MESENCHYMAL STEM CELLS GUIDE ENDOTHELIAL CELLS IN A MODEL ORGANIZED BY LASER ASSISTED BIOPRINTING

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Laser-Assisted Bioprinting: a tool to organize and control morphogenesis in Tissue Engineering

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INTRODUCTION

Tissue engineering (TE) is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue or organ function (1). Its goals are to develop new technologies for cell and molecular assembly, and therapeutic strategies for the replacement of tissues (2). In particular, the bone has a specific organization over different length scales were various cells, blood vessels, collagen fibers, collagen molecules, and dynamic processes take place. Vascularization of clinically relevant sized TE constructs remains a limit in the transfer from *in vitro* to *in vivo* systems (3). Immediate vascularization of implanted bone or graft substitutes is critical for their survival and function, due to the limitations in oxygen and nutrient supply. During culture, tissue constructs can be supplied with nutrients by medium perfusion, but *in vivo*, the vascular ingrowth is normally too slow to assure graft survival (4). Nonetheless, none of the different strategies that are commonly and currently used can fulfill all the requirements, emphasizing the need for a bone tissue engineering strategy integrating scaffolds, growth factors or cells (5).

Recent studies have been developed in order to solve the problematic presented when referring to bone tissue engineering. One approach to provide a functional blood supply to the bone defect would be the *in vitro* creation of vascular infrastructure that could be integrated with the patient's vasculature (4). Chen's group showed the potential of endothelial and mesenchymal 3D aggregates to neovascularize a mouse limb after ischemia induction (6), demonstrating the potential of these cells *in vivo*. Other groups are adding to their studies different technologies based on biofabrication, micropatterning and laser systems using or not scaffolds, to study human endothelial and mesenchymal stem cell interactions (7) (8).

The approach proposed by the BioTis Laboratory and TEAL group (Tissue Engineering Assisted by Laser) is to take advantage of the Laser-Assisted Bioprinting (LAB) technique. This is a computer-aided rapid-prototyping technique that has been adapted

toward the fabrication of 3D artificial tissue or organ, and that allows printing of cells with a good resolution in a high-throughput manner without any apparent damage to phenotype and genotype (9) (10). In order to overcome this approach, the aim is to print human mesenchymal stem cells and human endothelial cells in a collagen matrix in order to obtain well-defined patterns of printed cells with controlled distance between them. Various distances and patterns will be studied in order to favor the alignment of the post-printed cells comparing their behavior over time.

LITERATURE REVIEW

Bone Tissue Engineering: The problem of vascularization

One of the current limitations of tissue engineering is its inability to provide sufficient blood supply in the initial phase after implantation. This lack or insufficiency of vascularization can lead to cell death or to improper cell integration of tissue-engineered constructs. As the speed of vascularization after implantation is a major problem, to success in the application of TE for bigger tissues, such as bone and muscle, the difficulty of vascularization has to be solved. Among the various strategies to solve the problem of blood supply in bone defects, the *in vitro* and *in vivo* prevascularization appear as a potential for mimicking human tissues (11) (12).

The crosstalk between endothelial cells and mesenchymal stem cells activity

Mesenchymal stem cells (MSCs) are multipotent cells that have the ability to self-renew and differentiate into namely osteoblastic, chondrocytic and adipogenic lineages. Their rapid expansion in culture and high degree of plasticity has made these cells an excellent candidate for a variety of research and therapeutic applications (13) (14) (15). Endothelial cells (ECs) self-assemble into vascular tubes when they are surrounded by extracellular matrix and could be prime sources of modulators of bone development and function (16). They are also a critical component of blood vessels, functioning at the interface between the vessel wall and the components of the blood (17).

The clinical applications of MSCs have been restricted due to the limited understanding of the factors that regulate their activity and the lack of knowledge of the complexity of the interplay between these cells and components of their immediate microenvironment or niche. In an attempt to recreate this complex microenvironment, the use of 3D culture systems is gaining attention. Significant alterations in cell behavior have been identified, when they are grown in 3D compared to 2D conditions. These changes deal with cell morphology, differentiation capacity, replicative ability, cell signaling, as well as significant increases in their therapeutic potential (14) (18).

