BIO-469 Scientific Project Design in Regenerative Medicine and Diagnostics

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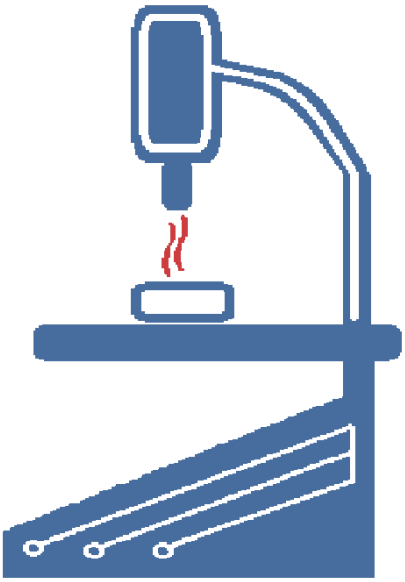
MATRILIGHT



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1 EXECUTIVE SUMMARY

Strategy

Organoids – three-dimensional (3D), self-assembled cells aggregates resembling an organ – are a very promising application of regenerative medicine, which could be used for various purposes, such as drug testing, disease studies, personalized medicine or animal models replacement. However, current organoids generation systems still present a lot of issues: they do not allow organoid vascularization *in vitro*, nor they do provide spatiotemporal control over the mechanical properties of the extracellular matrix. The resulting organoids are therefore much smaller and short-lived than adult organs *in vivo*. Moreover, they often cannot fully mature and present organ-like topological and functional features. Here, at **Matrilight**, we propose a revolutionary solution to these problems: a versatile, 3D organoids generation system. It enables high-resolution spatiotemporal control of the matrix mechanical and chemical properties, as well as vascularization. Say goodbye to your old culturing techniques. Make your organoids come true.

Market

Matrilight's target market is the 3D cell culture market, which was valued at 568 million USD and constituted 9% of the global cell culture market in 2014. Considering their respective compound annual growth rates, by 2020, the 3D market is estimated to capture 28% of the global one, thereby reaching a value of 2.7 billion USD. Among these, about 30% is expected to stand for scaffold-based gels, scaffolds and bioreactors. Moreover, Europe, our early market of interest, currently holds about 40% of the shares, which is expected to remain stable by 2020.

One of our major competitors in the 3D cell culture market is Corning, the leader in hydrogel scaffolds, with cell culture products accounting for 38% of their Life Sciences domain sales (326 million USD). Other potential competitors are Lonza, QGel, Ibidi, etc. However, Matrilight is the only company addressing matrix stiffness patterning, for the time being. Assuming Matrilight manages to capture only 0.2% of the European 3D market in 2020, when we expect to enter the market, this is estimated to represent 2.1 million USD.

Our product

Matrilight's flagship product will be composed of the **MatriScope**, a two-photon microscope enabling polymerization of a photocrosslinkable hydrogel at any location in the formulation. With MatriScope, it will therefore be possible to pattern the stiffness of the hydrogel in any desired 3D shape using a template drawn with a computer-assisted design software. The hydrogel will be embedded in the **MatriChip**, a multi-layered microfluidic chip allowing precise spatiotemporal delivery of biochemical factors. Additionally, the hydrogel properties will be chosen carefully so that it provides a safe and biomimetic environment for cell culture. Thus, our product will enable researchers and clinicians to grow 3D organoids in a spatiotemporally controlled environment, biochemically and mechanically speaking. Moreover, the combination of two-photon polymerization and microfluidics will make vascularization possible, thereby solving most of the issues presented by current organoid culture techniques.

Key financial data

Like any startup company, Matrilight will need financial support from partners and investors, in the form of academic grants for the early research stages. Then, funds will come from incubators and business angels, and gradually from Venture Funds. The registration of the company as well as the making of the protoype machine will cost about 900'000 CHF in the first year of the company. We expect to gain customers starting from the fourth year after company creation.

The gross benefit coming from the sale of one apparatus is approximately 284'000 CHF. However, in the long run, revenue will come from the sale of MatriChip, the hydrogel-containing microfluidic chip. A MatriChip costs around 2 CHF to manufacture, and could be sold at least 50 CHF. Our five-year business model plans that 1.6 MCHF of investment capital will be needed, and the business will be profitable from the first years of product commercialization.

2 INTRODUCTION AND OBJECTIVES

Since the discovery of stem cells, scientists have been dreaming of using these incredibly versatile cells for tissue engineering and regenerative medicine purposes, such as skin repair for major burn victims or bone marrow transplantation in patients with leukemia, today well-established. One of the most promising applications of stem cells, nowadays, is organoids generation; Lancaster and Knoblich [Lancaster and Knoblich, 2014] define an organoid as "a collection of organ-specific cell types that develops from stem cells or organ progenitors and self-organizes through cell sorting and spatially restricted lineage commitment in a manner similar to *in vivo*". Typical methods to generate organoids take advantage of the aforementioned sorting out and spatially restricted lineage commitment properties of cells during development. Indeed, culturing stem cells or organ progenitors in three dimensional matrix hydrogels supplemented with organ-specific growth factors is sufficient to observe cells self-organization into organ-like structures [Lancaster and Knoblich, 2014][Xinaris et al., 2015]. The great interest recently shown for this research field led to the development of more or less successful gut, liver, brain, retinal and kidney organoids, with the help of these methods. Eventually, organoids could be used by laboratories, companies and even clinics for drug screening and toxicity testing, disease and organogenesis studies, personalized medicine and, on the long run, organ transplantation and animal models replacement.

Nevertheless, current techniques for organoids generation still present many limitations that must be overcome before organoids can be used on a regular basis. First of all, *in vitro*, organoids lack vascularization. Therefore, nutrients transport is restricted to the outer cell layers of the organoid, which may impair its survival and dramatically limits its maximal size. Indeed, organoids' sizes are typically in the submillimeter range. The use of a spinning bioreactor, enabling better nutrients exchange, allows the organoids to reach a few millimeters in size, which is still much smaller than most adult human organs[Lancaster and Knoblich, 2014]. On the other hand, it has been shown that, upon transplantation, host vasculature can invade the grafted organoid[Xinaris et al., 2015]. However, this only constitutes an advantage for organ replacement, while most of the aforementioned applications necessitate viable *in vitro* organoids.

A lack of nutrients can also impact maturation of the organoids [Lancaster and Knoblich, 2014]. Indeed, most organoids currently generated recapitulate the early development of the organ of interest, but only some parts develop into their final, adult state. This obviously also impairs the function of the target organ. For example, even though retinal organoids can be close to a natural human retina morphologically speaking, they cannot express mature, light-sensitive photoreceptors for the time being [Lancaster and Knoblich, 2014][Xinaris et al., 2015]. Once again, transplanted organoids may be able to develop more than *in vitro* owing to the invading vasculature, but also possibly because they receive spatiotemporally controlled chemical and physical cues from the environment. Indeed, lacking this important spatiotemporal control constitutes another common issue for organoids cultured *in vitro*. Moreover, even though microfluidics are a useful tool to provide organoids with chemicals in a quite spatiotemporally controlled fashion, mechanical conditions are currently poorly controlled. Organoids can obviously be grown on more or less stiff matrices, but it is very challenging to make their mechanical properties vary in time and space. And the latter would precisely be helpful to model diseases; for instance, breast cancer can be characterized by an increasing extracellular matrix stiffness and it has been shown that the stiffness of the extracellular matrix (ECM) impacts stem cell fates[Mason et al., 2012] .

