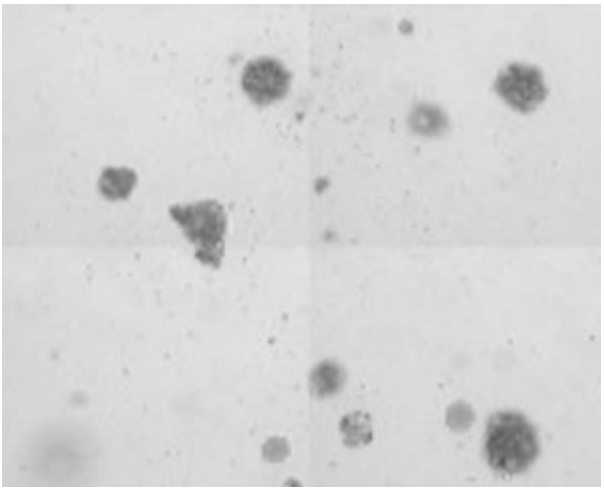


Figure 33 H. ovarian organoids derived from ascites

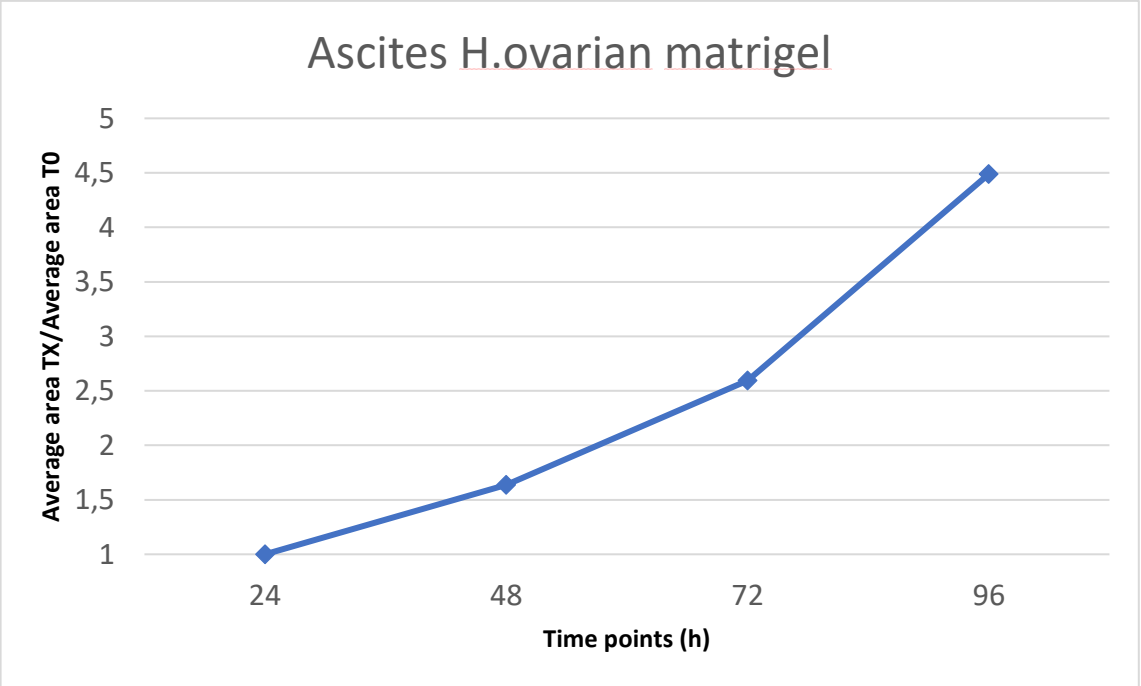


Figure 34 Graphic of the growth curve of human ovarian organoids in Matrigel following the ALI-method's protocol

As it is possible to observe from the growth curve, the growth of the two types of organoids is very similar. Indeed, the two curves have more or less the same slope. It is therefore evident that also for the culture of human ovarian organoids, the Ali-method lends itself to being a valid alternative to the classical methods of immersion culture. As in the previous experiment, where the different types of buffers were tested, in the following test the ability of the Ali-method to support the growth of ascites with a medium containing no growth factors was tested (Advanced DMEM). The two types of growth mediums, Human Ovarian and Advanced DMEM, were then compared using a growth curve obtained as in the previous tests.

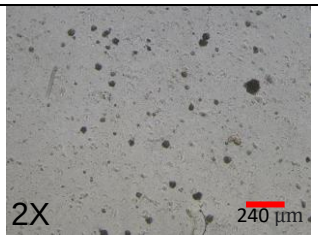
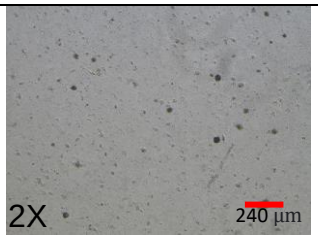
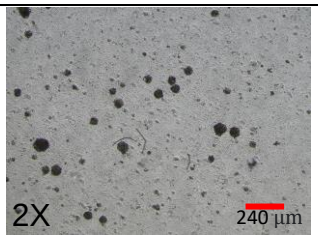
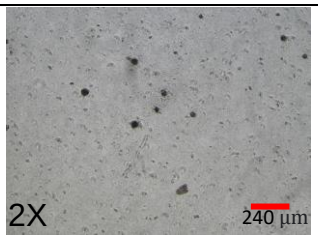
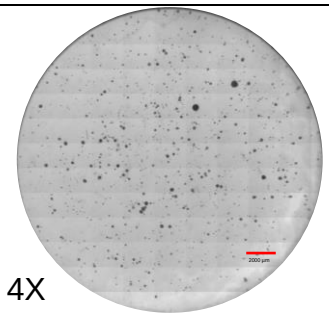
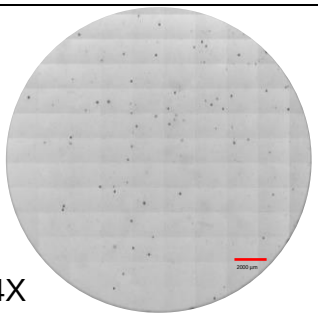
ALI method, ascites cultured with H.Ovarian medium	*Time(h)*	*ALI method, ascites cultured with Advanced DMEM*
	***24***	
	***48***	
	***72***	

Table 8 Time points of the wells containing H.Ovarian organoids derived from ascites cultured following ALI-method using Matrigel®, buffer 1 . Comparison between H.Ovarian medium and Advanced DMEM

From the images derived from different time points (table 8) it is shown a growth of ascites even in the medium without specific growth factors for the growth of this type of organoids. This could be due to the presence of components that mimic the habitat in vivo within the matrigel. In the case of the buffer test, on the other hand, the medium without growth factors was tested in a matrix composed entirely of collagen derived from rat tail. The collagen represents the 30% of the composition of matrigel. Although ascites growth is observed also in Advanced DMEM it is evident that this growth, both in terms of size and numbers, is much lower than the culture in the presence of Human Ovarian medium. The growth curve (fig 35) further highlights how the growth of ascites in Human ovarian medium is greater than in Advanced DMEM medium and therefore the first medium is to be preferred for the following comparative analysis.