Cells can communicate through three mechanisms when they are in coculture: (a) secretion of diffusible factors (either by the cells, or released from the ECM) that can activate specific receptors on the target cells, (b) gap junction communication that form direct cytoplasmic connections between adjacent cells, and (c) direct interaction between membrane molecules of the two adjacent cells (5). The communication is important to stimulate differentiation and angiogenesis by the coculture of endothelial cells with other cell types. Studies have shown that two-dimensional coculture of human MSCs and ECs induced osteoblastic differentiation of MSCs and stimulated the formation of a prevascularization (formation of a self-assembled network) (19).

As cell-to-cell communication and migration are concerned, the distance between both types of cells has been shown as an important parameter. One study showed that Human Bone Marrow Stem Cells (HBMSC) demonstrate strong distance-dependent directional migration toward Human Umbilical Vein Endothelial Cells (HUVECs), with highest extent of sprouting when these cells are placed at the closest (500 μm) initial distance from HUVECs, and decreasing as the channel-to-channel distance increased up to 2000 μm (7). Published examples of *in vitro* coculturing experiments also suggest that ECs drive MSCs towards the osteoblastic phenotype and that ECs could thus be considered as “osteoinductive” mediators in a coculture model (18).

The crosstalk has been studied in 2D, and in 3D by the use of scaffolds or matrices. The choice of biomaterials available for learning the dialogue between ECs and MSCs in regenerative medicine continues to grow rapidly in the purpose of mimicking the function of the extracellular matrix in supporting cell attachment, proliferation and differentiation (20). Despite of synthetic polymeric scaffolds, biomaterials based on extracellular matrix (ECM) molecules like hyluronan, fibrin, or collagen play a prominent role as substrates for cells in culture (21).

Among the different types of collagen, the collagen type I is the major component of the ECM in mammals, and it is strongly expressed in several tissues like tendons, ligaments, bone, or blood vessels. It represents appropriate biocompatible sources for degradable scaffolds in the human system and has specific mechanical and structural properties (20).

Bioprinting: a biofabrication technology

Biofabrication is the assembly of the many techniques that transfer biologically important materials onto a substrate, which covers a range of patterning length scales and varies depending on the application. Among the biofabrication techniques, the bioprinting is defined as the use of material transfer processes for patterning and assembling biologically relevant materials - living cells, extracellular matrix (ECM) components, biochemical factors, proteins, drugs, molecules, tissues, and biodegradable biomaterials - on a receiving substrate or liquid reservoir to accomplish one or more biological functions (22) (23). In addition, it is an automated approach with the potential for mass production of tissues that has multiple advantages and characteristics. It is simple to use, allows high-throughput generation of spatially and temporarily controlled complex constructs, and provides 3D complexity by multilayer printing (23) (24). When bioprinting is used at the finest scale, it is a molecular delivery mechanism, and at its coarsest scale it is required to reproduce great anatomical features, such as a vascular network (22).

Bioprinting technologies

Bioprinting techniques with biomedical applications are generally divided in three groups: nozzle-, printer-, and laser-based systems.

The nozzle-based systems are techniques based on a melting process; printer-based systems implement inkjet technology by using electrical signals to control the ejection of an individual droplet; and the laser-based subclass subsequently deposits light energy in specific predefined factors (25).

Laser-Assisted Bioprinting (LAB) has been particularly ambitiously studied and adapted toward the fabrication of 3D artificial tissue or organ printing (9). It is based on laser-induced droplet ejection and is basically composed of a pulse laser source; a target (ribbon) that is made of a thin absorbing layer of metal (gold or titanium) coated onto a laser transparent support, from which a biological material is printed; and a substrate that receives and collects the printed material (26). Depending on the optical properties of the bio-ink or laser wavelength, a laser absorbing interlayer is necessary to induce transfer and is thus placed between the support and the biomaterials to be printed (27). Organic material is prepared in a liquid solution, and deposited at the surface of the metal film. The laser pulse induces vaporization of the metal film, resulting in the production of a jet of liquid solution, which is deposited onto the facing substrate. Figure 1 shows the aforementioned principle of the laser-assisted bioprinting.