To adress those problems, we, Matrilight, have created an innovative device combining two-photon polymerization (2-PP), microfluidics and software engineering allowing to generate organoids in a versatile, spatiotemporally-controlled way. More precisely, we have developed a machine in which multi-layered, hydrogel-filled microfluidic cartridges, supplemented with specific cells, can be inserted. A microscope can perform 2-PP on the hydrogel, for example to create channels mimicking vasculature; flushing endothelial cells through these channels then leads to the formation of blood vessels. Similarly, 2-PP enables to change the stiffness of the hydrogel at very specific locations. The microfluidic system allows to create concentration gradients of various chemicals. Thus, any combination of stiffness variations and growth factors gradients is made possible and can ultimately be used to develop organoids.

Our technology is powerful in that it solves most of the problems explained above: first of all, it gives researchers a very precise spatiotemporal control of the conditions in which they grow organoids. What's more, vascularization is made possible; consequently, larger, longer-lived and potentially more mature organoids can be generated. The simultaneous control of matrix stiffness and chemical conditions also enables researchers to investigate the impact of various environmental factors in diseases development. Finally, the high resolution of 2-PP and microfluidics makes experiments easily and faithfully replicable.

3 MARKET ANALYSIS

Our product positions in the 3D cell culture market, which is itself part of the global cell culture market. First, the behavior of those two markets is investigated for the next few years based on 2013 and 2014 numbers.

In 2013, the global cell culture market was valued at 6'097 million USD [Transparency Market Research, 2015]. In the same report, the compound annual growth rate (CAGR) was estimated to be 7.1%. Hence, it is expected to grow up to 9'855 million USD by 2020. In 2014, the 3D cell culture market is estimated to consist of approximately 9% of the global one, with 568 million USD [Meticulous Research, 2015]. Given that the CAGR for 3D market is estimated to be around 30% by all the different sources, the market size in 2020 is estimated to be 2713 million USD. It is thus expected to capture 28% of the global market by 2020. Figure 1 shows the actual and projected proportion of the 3D cell culture market compared to the global one and the actual and projected distribution of this market per region. This shows the relative importance of the European Union (EU) market, which holds approximately 40% of the shares [Persistence Market Research, 2015], as well as the expected rising of the Asiatic market by 2020. Note that the CAGR might be a bit overestimated since the 3D market size for 2015 is now valued at 725 million USD, which represents a 23% increase compared to 2014. The numbers for each regions were extrapolated from [Persistence Market Research, 2015]. Note that the value reported for the US market size (3D) varies strongly between the different sources, so it has to be considered carefully.

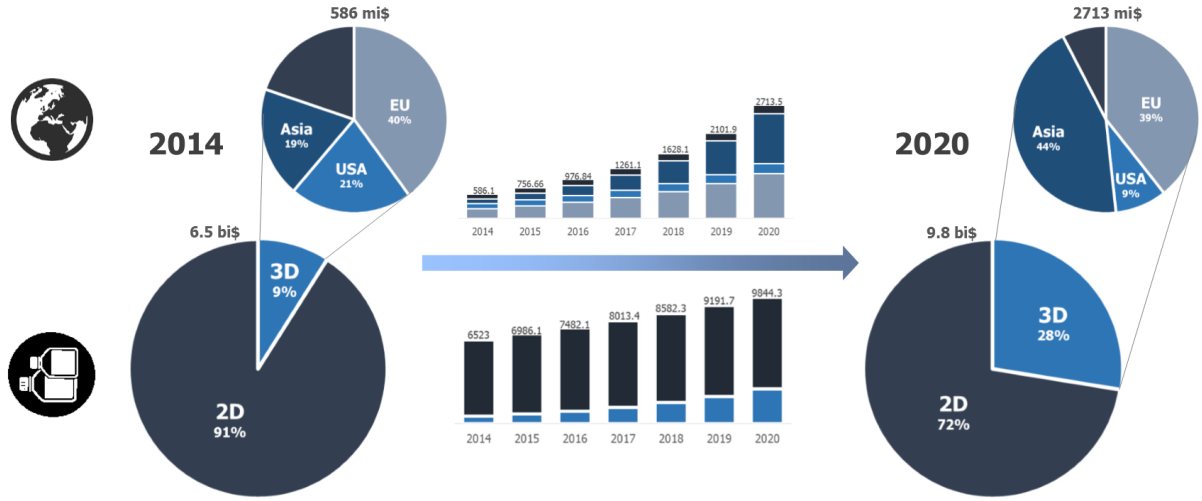

Figure 1 – Cell culture market sizes in 2014 and previsions for 2020 Global and 3D cell culture markets sizes are shown in the bottom part, 3D cell culture market is then segregated by region (EU, USA, Asia and rest of the world) and shown in the upper part. Their expected behavior for the next 6 years is computed based on the predicted CAGR provided by different sources (based on 2014 numbers).

The 3D market consists of significantly different domains. They are described here to know which proportion we would potentially intervene in. The gels, scaffolds, bioreactors and hanging droplets domain represents 43% of the 3D market, the remaining 57% accounts for cells and tissues [Hunter (BCC Research), 2015] domains. Scaffold-based systems represent 76% of 3D cell culture systems [Roots analysis, 2015]. Considering those two numbers, approximately 30% of the 3D market will stand for scaffold-based gels, scaffolds and bioreactors, which is the market our product targets. It represented 170 million USD in 2014 from which 70 were for EU market.

The three main applications of 3D cell culture are cancer research (40%), high throughput drug/toxicity screening (30%) and stem cells/tissue engineering/regenerative medicine (25%) [Comley, 2013]. But the latter is expected to grow fast in the next few years. To provide tools for those applications, it is generally needed that **a** the cells can be fluorescently imaged, **b** scaffolds are representative of human ECM and **c** scaffolds can be used with high throughput and automation processes [Comley, 2013]. The main consumables the clients are interested in are, in decreasing order of interest, **a** microplates supporting tissue generation, **b** hydrogel 3D scaffolds, **c** microwell inserts incorporating 3D scaffolds/structural 3D scaffolds and **d** devices for creation of 3D forms.

Key-players involved in the 3D market consist of large/diversified companies (such as Corning, BD Bioscience, Thermo Fisher Scientific and Lonza) and smaller/specialized ones (such as QGel, InSphero, 3D Biotek, 3D Biomatrix, Ibidi and Reinnervate). For the mentioned companies, table 1 summarizes the different technologies, products and applications listed above.

The business of some relevant/competing companies is investigated and the following summarizes our findings. First, we focus on the leader in hydrogel scaffolds, Corning. Then, specialized companies such as InSphero are studied as well to get a sense of the price at which we could sell our device.