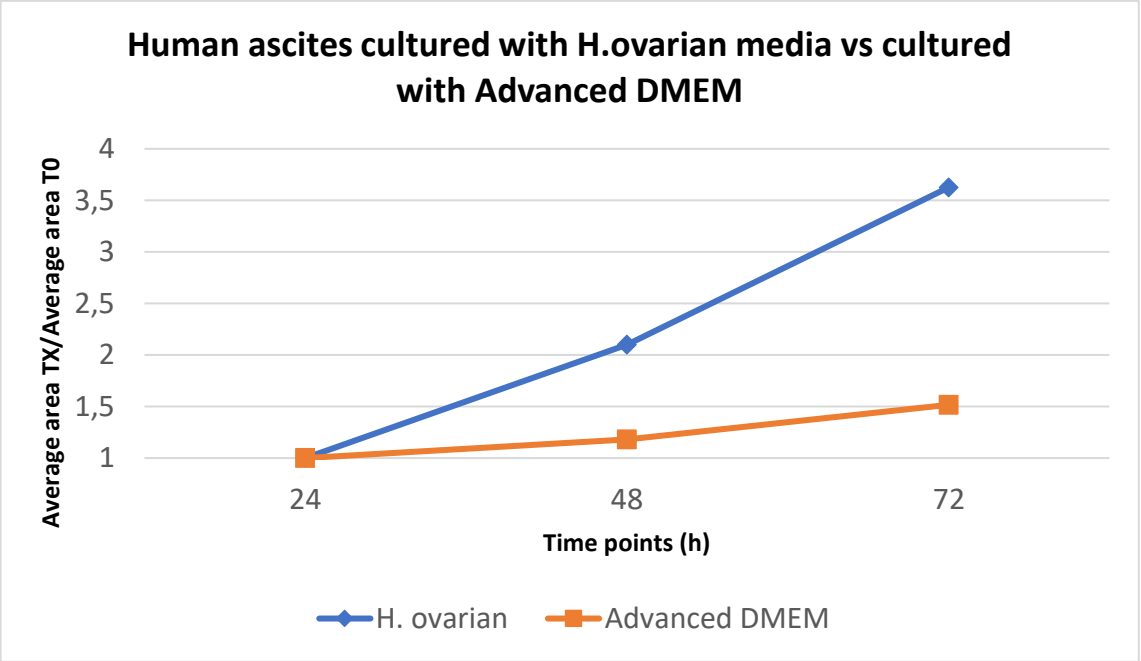


Figure 35 Graphic of the growth curve of human ovarian organoids in Matrigel following the ALI-method's protocol using H. ovarian medium and Advanced DMEM

In the next test, collagen reconstituted with buffer 1 was used again for the two layers. Since in the previous tests the collagen had already shown signs of failure after 48 hours, a further modification was made to the initial protocol, to improove its stability. Assuming that the collagen solution did not reach an adequate pH level for its gelation and, since increasing the pH level of the buffer solution did not bring substantial improvements, it was decided to add a volume of about 50 µl of 1M NaOH solution. In such a way, the reconstituted collagen reach a value of pH of about 10. Ascites were then plated onto a reconstituted collagen substrate using the method discussed above and using Human ovarian medium.

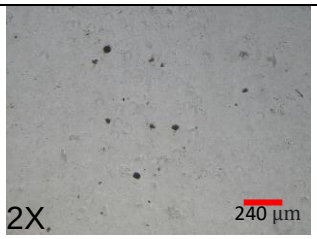
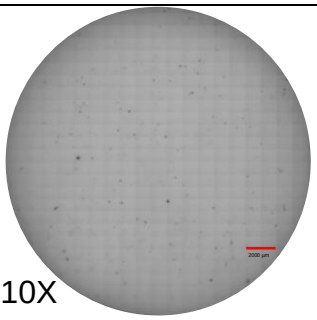
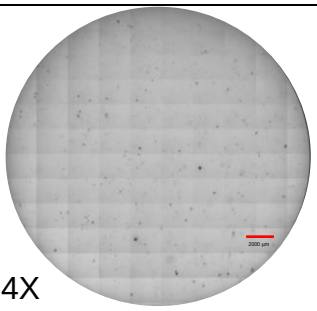
Time(h)	*Buffer 1 with Collagen H.ascites and H.Ovarian media*
24	
48	
72	

Table 9 Time points of the wells containing H.Ovarian organoids derived from ascites cultured following ALI-method using Collagen, buffer 1 and H. ovarian media

The development of ascites is noted since the time T0(24h) and the growth continues until the time T2(48h), when the experiment was suspended. However, there is an inequality in size and number between ascites in matrigel culture and ascites in collagen culture, the latter having a slightly smaller size and present in smaller quantities. Moreover, the growth trend is similar to that of the previous test curve for ascites culture in matrigel using human ovarian medium (fig 36). As for the tightness of the collagen layers, it can be seen from the images that these are still gelled and do not give any sign of subsidence, confirming the thesis of the need for a more basic environment for our specific type of collagen.

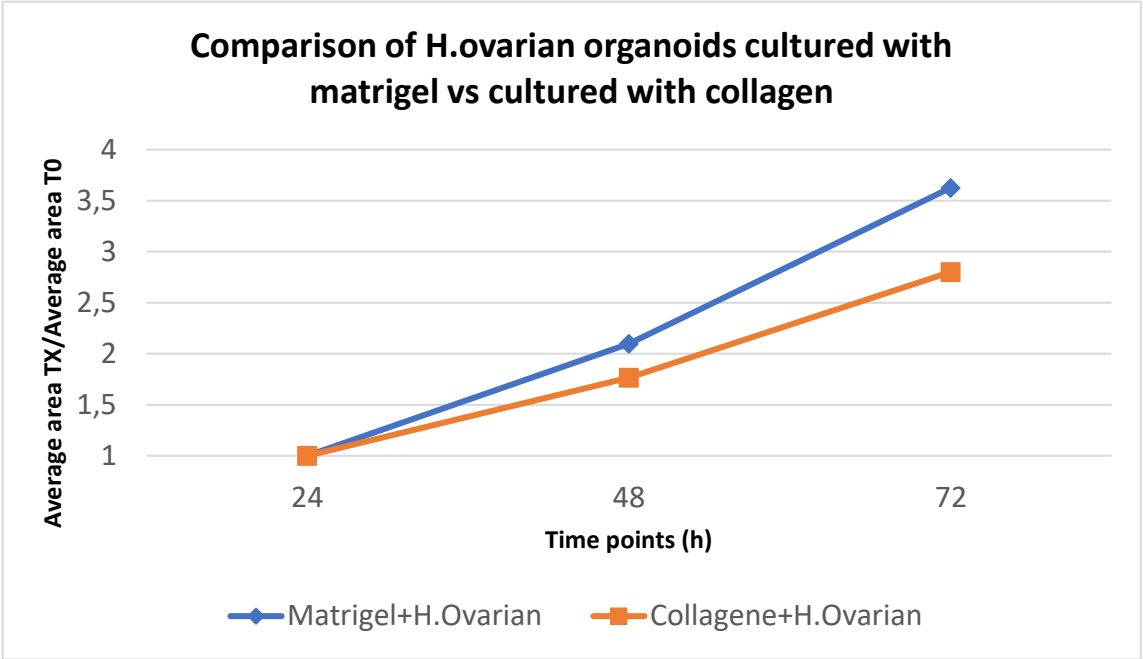


Figure 36 Graph of the growth curve of human ovarian organoids in matrigel and collagen following the ALI-method protocol

In light of the previous results, it was decided to use collagen as a matrix to make up the two layers for the comparative analysis, as far as the ALI-method is concerned. Although collagen results in ascites, of relatively smaller size and with a smaller population, it offers the advantage of having a known combination (collagen proteins) and of having a much lower cost than Matrigel.