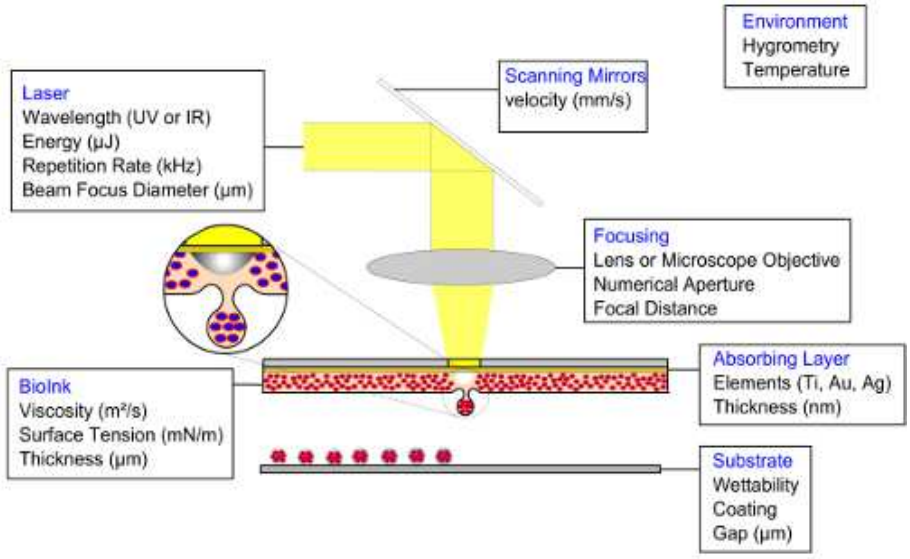

Figure 1: LAB schema (28)**.**

Some parameters control the volume of the printed droplets, such as: the thickness of the layer of the bio-ink coated onto the ribbon, the surface tension and the viscosity of the bio-ink, the wettability of the substrate, the laser fluency, and the air gap between

the ribbon and the substrate (26). However, it was found that the volume of deposited material depends linearly on the laser pulse energy, and that threshold energy has to be overcome for microdroplet ejection to occur (28).
This technique has been interesting over the others because of its high spatial resolution and because it is a non-contact and orifice-free technique; and since its usage, it avoids contamination and clogging problems (22) (29).

Physical and biological aspects of bioprinting

In all bioprinting methods, the effect of the transfer process on the biological material used is a very important aspect. This is why, bioprinting must deal with two biological problems: 1) the toxicity and damaging for cells and their DNA, 2) printed constructs must be able to rapidly evolve into a mechanically stable tissue (24).

In addition, before and during printing, cells and molecules must be carried in a fluid vehicle that requires consolidation shortly after printing, what implies a viscoelastic behavior. This phase change must occur without damage to the cells or complex units within the fluid, which presents a considerable challenge for future development of biomaterials (24).

Demonstration of cell viability during and immediately after printing is a priority task for bioprinting because printed constructs must be suitable for perfusion and be able to survive *in vitro*. Currently, several bioprinting technologies have attained viabilities exceeding 90% compared to the controls of non-printed cell cultures indicating the broad future applicability of bioprinting to various cell types ranging from neurons to stem cells (24) (23).

OBJECTIVES

According to the literature review and the potential of the bioprinting technology, the objectives of this work were:

- To print MSCs and ECs in monoculture and coculture with a specific pattern, using the Laser-Assisted Bioprinting.
- To demonstrate that this technique allows the printing of alive and functional cells.
- To demonstrate the effect of a coculture model against monoculture models.

Mesenchymal Stem Cells guide Endothelial Cells in a model organized by Laser-Assisted Bioprinting.

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Abstract

The construction of complex tissues including a vascular network is a challenging issue in tissue engineering. The lack of vascularization during the *in vitro* growth and development of bones remains one of the main problems that must be overcome. *In vitro* prevascularization by the use of endothelial cells (ECs) and mesenchymal stem cells (MSCs) in coculture is acquiring much more importance. However, tissues are a combination of small repeating units assembled together. To allow in vitro building of tissue-like structures there is a necessity to create and manipulate the microenvironment consisting of biomolecular gradients and cell-cell interactions. The aim of this study is to use the laser-assisted bioprinting technique to investigate the optimal pattern and distance at which human umbilical endothelial cells (HUVECs) and human bone marrow stem cells (HBMSCs), alone or in coculture, will form an aligned structure and hence a functional micro-vascular structure. Both HUVECs and HBMSCs showed a beginning of an alignment at 6 and 24 hours, respectively; while in coculture they showed a complete alignment at 24 hours where HBMSCs conduce HUVECs to stay over them. Specific markers of ECs, such as von Willebrand Factor (vWF) and CD31 were expressed post-printing in monocultured and cocultured HUVECs. HBMSCs guide HUVECs organization in a coculture model. The bioprinting is a promising tool to study cell behavior with great accuracy as well for developing innovative strategies for tissue fabrication.