	Technology				Product			Application		
Company	hydrogel scaffolds	structural scaffolds	biomimetic constructs	scaffold free	microplate	well insert	microfluidic device	tissue creation	stem cell focus	drug screening
Corning	✖	✖		✖	✖	✖			✖	
Lonza	✖	✖	✖	✖	✖			✖	✖	
Thermo Fisher Scientific				✖	✖				✖	
QGel Bio	✖				✖			✖	✖	✖
3D Biomatrix				✖	✖			✖	✖	
Reinnervate		✖			✖	✖		✖	✖	
3D Biotek		✖				✖		✖	✖	
Ibidi	✖				✖		✖		✖	
InSphero			✖	✖	✖			✖	✖	✖

Table 1 – A few key players involved in the 3D cell culture market Technology, product types and applications

In 2014, Corning sales in the Life Sciences domain account for 9% of their total sales [Corning Inc., a]. This represents 862 million USD, from which 38% account for cell culture products (326 million USD) and the remaining 62% for labware products (536 million USD). In their 2014 annual report, they explain that a net increase in the sales for their Life Sciences department between 2012 and 2013 (+ 192 million USD) is attributable to the acquisition of Discovery Labware, a former division of BD Bioscience, which sells Matrigel, Falcon and Gentest. This shows well the importance of cell culture products such as Matrigel in their business. Considering the numbers stated above, it represents 3.5% of their total sales in 2014, for 326 million USD. In their 2015 proxy statement [Corning Inc., b], they identify Thermo Ficher Scientific as their main concurrent in the Life Sciences segment and provide some numbers to compare their performances. Their Sales CAGR for 1 year is 1% (13% for 3 years) compared to 29% (13% for 3 years) for their concurrent and their net profit after tax (NPAT) is -5% (13% for 3 years) compared to 48% (23% for 3 years).

InSphero, a swiss company founded in 2009, is more focused on drug testing and uses a scaffold-free technique. Rather than only selling a media, they also sell cells and organoids. They even propose (since October 2015) a new service 3D InSight^{TM} 14 Day Hepatotoxicity Testing, which consists in performing drug toxicity testing in their hanging drop liver organoids for 990 USD per compound and sending the results to the customer. Similarly, 3D Biomatrix also sells equipment for scaffold-free 3D cell culture (hanging drop technique). On the other hand, Reinnervate sells Alvetex, which consists of a highly porous polystyrene scaffold and 3D Biotek's first product is 3D Insert, a porous scaffold as well. In July 2015, Lonza reached an agreement with TAP Biosystems and is now selling the RAFT^{TM} 3D Cell Culture System. It allows to generate matrices with high collagen density, which is closer to the *in vivo* environment compared to standard products.

Given the predicted expansion of the 3D cell culture market and the rising interest in this domain, it seems that the market will not saturate anytime soon. There are some large companies involved and some of them acquired smaller specialized ones (see Corning and Lonza), which shows the potential of such products. What's more, we found no company addressing patterning of matrix stiffness. Generally, only the global stiffness properties of hydrogel scaffolds can be controlled, and it can not be done precisely. If we manage to show the benefits of such a technology, it is highly probable that it will meet customers' needs. If we manage to capture only 0.2% of the 3D market, this will potentially represent 2.1 million USD for EU market in 2020 (expected market entry).
Based on this analysis, we will target pharmaceutical companies and laboratories as customers.

In the long run, if our product satisfies certain particular requirements, we hope that it will be able to replace in part animal models used in the process of drug discovery, which is a huge market.

4 STAKEHOLDERS

4.1. Internal stakeholders

Board members will firstly consist of the first team members (CEO, CTO, CFO), influent people who can act as counselors, and the first investors who would like to take a decisive part within Matrilight. As the company grows, board members will switch place with influent decisioners within our most important shareholders.

First shareholders will be composed of the first team members, as well as relations willing to take part in the company. Gradually, as the funding rounds go, the available stock will be diluted between more shareholders. In the beginning, capital will come from academic grants (which are not retributed in stock), but also accelerators and incubators, which will provide short-term money as note or stock, as well as counseling and relationships with important people in the field. In the second funding round, Venture Capitals (VC) and business Angels will share Matrilight's stock with the company owners. After the third funding round (Series A), most shareholders will be Venture Capital Funds. Since the product quality depends on the intensity and quality of Research and Development, highly qualified personnel has to be hired. Before sufficient funds are collected, the employees could also be partly paid in shares of stock (i.e a self-owned company), and therefore also being shareholders and be able to sell them out when the company grows.

4.2. External stakeholders

We will partner with key researchers well-known in the field of organoids. We will provide them with our machine, so that they can use it in their research and mention our company in their publications. We will contact the following: Madeline Lancaster, group leader at the MRC Laboratory of Molecular Biology in Cambridge, U.K. Her group is working on cerebral organoids. James Wells who works on the endoderm at Cincinnati's Children, Ohio, USA. Jürgen Knoblich from the Institute of Biotechnology (IMBA) in Vienna, Austria. His group also focuses on the brain. Calvin Kuo from Stanford University in California, USA, who works on cancer organoids. Hans Clevers from the Hubrecht Institute in the Netherlands. His group develops many different epithelial organoid cultures. We could also partner with Matthias Lütolf, whose group at the Swiss Federal Institute of Technology of Lausanne (EPFL) works on relevant subjects such as stem cell niches, hydrogel patterning, and microfluidic devices.

In the early years of Matrilight's development, we will find people and companies that could also invest funds in our startup and help it start its growth. We could partner with A3 Angels, aasa, Aravis or aventic partners. They are active in the field of biotechnologies in Switzerland. They will provide us with money as well as support, network and exposure, and possibly housing for our developing startup company. Their experience in business administration and startup company is very valuable. In our time plan, we would already have developed our prototype from grant money (in academia), and will be able to move forward with pilot studies. Furthermore, the grants' source, in our case EPFL, will take ownership of our patent. In order to successfully launch our products, EPFL should provide a licence for device commercialization.

Customers can be companies, mostly pharmaceutical companies that can use our product for toxicity testing, cancer diagnostics, preclinical trials. By making organoids very similar to real organs, it is possible to replace animal models and animal testing to some extent. As they are generated using human cells, organoids can be closer to real organs than animal models. Furthermore, the maintenance of animal facilities is relatively costly and it may be more expensive than organoid culture. In addition, there is no ethical issue with the use of organoids for research and drug testing. Customers can be privately-owned or governmental laboratories, who would use our product for basic research on organ development, signaling pathways or vascularisation. It can also be clinics or hospitals.

It is important to pursue a fruitful relationship with suppliers and original equipment manufacturers (OEMs), since most of our product will use highly technological and costly parts. Furthermore, the final cost of the apparatus will greatly depend on them. There are numerous injection molding companies that we could choose, but much fewer businesses are able to provide the two-photon microscopy parts.

5 BACKGROUND

5.1. Microfluidics

Microfluidics techniques have given scientists a mean to precisely control the environment in 3D cell-culture. Fluids are spatially controlled in channels, which allows for the integration of specific gradients and flows. The creation of molecular gradients is extremely straightforward, thanks to the laminar character of the flow in such small channels. Van de Meer *et al.* studied the difference in the response of endothelial cells to a homogeneous solution of vascular endothelial growth factor (VEGF), or to a gradient of VEGF [van der Meer et al., 2010]. Cells were seeded in a microfluidic channel and exposed to either solution. They showed that cells migrated more when exposed to the gradient of growth factors. They also observed the importance of shear stress on cells, by testing different flow rates.