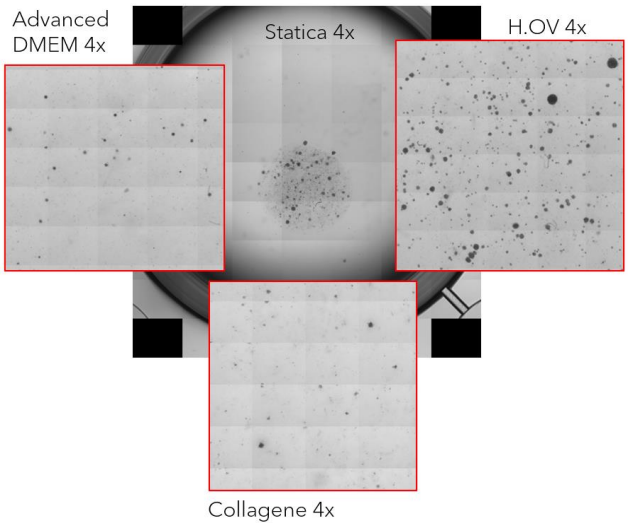
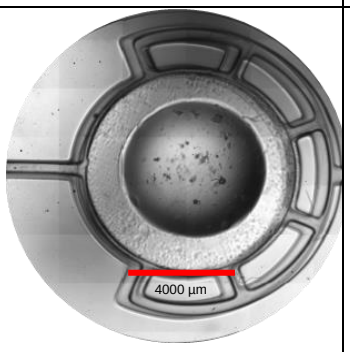
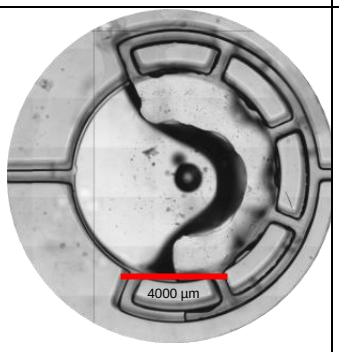
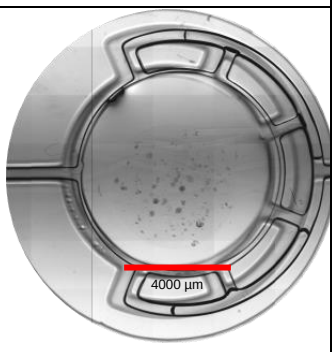
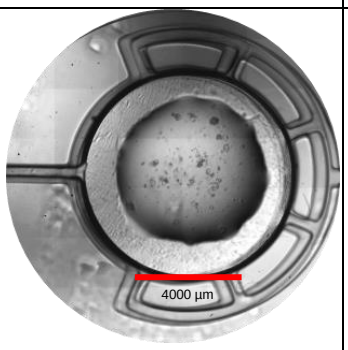
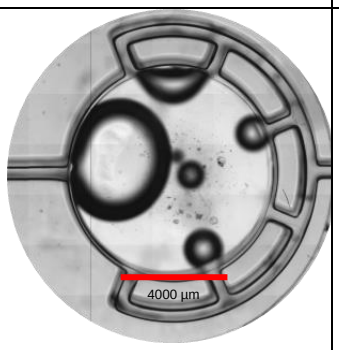
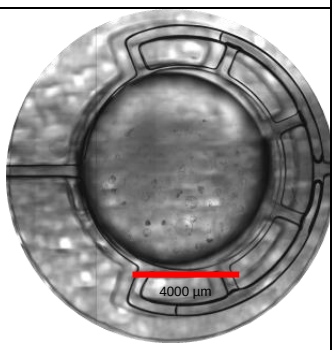
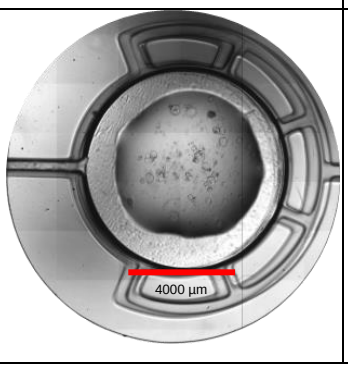
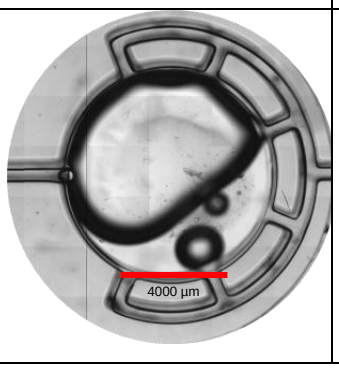
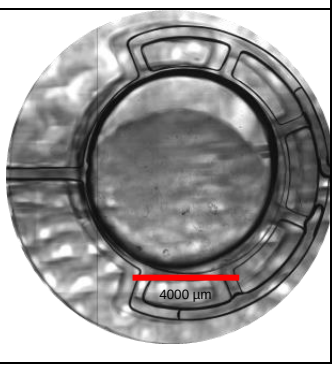


Figure 37 Comparative images of Human ovarian organoids

Flow velocity analysis for the microfluidic technique

An analysis on the growth of organoids with different flow rates (1 µl / min, 5 µl / min and 7 µl / min) is proposed below. Murine hepatic organoids were used for the test. These were cultured using the microfluidic system discussed in the materials using mouse ovarian medium. Also in this case, a growth curve is proposed which compares the growth of the organoids subjected to the three different flows (fig 42)

Time (h)	*1 µl/min (4X Zoom)*	*5 µl/min (4X Zoom)*	*7 µl/min (4X Zoom)*
0			
24			
48			