Interfaces in organs where exchanges with the environment occur can also be modeled in microfluidics thanks to compartmentalization. For example, Huh *et al.* managed to reconstitute the alveolarcapillary interface of the human lung in a microfluidic device [Huh et al., 2010]. A membrane divides a channel, and air is flown through one side while a blood-like fluid goes through the other side, which leads to cell differentiation.

Additionally, microfabrication can be used to add physical constraints. For instance, Cao *et al.* used discontinuous microwalls in microchannel scaffolds to regulate the orientation and phenotype of smooth muscle cells [Cao et al., 2010].

Ann M Taylor *et al.* were able to control the polarization of individual central nervous system axons with microfluidics [Taylor et al., 2005]. The device of interest consists of two chambers connected by microchannels. Embryonic neurons are seeded in a chamber and axons are forced to grow in the channels.

Hydrogels are used as artificial matrices for cell culture in microfluidics. Their structure and shape can be controlled in 3 dimensions using lithography, lasers or 3D bioprinting. Sung *et al.* used a 3D hydrogel scaffold to produce an intestine villi model which exhibits an *in vivo*-like differentiation pattern [Sung et al., 2011].

The field of microfluidics is evolving very rapidly, and 3D cell culture is extremely advanced. However, most of these "organs on chips" are static, and there is a need for a more dynamic approach, as pointed by Verhulsel *et al.* in their review of the field [Verhulsel et al., 2014].

5.2. Vascularization

One of the main limitations in organoid generation resides in the necrosis of the core cells once the organoid reaches a certain size. This necrotic core is a result of the limited diffusion of oxygen and nutrients to these deeper cells which triggers their apoptosis and thus leads to unfunctional organoids of limited size. In the past years, studies have been led and several approaches have been used to overcome this issue.

For example, McGuigan *et al.* designed a method that consists in encapsulating cells of interest into small tubes (2mm in length) of collagen gel [McGuigan and Sefton, 2006]. These tubes are then confluently seeded with Human Umbilical Vein Endothelial Cells (HUVEC) cells to form a module. Random assembly of these modules forms a construct through which medium or blood can be flown. HUVEC cells induce a low thrombogenicity and a high cell density can be reached with this technique. By doing so, the authors could obtain a pseudo tissue. However, this technique could not be used to induce vascularization of complex organs where spatial segregation and differentiation are needed.

Another group approached the issue by limiting the thickness of the organoid [Kusumi et al., 2009]. They grew sheets of hepatocytes and stacked them, intercalating a porous membrane thus allowing medium to flow in between and reducing the distance of diffusion.

Lancaster *et al.* developed a new technique for organoid culturing that uses constant shaking in order to increase diffusion and to allow organoids to have bigger size. In the case of mini brains, they could observe differentiation of brain areas such as the cortex and the hippocampus [Lancaster et al., 2013].

Even though these studies improve the culturing of organoids, they fail to mimic natural organ vasculature, which prevents full growth and differentiation.

A very promising approach has been established by Miller *et al.* [Miller et al., 2012]. They molded a 3D mesh made out of carbohydrate glass. They then casted a hydrogel containing cells of interest which degraded the carbohydrate glass after gelation. The dissolved glass yielded a 3D perfusion network that was seeded with endothelial cells. It allowed perfusion

of the whole hydrogel, thus bringing the right amount of nutrients and oxygen to all the cells within it. They even observed endothelial cells sprouting from the perfusion network which recalled *in vivo* angiogenesis.

Therefore, a technique that allows biological tissue to help itself by its intrinsic mechanisms seems to be an effective approach to use in the field of organoids.

5.3. Control of matrix stiffness and applications to organogenesis

In vivo, cells are embedded in a complex environment called the extracellular matrix (ECM). The ECM interacts with cells in a bidirectional and dynamic way. It provides spatiotemporal signals that influence cell fate (proliferation, migration, differentiation, etc.). These signals can be mechanical, topographical and/or biochemical. To culture cells *in vitro* in physiological conditions, it is essential to reproduce the characteristics of the ECM.

Matrix stiffness plays a key role in many aspects of the cell behavior. It is known to affect cell migration in absence of any ligand density or chemotactic factors. This phenomenon is called durotaxis [Lo et al., 2000]. It is similar to chemotaxis, where a gradient of concentration drives cell movement, but with an extracellular matrix rigidity gradient. Usually cells migrate up the gradient, from soft to stiffer matrices. It is also of strong interest to us that it directs stem cell lineage specification [Engler et al., 2006]. Indeed, soft matrices are neurogenic, stiff ones are myogenic and rigid ones are osteogenic. It was also shown that endothelial cells accumulate preferentially on stiffer regions of substrates [Gray et al., 2003]. In addition, matrix stiffness also modulates focal adhesions, cell-cell assembly, cytoskeletal assembly, traction forces and proliferation [Mason et al., 2012].

It has to be noticed that some previously mentioned studies [Lo et al., 2000][Gray et al., 2003] were limited in the way they cultured cells, namely only on 2D substrates. There are differences in how stem cells morphology and differentiation are affected by stiffness if the environment is 2D or 3D, as summarized by Lv *et al.* [Lv et al., 2015]. Also, the scaffold polymer choice (polyacrylamide) may not have been optimal biomimetically.

A 3D model based on collagen was successfully proposed by Hadjipanayi *et al.* [Hadjipanayi et al., 2009]. It consists of a matrix with a continuous stiffness gradient. It is produced by compressing collagen gels to produce sheets of constant thickness but varying stiffness. Also, with the use of photoinitiated stiffening of the matrix, it was shown that mesenchymal stem cell fate was regulated by stiffness gradients in 3D matrices [Tse and Engler, 2011]. There were some attempts at patenting methods for creating 3D matrices with tunable elasticity to enable the regulation of stem cells differentiation, but they were abandoned [Rehfeldt et al., 2010].

Successful applications to organoids generation were also demonstrated. Recently, vascularized liver buds were generated by co-culturing mesenchymal and endothelial stem cells after they were transplanted into mice by Takebe *et al.* [Takebe et al., 2013]. By mimicking the endodermal sheet-like delamination occurring in organogenesis, they were able to initiate 3D organ buds condensation. Very recently, the same group elicited the importance of substrate stiffness for the self-condensation of those initially 2D cultures and showed that it is maximized by soft environmental conditions [Takebe et al., 2015]. Those results highlight how matrix mechanical properties affect assembly of multiple cell types [Wrighton and Kiessling, 2015].

5.4. Two-photon polymerization

Over the past few years, hydrogel scaffolds have been a material of choice to provide an environment mimicking the ECM. Hydrogels have mechanical properties similar to those of many biological tissues. Cells can be either seeded onto prefabricated porous scaffolds or encapsulated during scaffold formation [Nicodemus and Bryant, 2008].

In order to provide cells with a proper environment, the hydrogel needs to fulfill some requirements. It must be degradable so that the cells can replace it with their self-produced ECM. The degradation products must not affect cell viability. Many properties of the gel, such as the mechanical strength, swelling ratio, crosslink density, hydrophobicity, degradability, can be tuned in order to design suitable environments. Hydrogels have the ability to absorb large amounts of water and they are generally permeable to nutrients. However, thick constructs can act as a barrier to oxygen and nutrients diffusion. Hydrogels with a porous architecture have been designed to overcome this limitation [Desai et al., 2012].

2-PP is a technique allowing the production of 3D structures at multiple length scales and at high resolution. It allows the fabrication of feature sizes ranging from 65nm to approximately 1cm. It relies on the principle of two-photon absorption. Femtosecond (fs) laser pulses are focused precisely where the polymerization is required. At this focal point, a photoinitiator becomes excited by absorbing two photons and, upon excitation, generates reactive radicals that locally initiate the polymerization. It can thus take place at any point in the formulation. This allows patterning of 3D structures by successively illuminating the points to be polymerized [Torgersen et al., 2013].

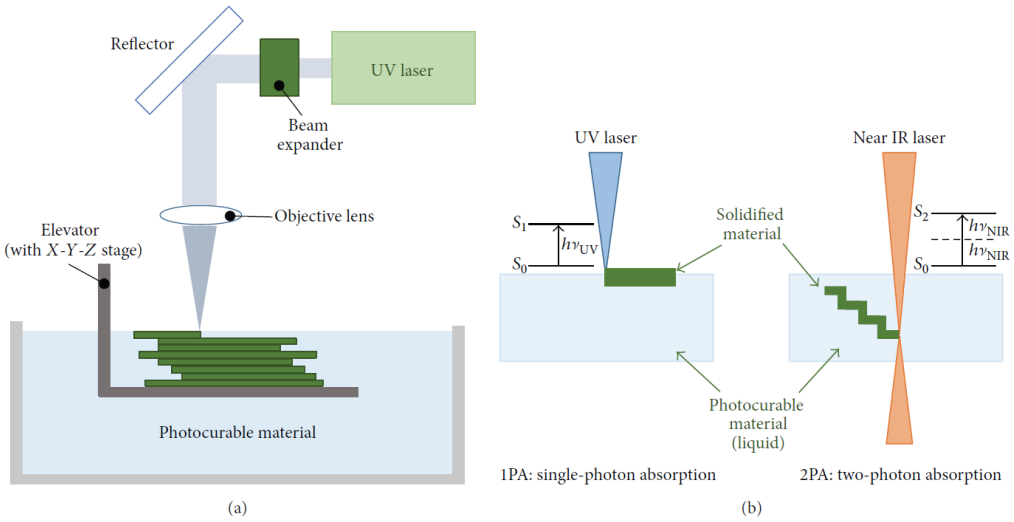

Figure 2 – (a) Description of the two-photon polymerization process. (b) Comparison between single- and two-photon polymerization. [Lee, 2015]

2-PP offers a mild gelation process which is important for cell survival. Near-infrared (NIR) light is used to induce hydrogel polymerization since it penetrates the monomer solution as shown in Figure 2. Furthemore, it prevents damaging the cells because the light absorption of biological tissue is very low in the NIR spectral range [Torgersen et al., 2013]. The main limitation of 2-PP for patterning 3D structures is the processing time. It might take hours or even days to produce millimeter-sized structures. To overcome this issue, research is ongoing to speed up the chemical process and the processing of the formulation by the lasers. In 2013, Torgersen *et al.* reported writing speeds up to 500 mm s^{-1}. Using a dynamic mask, it is possible to generate different structures simultaneously with a single laser. This can further reduce the processing time [Torgersen et al., 2013].

6 PRODUCT DEVELOPMENT PLAN

6.1. Timeline

Figure 3 shows the principal milestones of Matrilight's development for the upcoming years. The first year will be dedicated to applying to grants, awards and other pre-seed funding, and starting out with the research and development of the first prototype. Ideally, this would be done in the context of a PhD. We will then find business angels and officially create the company, while working on the prototype, followed by our proof of concept with intestinal organoids. After another year, we will find Venture Capitals. Once we are done with our proof of concept, we will be able to partner with laboratories for pilot studies. They should last two years, during which we would also start working on the industrialization of our product. Next, we will enter the market, which will be accompanied by marketing work. A year later we will start finding late-stage Venture Capitals, which will allow us to further improve our product.

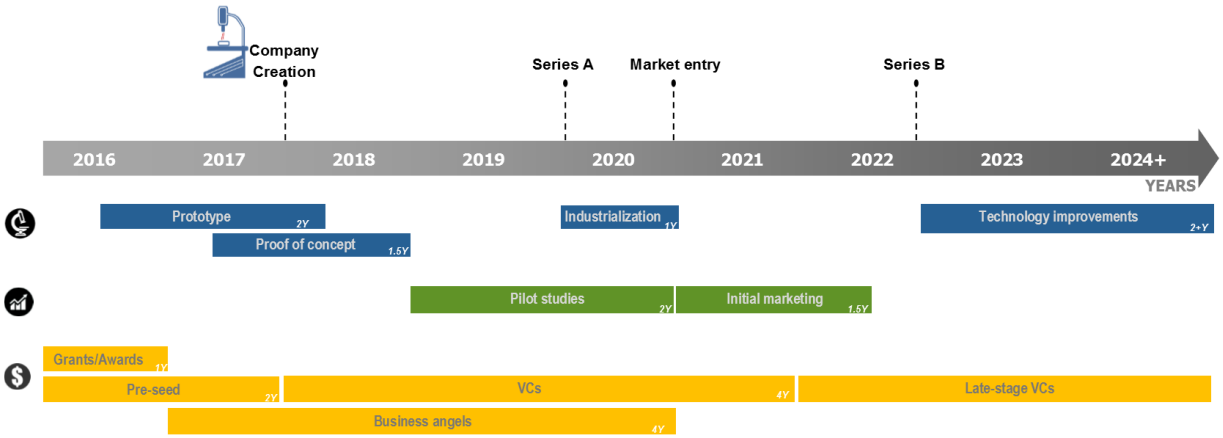

Figure 3 – Timeline Technological aspects are shown in blue, marketing is in green and funding is represented in yellow.

6.2. Development

6.2.1. MatriScope design

The aim of our device is to create and develop organoids in a controlled environment. 2-PP is used to control the mechanical properties of the environment in a spatiotemporal way. The organoid and the hydrogel are contained inside the chamber of a microfluidic chip, which allows a spatiotemporal control over the biochemical signals flown into it. Figure 4 shows the elements composing the MatriScope. The femtosecond laser generates pulses inducing polymerization at the focal point. The acousto-optic modulator (AOM), the $\frac{\lambda}{2}$ adjustable waveplate and the polarising beam-splitter modulate beam intensity. The microscope objective focuses the fs-laser into the sample contained inside the chamber of the microfluidic chip. The galvo-scanner is used to scan the beam within the sample in order to reproduce patterns created with a computer-aided design (CAD) software. The charge-coupled device (CCD)-camera allows monitoring of the 2-PP process as well as live cell imaging. Piezo-driven XY- and Z-stages allow to position the region of interest. The microfluidic chip is located at the place mentioned as "specimen" in Figure 4. The handling of the microfluidic chips will be automated so that many chips can be stacked and placed under the microscope when needed. This allows the culture of several organoids in parallel with only one device (but as many chips as organoids). Cell culture is enabled inside the MatriScope so that the stiffness of the matrix can be changed at anytime. The chips that are being used (i.e. that contain growing organoids) are kept inside an incubator in order for them to stay at constant temperature.