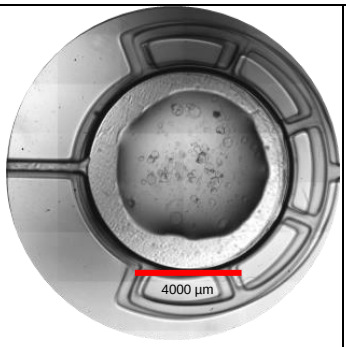
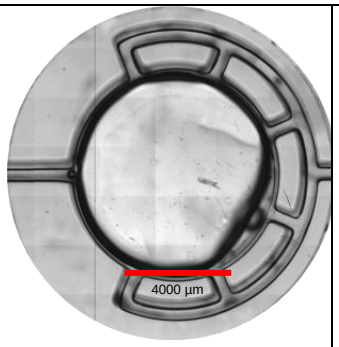
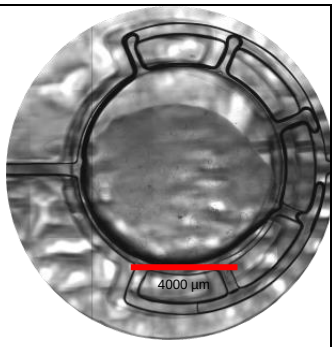
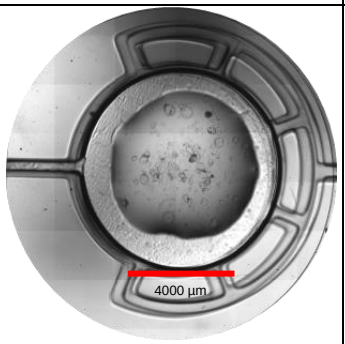
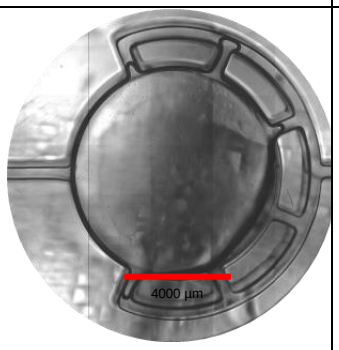
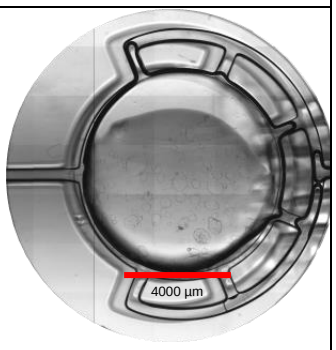
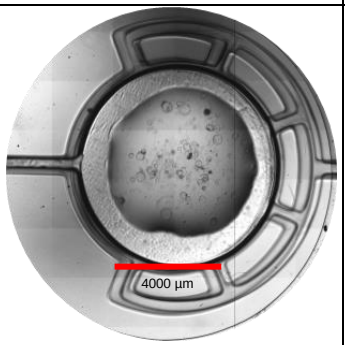
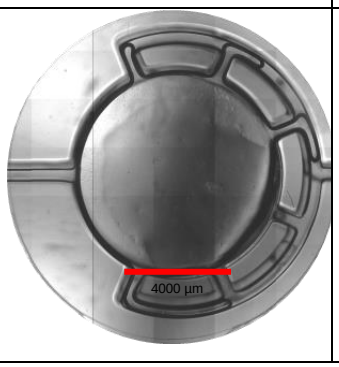
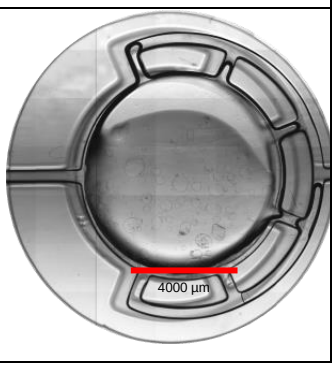
72			
96			
120			

Table 10 Time points of the wells containing murine liver organoids cultured following Organ-on-chip procedure using Matrigel® and H.Ovarian mouse medium. Comparative analysis between three different flux velocities

At time T1(24h) we witness the formation of organoids in each of the 3 conditions, in particular they appear larger in the condition with a flow of 7 µl / min. This trend is maintained throughout the experiment as can be seen in the growth curve in fig 42. The shapes of the organoids appear regular in low flow conditions (1 µl / min) (fig 38) while with increasing speed they appear more elongated. (fig 39, 40)

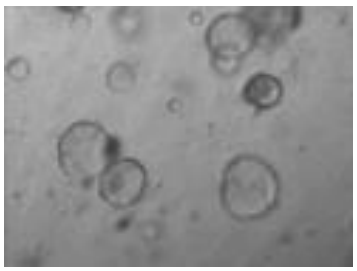


Figure 40 Organoids grown at flow rates of 1 µl / min

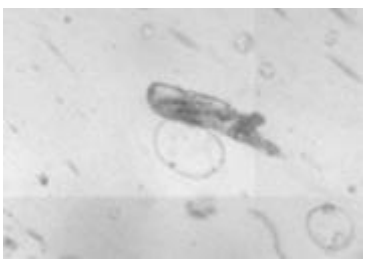


Figure 39 Organoids grown at flow rates of 5 µl / min

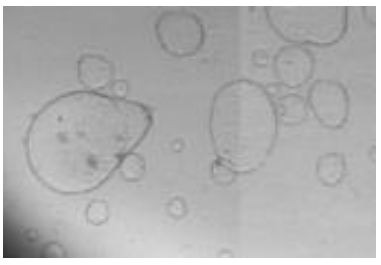


Figure 38 Organoids grown at flow rates of 7 µl / min

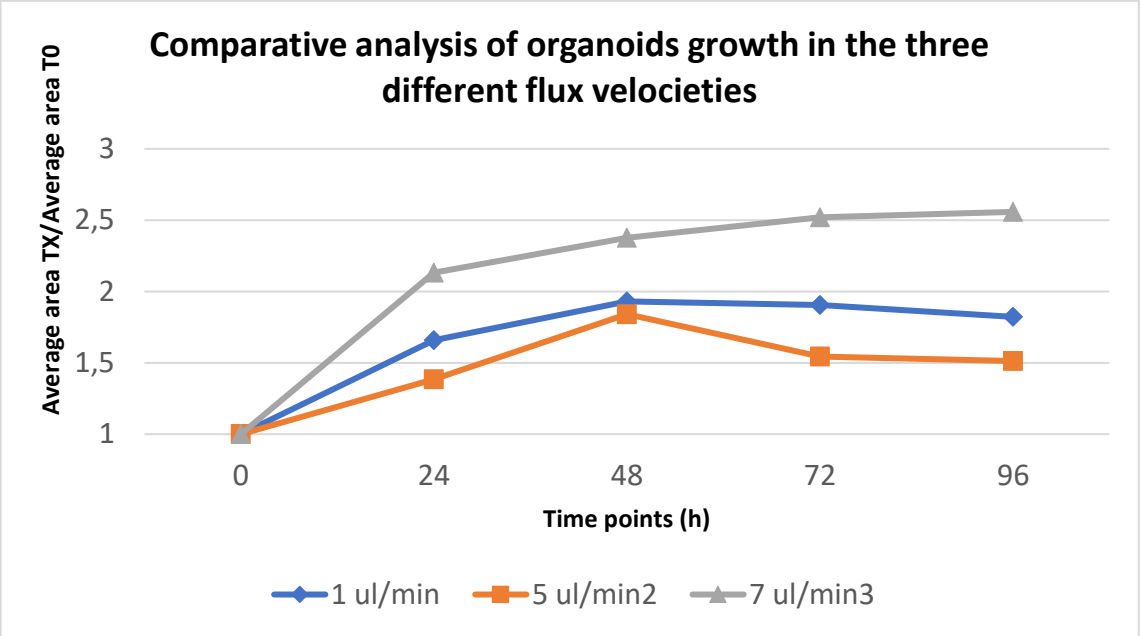


Figure 41 Growth curves of organoids grown in the three different flow rates

As can be seen from the growth curve, shown above (fig 41), and from the images proposed, it would seem that the flow condition that provides the best conditions for the growth of organoids is that of 7 µl / min. From the images, however, it can be noticed how the high flow velocity caused the drop of matrigel to yield, thus leading the organoids to migrate towards the channels from where the media flowed towards the waste.(fig 42)