6.2.2. MatriChip design

Figure 5 shows the design of the MatriChip, the microfluidic chip containing the organoid. The size specifications as well as the number of channels described here might change depending on the prototyping stages and to allow larger organoids generation. It has the size of a standard microscope slide, i.e. 76mm $\times$ 26mm and is 7mm thick. It contains a hollow chamber measuring 5000μm $\times$ 5000μm $\times$ 5000μm where the hydrogel (and later the organoid) is localized. The front and rear walls respectively contain 9 inlets and 9 outlets connected by 100μm $\times$ 500μm circulation channels and spread as 3 rows of 3. These will be controlled with diaphragm valves in order to control temporally the input and output flow and mix different

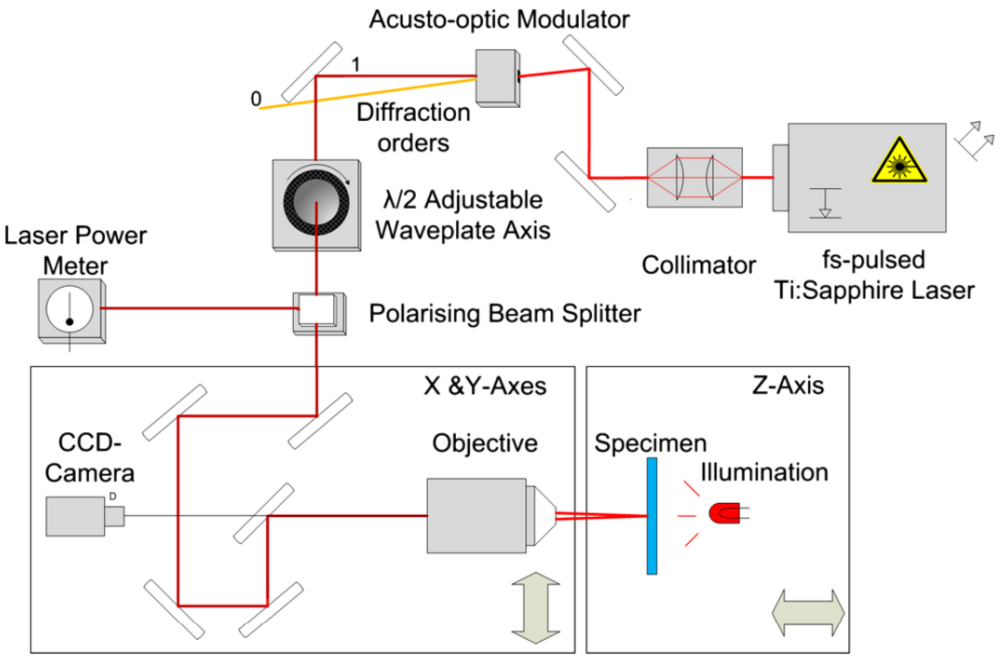

Figure 4 – **Elements of a two-photon microscope setup** In the MatriScope, the microfluidic chip will be located at the place mentioned as "specimen". [Torgersen et al., 2012]

compounds. Inside the chip, each of these channels is divided into 4 channels in order to distribute the flow over the entire hollow chamber. At later stages, the number of channel divisions might be increased in order to reach an interchannel distance that allows fast diffusion. The prototyping stages will enable us to determine the best design for the MatriChip chamber. Indeed, the chamber is either located completely inside the chip or it can constitute a hollow cavity located at the surface of the chip. In the first case, the hydrogel would be injected through the circulation channels until it fills the chamber. In the second case, the hydrogel would be poured directly in the chamber. The chamber would then be covered by a coverslip to allow oil-immersion microscopy.

The chip is made of Cyclic Olefin Copolymer (COC). The choice of the polymer is dictated by the requirements of our application. Because of 2-PP, the polymers must be transparent to NIR light. It also needs to be transparent to visible and ultraviolet (UV) light for live cell imaging. COC fulfills these requirements, while having a very low autofluorescence. Furthemore, it can be used for hot embossing and injection molding, two manufacturing processes that are very cost-effective for the production of a large number of pieces.

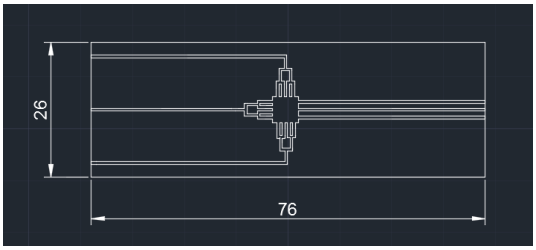

Figure 5 – **Microfluidic chip design** The chip is designed in a CAD environment with the correct specifications and dimensions. In the center, there is the chamber for the hydrogel ($5 \times 5 \times 5$ mm). It is connected by 500×100 μm square channels. Three replicates of the same design are superposed to form the final chip which consists of 3×3 inlet and 3×3 outlet channels.

6.2.3. Hydrogel

To ensure biocompatibility and efficiency of 2-PP, the hydrogel inserted into the MatriChip will need to fulfill the requirements detailed previously (see 5 Background). In particular, it must be degraded by the cells in a non-toxic way. Several tests will be performed during prototyping to identify the best hydrogels for different cell types. We will also choose the photoinitiator very carefully, so that the radicals it produces are non-cytotoxic. Moreover, mechanical properties of the formulation will be optimized by choosing the appropriate multifunctional crosslinker and prospective diluents.

6.2.4. Software

Software requirements and specifications are described hereafter. The software should allow extensive control of the different parts of the MatriScope. Its main roles will be to control the microscope (spatiotemporally) and the microfluidic interface. Image acquisition should also be addressed. For this, the open source software ThorImageLS might inspire our developments. A CAD environment, or at least 3D software compatibility (AutoCAD, Maya...) shall be implemented for designing the polymerization patterning. The conversion from magnetic resonance imaging (MRI) or computed tomography images directly to proper 3D scaffold models shall be enabled. Such models could be proposed in a database where users can submit their own creations, and share or sell them to the community. Some value might be created from these files, since we could get a commission each time a model is used. The software should be used for calibration purposes, and get access to a large panel of parameters, but it should contain a user-friendly mode, designed for biologists and researchers, with direct control over a limited array of parameters.

6.2.5. Intellectual property

In order to protect our technology, we plan to patent both the chip and the machine. The software will be covered by copyright (at least in Switzerland). A patent attorney will be contacted to help us in the patent filing, and specifically to set up the claims. However, a draft version is provided here.

Firstly, target states where our inventions should be protected are identified. They consist of Switzerland and EU at first because of the market size, as well as of the US since it will be risky to leave it unpatented there. Later, Asian countries will have to be taken into account since it is expected that their market will grow very rapidly.

Then, an extensive search in the prior art will have to be done to know exactly up to which point what we propose is new. The 2-PP in itself is subject to many publications, hence it is not patentable. Concerning the precise patterning of hydrogel stiffness, we only found one patent [Rehfeldt et al., 2010]. They use monofunctional thiol modifications of hyaluronic acid to tune the elastic modulus of the hydrogel, but do not have any spatial control, nor make use of a photocrosslinkable polymer. Moreover, their patent application was abandoned.

Patent application for MatriChip

Summary: Our invention consists of a microfluidic chip establishing an environment for the formation of organoids in 3D gel matrices. It is compatible with both 2-PP and microscopy. Moreover, the control of matrix stiffness is allowed. A more complete description is provided in chip design (see 6.2.2).
Revendications: The chip possess several inlets matching to an empty chamber where a hydrogel is inserted. The inlets are connected by channels inside the gel. Pumps enable to pulse fluid and cells through them. The inlets and oulets are organized in a multilayer manner, permitting to control spatially inputs and outputs, and allowing the creation of chemical gradients through the hydrogel. Valves are implemented in order to control temporally the input and output flow and mix different compounds.
Embodiment: Preferred embodiment is roughly defined in chip design (see 6.2.2). Some alternative versions will be submitted by varying the size of the channels, their numbers or the number of valves for instance.

Patent application for MatriScope

Summary: Our invention consists of a machine enabling to control mechanical properties of a 3D matrix precisely in order to generate organoids that are closer to nature, as well as generating organoids with a user-defined shape. A more complete description is provided in machine design (see 6.2.1).
Revendications: The apparatus makes use of a two-photon microscope to achieve polymerisation of a photocrosslinkable hydrogel, thereby enabling spatial patterning of the matrix stiffness and etching complex channel networks through the hydrogel. The two-photon microscope also serves as an imaging tool and can be used as a confocal microscope.
Embodiment: Preferred embodiment is defined in machine design (see 6.2.1). Some alternative embodiment might offer other designs, by replacing the microscope, including other lasers, etc.

6.2.6. Prototype

With the money from grants and ventures, we are going to build a prototype of our product. As our product development will start in academic laboratories, we intend to perform those first steps at EPFL, as part of some of the founders' PhD or postdoctoral degree. In addition to its high-tech facilities, EPFL presents the advantage of being located in Switzerland, namely the market of interest for Matrilight's early years. Moreover, Matrilight founders already have interesting contacts in this institution, where several laboratories might be interested in taking part in MatriScope and MatriChip development. In particular, we could contact the Laboratory for Regenerative Medicine & Pharmacobiology (LMRP), the Laboratory of Stem Cell Bioengineering (LSCB) and the Microsystems Laboratory 4 (LMIS4).

The prototype of the MatriScope will have similar dimensions as the final version. The first step in its fabrication will consist in the two-photon microscope fabrication. Preliminary negotiations with source parts producers will have been made in order to obtain price reductions for future bulk orders. In the meantime, a software engineer – ideally an EPFL student or intern – is going to code the interface that will enable programmed spatiotemporal control of the microscope. In the end, this interface will be used to pattern the hydrogel into any 3D structure loaded from a CAD file.

As for the prototype of the MatriChip, we intend to optimize its design by investigating several fabrication techniques, which could be performed at the Center Of Micronanotechnology (CMi), EPFL. Alternatively, or at later stages of the prototyping process, we could collaborate with companies specialized in injection molding, such as Makuta Technics, to develop a mold with our final cartridge design and mass produce MatriChips. In parallel, several hydrogels verifying the properties mentioned above are going to be tested for 2-PP and 3D cells culture, in order to find the best suited one for organoids generation. Once the first prototype is finished, we will use it to generate intestinal organoids as a proof of concept.

6.2.7. Proof of concept

Background

Proliferation and differentiation of intestinal stem cells (ISC) is tightly regulated by spatial gradients of components of specific signaling pathways such as TGF-β, Wnt, Notch, etc. Wnt and Notch signaling control proliferation and undifferentiation, Endothelial Growth Factor (EGF) promotes proliferation and Bone Morphogenetic Proteins (BMPs) control differentiation [Date and Sato, 2015]. For example, BMP is expressed at the top of the crypt and Wnt at the bottom, thus creating a gradient that will polarize the tissue from crypt to villus. The ISC's niche is composed of subepithelial fibroblasts that secrete signaling molecules such as BMP, Gremlin, TGF-β, Wnt, and R-spondin. Matrigel (the gold standard culture medium for intestinal organoids) is derived from Engelbreth-Holm-Swarm (EHS) tumor and mimics the basal membrane [Kleinman et al., 1986]. It contains mainly laminin, collagen IV, entactin, and heparan sulfate and some growth factors like TGF-β and fibroblast growth factor (FGF).

Protocol

We will engineer a hydrogel precursor containing cell binding sites homologous to the basal membrane molecules. We can thus provide a safer environment for clinical trials compared to Matrigel. Moreover the hydrogel will be enriched with photoinitiators able to cross link upon two-photon illumination, thus allowing gelation. The hydrogel polymer will also contain matrix metalloproteinase (MMP) sites allowing degradation and remodeling of the matrix by the cells.

The hydrogel precursor will be injected into the chip chamber and a 3D perfusion network will be created by 2-PP based on CAD models. Microfluidic channels will allow perfusion within the chamber. ISCs will be seeded in the hydrogel through one of the channels, left incomplete. Then, the construction of the pattern in the hydrogel will be triggered by 2-PP. The pore size should be smaller than 2μm in order to mimic at least a 50% Matrigel hydrogel that will provide enough stiffness for cell adhesion and migration [Zaman et al., 2006].

The 3D perfusion network will be seeded with endothelial cells that will reach confluence within a few days, giving rise to a vascular system able to sense and grow with the organoid itself. By doing so, Matrilight will provide a tool allowing a spatiotemporal control over the delivery of chemical and mechanical cues from the environment. In order to mimic ISC niches, signaling molecules released by subepithelial fibroblast can be delivered through the appropriate channels, thus creating a gradient from the periphery to the center of the gel. BMP will then be provided through central channels, thus mimicking the natural gradient that induces cell differentiation.

The vascular system will generate new vessels according to hypoxic signals from the organoid core, thereby allowing an *in vivo*-like vasculature. We can also imagine injecting vascular endothelial growth factor (VEGF) through the hydrogel core channel in order to attract endothelial cells.

Histological analysis will determine the nature of the grown organoid and provide important information such as its differentiation states. By genetically modifying the ISCs with reporter genes such as Green Fluorescent Protein (GFP), one can also monitor the evolution of differentiation and spot specific cells that will express GFP through real-time lineage tracing.

6.2.8. Design optimization for industrialization

Once we will have developed our prototype and gotten feedback from our partner laboratories, we will optimize the design of the MatriScope for industrialization. It will have to fit the requirements from the laboratories and to be cost effective for production.

Injection molding will be the manufacturing process of choice for the mass production of the chips since it is very cost effective.

6.3. Business Model

Our product will be sold in a package that includes shipping, onsite device calibration and testing, training of personnel and troubleshooting support. The main source of revenue will then be the cartridges, which need to be changed for each new organoid to be generated.

6.4. Marketing

Pilot studies will be achieved by our partner laboratories. We will provide them with our machine, so they can use it for their research and mention the use of our product in their publications, some of which we could co-publish. The aim is to find key partners with a renowned name in the field to obtain a large marketing impact. Matrilight will also be presented in congresses related to cell culture devices as well as regenerative medicine, and there will be press releases concerning noteworthy milestones. We will of course have a website, with a blog and quotes from our partners, and we will be active on various social media.