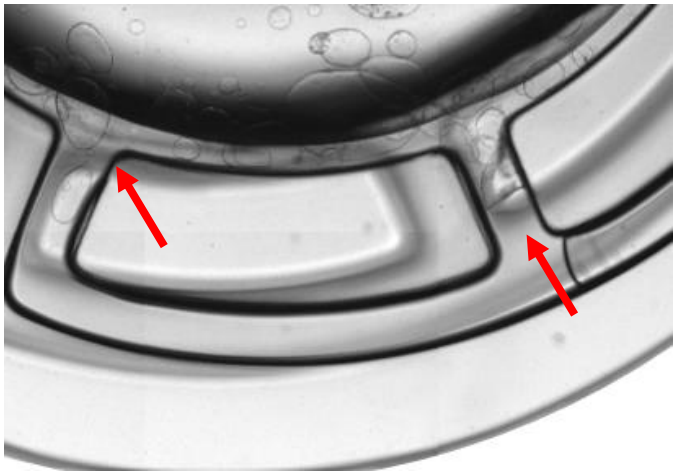


Figure 42 Failure of the drop of Matrigel with consequent loss of organoids

The weakening and consequent migration of the matrix inside the micro channels could cause clogging of the latter with the consequent compromise of the entire experiment. This could be a problem because the experiment is not monitored 24 hours a day but rather relies on automated devices that adjust the various parameters continuously and automatically take images at regular intervals of time. This phenomena also causes the loss of organoids, making the results unreliable.

Another good result is given by the chip with a speed of 1 µl / min, the growth is constant and the drop of matrigel does not appear to deteriorate significantly during the time of the experiment. It is also possible that the larger size of the organoids of the flow of 7 µl / min derives from the fact that the drop, losing its stiffness due to the stress caused by the high flow velocity, has favored a more flattened growth of the organoids. These organoids can also be spread on the bottom of the chip making them to appear bigger. For the above reasons, it was decided to use 1 µl / min as a flow rate because this solution is a good compromise between system stability and good organoids growth.

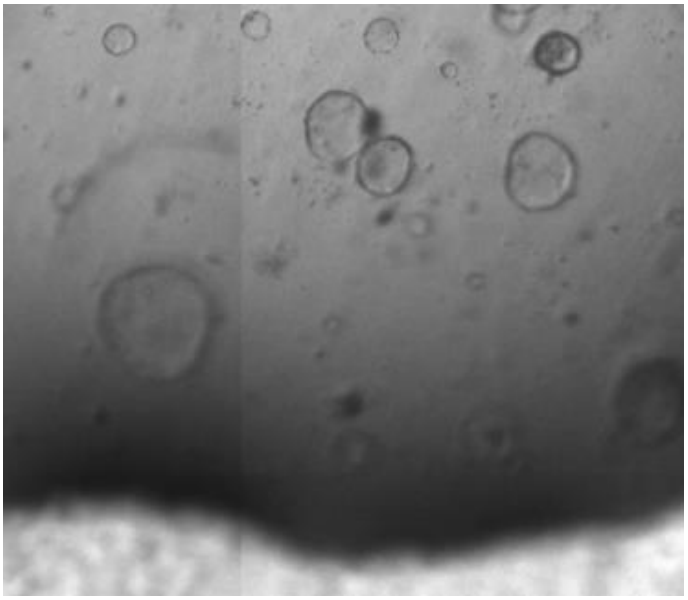


Figure 43 Drop edge of Matrigel with flow rate 1 µl / min

Comparative study between ALI-method, microfluidics and matrix immersion

In the following test, the three different methods discussed in the previous pages were assessed by plating ascites and using human ovarian medium as a culture medium. As regards the microfluidics technique, the applied flow was adjusted at a rate of 1 µl / min and the matrigel was used as a matrix. Matrigel was also used for the immersion culture which acted as a positive control. In the case of ALI-method, on the other hand, collagen was used as a matrix because it is more economical, it posses a defined chemical composition and therefore it is able to be competitive with matrigel. As for the previous tests, photos were taken using the optical microscope Nikon eclipse TS2R for the analysis of the immersion technique and ALI-method, while for the microfluidics the Evos auto 2 microscope with the incubation chamber was used. The results were then compared by means of a growth curve and observation of the images.

In the following pages were shown the results obtained for these three different culture techniques.

Immersion culture technique

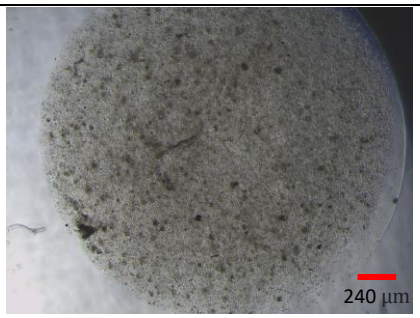
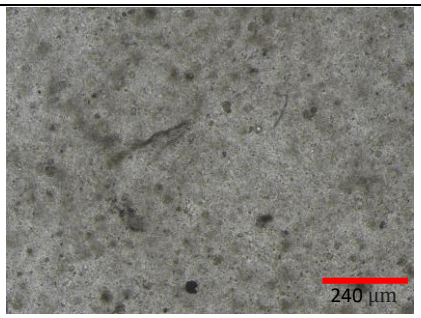
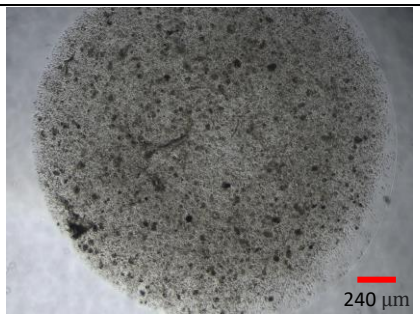
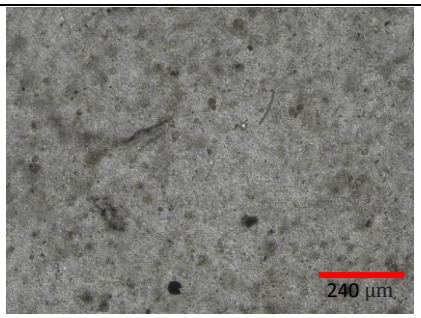
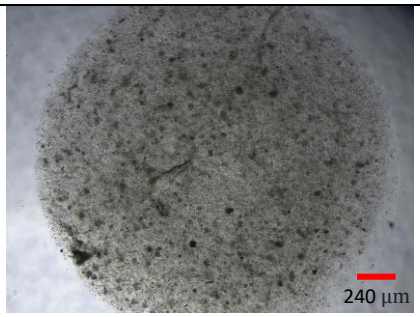
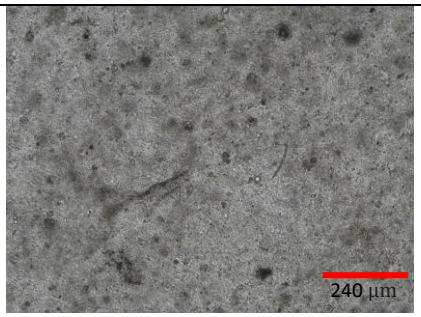
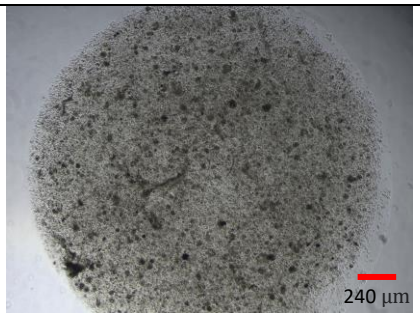
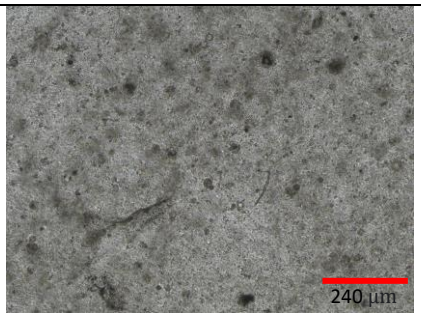
Time point (h)	*H.ovarian organoids immersion culture (2X Zoom)*	*H.ovarian organoids immersion culture (4X Zoom)*
24		
48		
72		
96		

Table 11 Time points of the wells containing H.Ovarian organoids derived from ascites cultured following immersion culture technique using Matrigel®

ALI-method

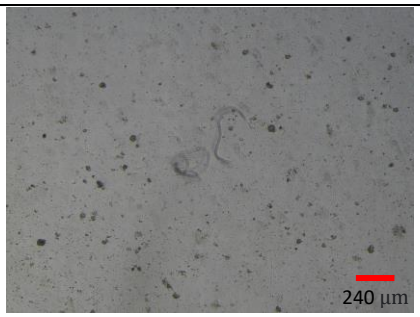
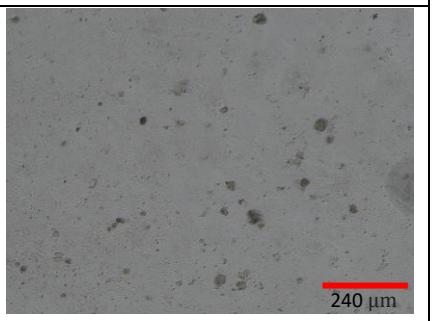
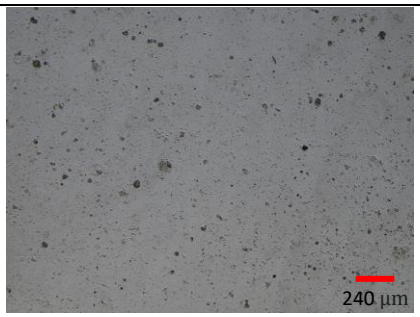
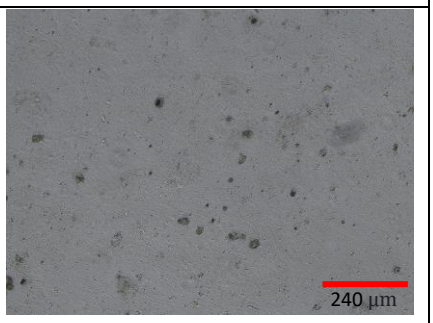
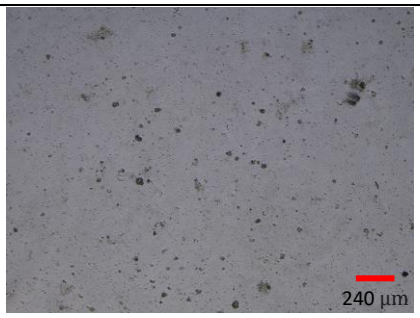
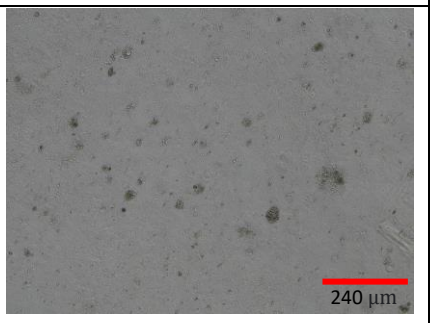
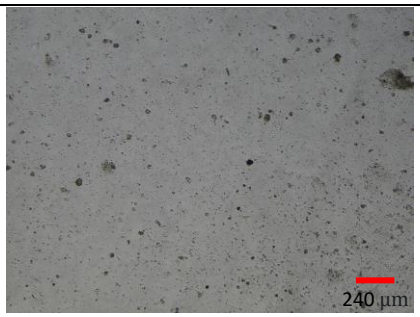
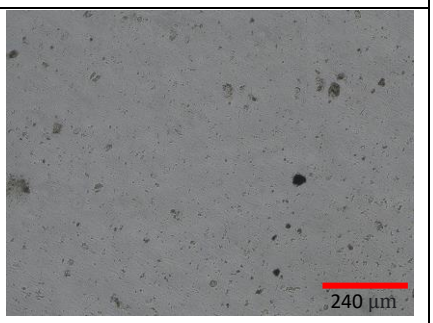
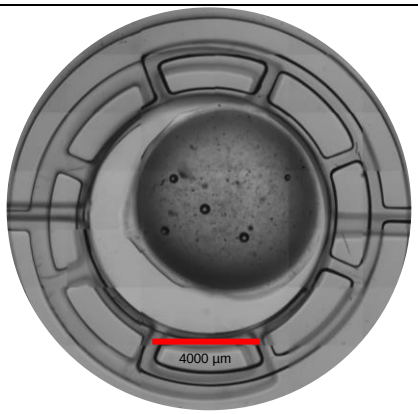
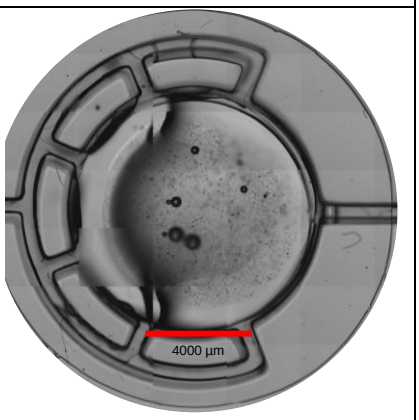
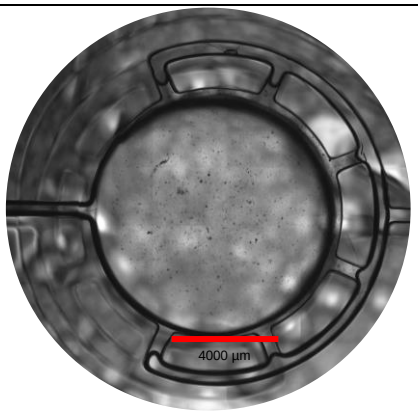
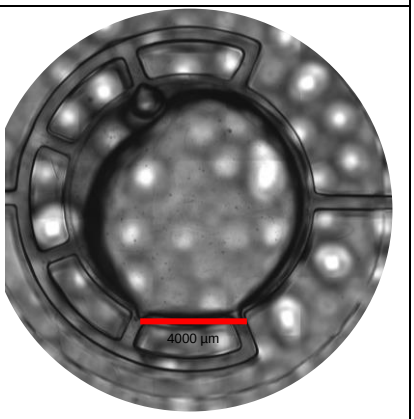
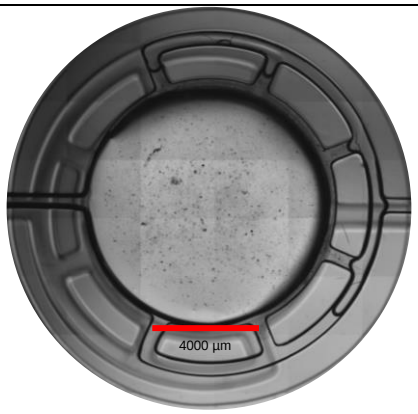
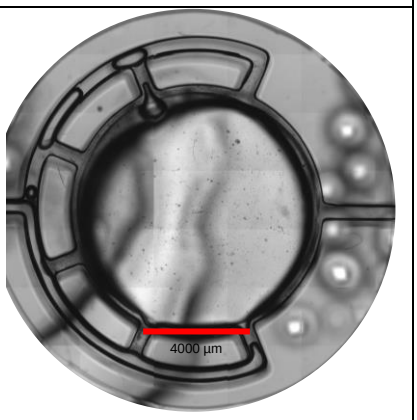
Time point(h)	*H.ovarian organoids ALI-method (2X Zoom)*	*H.ovarian organoids ALI-method (4X Zoom)*
24		
48		
72		
96		