6.5. Funding

6.5.1. Grants and pre-seed capital

Research and development grants support the academic research of PhD students and postdoctoral researchers. As the development of a product such as the MatriScope takes years and is costly, we will start the development in academic laboratories in order to benefit from these grants. Then, pre-seed grants and money from founders and their relatives will help to develop a prototype. It may also cover the costs of the startup foundation. This can be provided by an Innogrant, a fund that aims at helping technology transfer at EPFL, up to 100'000 CHF. We will apply to a Venture Kick as well as other incubators or accelerators for our pre-seed capital. It would help us build the initial team and provide us with the support (providing additional value in the form of coaching and mentorship, and access to a network) we need, as well as 130'000 CHF.

6.5.2. Venture Capital and business angels

The second round of funding will come from business angels, who are individual investors investing their own capital, often less than 50'000 CHF per person, often as stock, and sometimes as note. Some do that on a full-time basis as professionals and can be called micro Venture Capitals. At a later stage (after developing a prototype, before the company's market entry), we will try to find Institutional Venture Funds, which we can call the third round of funding or Series A. After launching our flagship product, we will look for larger Venture Capitals for a more sustainable and long-term development.

6.6. Future (5-10 years plan)

While the first version of our product will be commercially available, the R&D department of our startup will continue to improve it and add new features, mostly software-based. For example, an intelligent and automated control of the growth factors delivery could be implemented. The type of growth factors delivered, their concentration and their target area within the hydrogel could be adjusted by an algorithm based on cellular markers (visualized with two-photon microscopy). The same type of control could be applied to the stiffness of the hydrogel. In this case, the local siffness could be adjusted based on cellular markers of the neighbouring cells. This would mimic the bidirectional talk between cells and ECM *in vivo*, before the remodeling of the hydrogel by the cells. The R&D department will also work on improving the writing speed for the patterning. It may also develop or integrate new hydrogels and new photoinitiators.

Customer feedback will help us adjust the design of the device and the software user interface.

Costs for our startup can be divided in one-time fees needed to assemble the first team and register as a company, annual costs (salaries and charges), and costs needed to build the first prototype and pilot studies.

7.1. One-time starting fees

These costs will be funded by accelerators, incubators and business angels.

Type	Description	Approximate costs
Registration	In Switzerland, the registration and launch of a business costs between 2'000 CHF and 15'000 CHF for a SA [Administration suisse, 2015]	8'000 CHF
Hiring fees	Meetings with candidates, advertisements,...	3'000 CHF
Office equipment and supplies	Computers, software licences, desks	30'000 CHF
Lab equipment and supplies	Used to prepare the hydrogels and organoid culture.	50'000 CHF
Patent fees	Patent submission fees for important countries (Switzerland, Europe, USA, China, Japan) [WIPO, 2015]	20'000 CHF
TOTAL		**111'000 CHF**

7.2. Annual costs

These costs will be funded by business angels (first years), VCs, and revenue from sales.

Type	Description	Approximate costs
Insurance	For a SA, professional risk (liability, theft, natural damage) insurance is required.	10'000 CHF a year for a 5-members startup.
Office and facilities	A 6-person (120 m2) office in a suburb in Switzerland.	5000 CHF per month, therefore 60'000 CHF / year. [Homegate, 2015]
CEO / Engineer	The CEO is also working as an engineer [Office fédéral de la statistique, 2010]	100'000 CHF
CFO / Sales / Marketing		100'000 CHF
CTO / Engineer		100'000 CHF
Software Engineer		90'000 CHF
Hardware Engineer		100'000 CHF
Accounting (part-time)	It will be done by a fiduciary [Contagest, 2015]	20'000 CHF
Hardware maintenance and consumables	Computers, communication, printers, website, labware	40'000 CHF
Marketing fees	Mailing-lists, prospects, calls, meetings, travels.	15'000 CHF
Patent renewal fee	Cost is increasing every year. This is an average with solicitor's fees. [Epoline, 2015]	2'000 CHF
Taxes	Around 20% of the net earnings and 1% of the capital. [Ville de Lausanne, 2015] For the first year no benefice is expected.	10'000 CHF
TOTAL		**647'000 CHF**

7.3. Prototype costs

We can assume that the final model will cost approximately 50% of the prototype cost (we aim for a CHF 200'000 net cost) due to better sourcing and design. Gel cartridges will be manufactured from injection molding. The cost of the mold is around 30'000 CHF and must be replaced every two years.

Type	Description	Approximate costs
Two-photon microscope	Also contains the laser system and CCD camera. Many manufacturers offer Original Equipment Manufacturer (OEM) products or are open to partnerships. [LabRigger, 2011] [Biocompare, 2012]	340'000 CHF
Microfluidics	Around 1000 CHF per channel, at least 4 pumps are required by the design. Diaphragm valves are 100 CHF per channel, 9×2 channels are required. Tubing and connections could be approx. 2000 CHF. Incubator. [Parker Inc., 2015]	25'000 CHF
Control system	Motors, clamps, D/A drivers, sensors, power stage. Could be done with an Arduino platform for control (standardized).	20'000 CHF
Metalwork		2'000 CHF
Chip prototype	Laboratory fees.	5'000 CHF
Proof-of-concept fees	Contact with university labs, travel costs, material costs.	20'000 CHF
TOTAL		**412'000 CHF**

7.3. Expected sales

Example of revenue from a customer ordering a machine and 3 years worth of MatriChips.

Description	Costs	Selling price	Benefits
MatriScope	200'000 CHF	490'000 CHF	290'000 CHF
Shipping + calibration fees by technician	6'000 CHF	0 CHF	-6'000 CHF
360 MatriChips (with hydrogel)	720 CHF	18'000 CHF	17'280 CHF
TOTAL	**206'720 CHF**	**508'000 CHF**	**301'280 CHF**

7.4. Budget for five years

We model that annual fees will grow by 10% a year after the first three years. We also model that in the first two years, salaries will be significantly lower (resp. 40% less and 20% less) in order to help with the growth of the company.

Year	Description	Costs	Sales
Year 1	Business starting fees	111'000 CHF	0 CHF
	Annual business fees	388'000 CHF	0 CHF
	Prototype and Proof-of-concept	412'000 CHF	0 CHF
Year 2	Annual business fees	518'000 CHF	0 CHF
	1 x Matriscope lent to partners	206'000 CHF	0 CHF
	120 MatriChips sold	240 CHF	6'000 CHF
Year 3	Annual business fees	647'000 CHF	0 CHF
	1 x Matriscope lent to partners	206'000 CHF	0 CHF
	240 MatriChips sold	480 CHF	12'000 CHF
Year 4	Annual business fees	711'000 CHF	0 CHF
	2 x Matriscope sold	412'000 CHF	980'000 CHF
	500 MatriChips sold	1'000 CHF	25'000 CHF
Year 5	Annual business fees	782'000 CHF	0 CHF
	6 x Matriscope sold	1'236'000 CHF	2'994'000 CHF
	1200 MatriChips sold	2'400 CHF	60'000 CHF
TOTAL		**5'633'120 CHF**	**4'023'000 CHF**

Therefore we need to find approximately a 1.6 MCHF capital in order to start to make benefits after 5 years. These funds will be provided by incubators, business angels and VCs.

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