Table 12 Time points of the wells containing H.Ovarian organoids derived from ascites cultured following ALI-method using Collagen, buffer 1 and H.Ovarian medium

Organs-on-Chip

Time point	*H.ovarian organoids Organ-on-chip (4X Zoom)*	*H.ovarian organoids Organ-on-chip (4X Zoom)*
24		
48		
72		

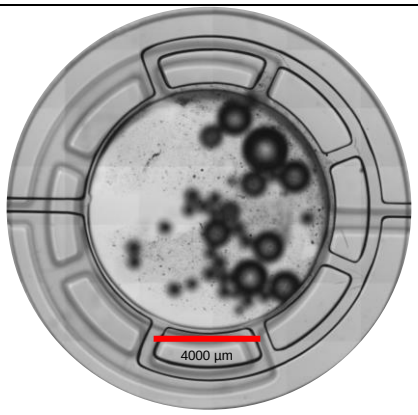
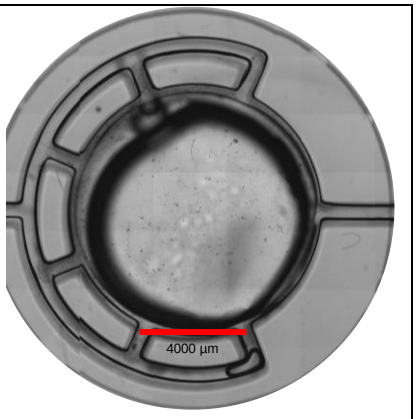
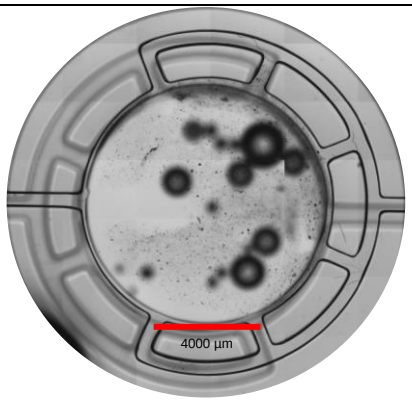
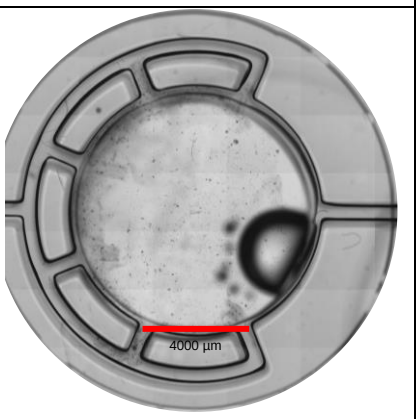
96		
120		

Table 13 Time points of the chips containing H.Ovarian organoids derived from ascites cultured following Organ-on-chip technology using Matrigel® and H.Ovarian medium

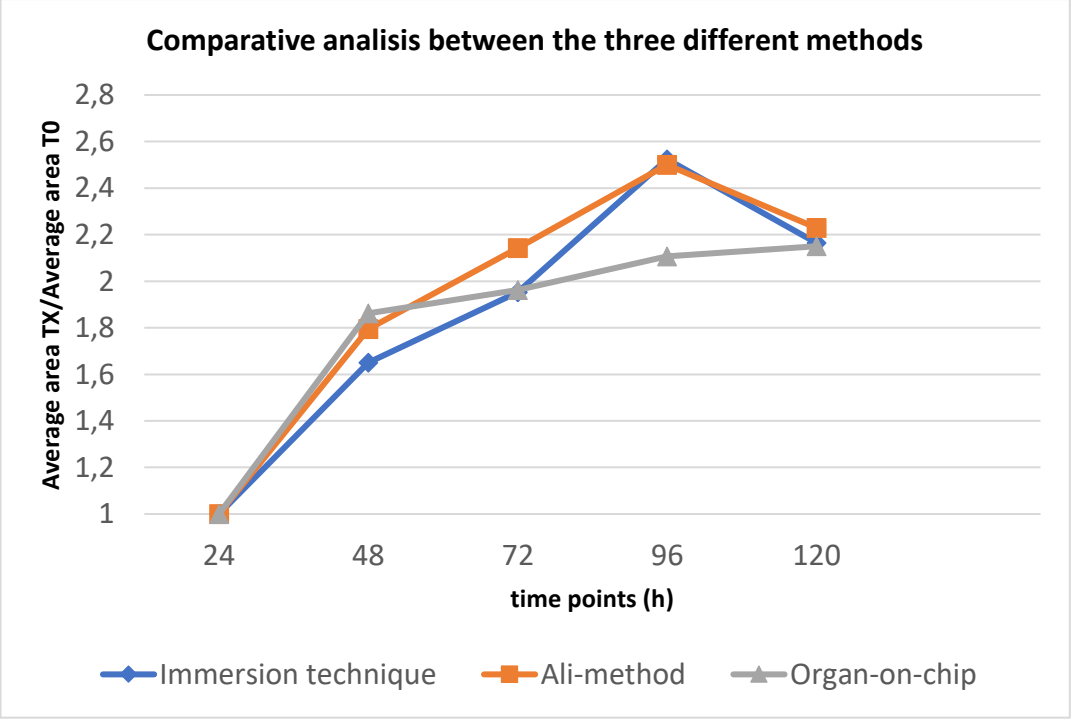


Figure 44 Growth curves of human ovarian organoids grown with the three proposed methods

In the proposed images it can be seen how organoids culture has been a success in all three proposed methods, albeit with different characteristics. The collagen layers in the ALI-method held up and showed no signs of collapse even in the last time point, confirming the effectiveness of the proposed variation to the original protocol. As already demonstrated in the previous test, collagen provided a good substrate for the growth of human ovarian organoids derived from ascites leading to a trend of increase in size similar to that recorded in the case of the immersion culture method. In fact, there is an increase with respect to the dimensions of T0(24h) by a value of 2.5 both in the immersion method and in the ALI-method. However, it appears evident that in immersion culture the acites are larger and in greater number than those cultivated with ALI-method. As regards the "density" of ascites in ALI-method, the dimensions of the well (6 multiwells plate) is larger than the immersion culture well (24 multiwells plate). This difference in dimension could make the ascites population appear more dispersed. In microfluidics, although the flow rate has been adjusted to a minimum (1 µl / min), the problem of failure of the drop of matrigel in both chips has nevertheless occurred. This could be due to a variability of the matrigel lot or to a involuntary dilution of the drop of matrigel during plating. In any case, this problem of drop failure did not compromise the growth of organoids. In figure 44 it is possible to observe how the growth trend appears constant throughout the experiment without particular peaks. It is interesting to note in figure 44 how in the last time point the growth curves converge at approximately at the same value, suggesting that the three culture methods proposed are all valid for the cultivation of organoids and that they have particular characteristics that make them suitable for different purposes. For example, the trend of microfluidics suggests that this methodology is suitable for experiments that last for long periods of time and therefore require a growth that is not too fast but rather constant over time.

Conclusions

The use of organoids has made an important contribution in the field of medical research as it provides an excellent experimental model capable of simulating the environment that would be found in an in vivo model. As illustrated in the previous pages, over the years, from the first organoid culture to the present day, different types of organoids have been created that are able to simulate different types of organs and neoplasms. To create specific types of organoids, specific growth factors in combination small molecules and a synergistic use of matrices capable of effectively simulating the stem cells niches were used. During the last few years, the use of matrigel combined with immersion culture has established itself as the main method, rapid and not difficult, to be implemented for the culture of organoids. The identification of alternative methods, that have characteristics capable of obtaining more relevant results in specific areas (such as pharmacological research, the study of the development of tumor forms and long-term investigations on the growth of specific organs) appears to be of fundamental importance. During the tests carried out previously, it was highlighted how the three different types of culture proposed are valid and have strengths and weaknesses that make them suitable for specific fields of use. It was also seen that although matrigel represents a highly effective, stable and reliable matrix for organoid culture, it exhibits structural variability , from batch to batch, due to its biological nature and its not fully known chemical composition. This variability was striking in the case of the microfluidic technique test where on two chips, at the minimum flow, the drop of matrigel did not hold up when in the previous tests it had shown excellent tightness. It is therefore interesting the test carried out using collagen as a scaffold. It provides a substrate with little variability over time, because of the quasi-total composition of collagen proteins. In the case of the ALI-method it has proven to be a valid and cheaper substitute of matrigel while providing smaller organoids. Having obtained organoids of smaller dimensions is however not to be considered a problem, as in some researches where a very long time is required for the study of certain development mechanisms, the large dimensions of organoids can give rise to necrotic phenomena in their nucleus. Moreover big organoids can lead to excessive nutrient consumption as seen in the ALI-method test using mouse ovarian organoids and matrigel. In ALI-method, the use of a

larger well and inserts provides great advantages for the regulation of nutrients and rapid change of experimental parameters which, on the other hand, is more difficult in immersion cultures. A further improvement in the realism of the simulation of the environment that would be found in vivo conditions, it is given by the Organ-on-chip technique. This technique, was found to be suitable for supporting the growth of different types of organoids with a constant growth trend over time. This trend suggests that this technique is particularly suitable in studies where it is essential to maintain the system for long periods of time, such as the study of the development of organs from their origin. The strength of this technology, which combines microengineering and biology, is the ability to precisely adjust nutrients and any drugs to be tested throughout the experiment. The equipment supplied to the laboratory also made it possible to regulate the flow of the culture medium that flowed inside the chip, thus allowing us to study the role of shear stress within the intratissutal microenvironment. Despite its strengths, the microfluidic system, that was tested in this thesis, showed several critical points due to the setup and perhaps from errors during the setup of the system. These problems did not occur in the other two systems. Furthermore, the variability of the density between different batches of matrigel was found to be critical. This difference determines partial yielding of the drop during the comparative test. During the experiment to find the optimal flow rates, one of the chips was affected by a mold contamination because the system, outside the biological hood, is not completely sterile.

In conclusion, the immersion technique with the use of matrigel is to be preferred for experiments that require a rapid execution and without particular need to control the culture conditions (i.e IC50 analysis). But after a short time the organoids saturate the drop and begin to deteriorate probably due to the phenomena of apoptosis and necrosis. The ALI-method protocol, on the other hand, allows a more prolonged growth over time and also a better regulation of the experimental parameters through the use of an insert. However it was necessary to modify the original protocol using medium with growth factors and obviating the stability problems of the collagen gel making the matrix more basic by adding an extra 1M NaOH solution. A further step in duration over time, thanks to the continuous flow of nutrients and the versatility of the experimental parameters, has been demonstrated by the microfluidic technique which, however, needs more care and more experience in order to be used effectively. For the realization of future set-ups

that include Organ-on-chip technology it is necessary to provide a more stable system with fewer variables. The first step to obtain a more stable system could be the implementation of a bubble trap in the system. This tool would allow, in the case of our setup, to eliminate the presence of bubbles from the chip. Eliminating the bubbles would prevent the media from being exposed to the air due to the bubbles. It may also be useful to test the stability of collagen in this system in order to reduce system costs and achieve a standard system. It would be advisable to prepare a biological hood where to insert the microfluidic system to eliminate the risk of external contamination. With the stability issues resolved, a further step would be the creation of interconnected chip systems. By growing different types of organoids in each different chip, it may be possible to simulate the passage of a drug through different organs. By passing a drug through various types of organoids it may be possible to realistically simulate its interaction with the various simulated organs. The metabolization of the drug, and the formation of maetabolites, could introduce new variables in the experiment that could provide more concrete and precise results.

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