1. INTRODUCTION

The construction of complex tissues including a vascular network is a challenging issue in tissue engineering (8). The vascular network acts as a transport system for hormones, waste products, and toxic and functional substances (2). After implantation, the lack of vascularization during the *in vitro* growth and development of bones remains one of the main problems that must be overcome due to the limitations in oxygen and nutrient supply that lead to cell death in the tissue engineering constructs (7) (5). Only

few tissues can be supplied by nutrients diffusion from auxiliary blood vessel systems that are further away. In the particular case of *in vivo* bone regeneration, the capillaries that are subdivided in the tissue must be at an optimal distribution that is related with the diffusion of oxygen. This is the reason why diffusion processes are limited due to the insufficient distances between the capillaries (30). Nowadays, several strategies for enhancing vascularization are under investigation. Some approaches emerge like the scaffold design to promote angiogenesis, the *in vitro* prevascularization (31), or the inclusion of angiogenic factors (11). The *in vitro* prevascularization is acquiring much more importance, while various studies are focusing on this strategy by using vascular endothelial cells (ECs) and mesenchymal stem cells (MSCs) in coculture. The formation of 3D prevascular structures (5); coculture systems to modulate MSCs expansion and osteogenic differentiation *in vitro* (32); and the formation of tubular networks from 3D aggregates formed on Matrigel® (6) are among these studies. The aim of these approaches is the acceleration of functional anastomosis that could join to the patient's vasculature and regenerate bone defects (7) (11).

Tissues are a combination of small repeating units assembled over several scales. During tissue development, most mechanisms of pattern formation are based on spatio-temporal heterogeneity inducing the formation of local environment (12). The necessity of tools to create and manipulate the microenvironment including the location and shape of biological gradients are essential. A variety of techniques have been developed to engineer free-scaffold tissues in three dimensions by assembling blocks mimicking those units in a bottom-up or modular approach (33) (12). Among them, bioprinting has emerged as a rapid prototyping technology for patterning and assembling biologically relevant materials. This technology provides the opportunity to program biological material organization into 3D to evolve positively towards functional tissue by promoting self-organization processes (34). It thus allows studying the relation between geometries or patterns of cells and tissue function (26).

In this study, human umbilical endothelial cells (HUVECs) and human bone marrow stem cells (HBMSCs) were printed to study the effect of the distance on their auto-organization. They were thus deposited into a collagen type I matrix using a Laser-Assisted Bioprinter. Cells were printed in monoculture and coculture using a specific pattern of 250 µm between dots of cells of the same segment, and 500 µm or 1000 µm between dots of two consecutive segments. The cell viability and cell behavior were investigated along 2 days post-printing searching the time point at which cells were able to form an aligned structure. It was shown by immunofluorescence that the orientation of the actin fibers is in accordance with the cell spreading and orientation, promoting the aligned structure. It was also demonstrated by the same technique that HUVECs were not changing their phenotype by showing the specific expression of von Willebrand Factor (in mono- and coculture), and of CD31 (in monoculture). Finally, cell distribution within collagen matrices after the printing process, was investigated.

2. MATERIALS AND METHODS

2.1 Isolation and cell culture

2.1.1 HBMSCs

Human bone marrow stem cells (HBMSCs) were obtained from human bone marrow aspirated from the iliac crest of healthy donors undergoing hip prosthesis surgery. Cells were separated into a single suspension by sequential passages through syringes fitted with 16-, 18- or 21-gauge needles. After centrifugation, the pellet was cultured in Minimum Essential Medium Alpha Modification (α-MEM; Gibco, Paislay, United Kingdom), and supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Lonza, Verviers, Belgium). Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37ºC (35).

2.1.2 HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described previously and transfected with Td-Tomato fluorescent protein

(36). They were grown in Iscove's Modified Dulbecco's Medium (IMDM; Gibco), and supplemented with 20% (v/v) FBS (Lonza) and 0.4% ECGS/H (PromoCell, Heidelberg, Germany). Flasks were coated with gelatin (0,2% in MilliQ water) few minutes before seeding the cells. Transfected cells were used depending on the experiments for following their fate within the cocultures.

2.1.3 Coculture

Cells in coculture were seeded at a 1:1 ratio using 50% of IMDM with 20% (v/v) FBS and 0.4% ECGS/H, and 50% of α-MEM with 10% (v/v) FBS (37).

2.2 Laser-assisted bioprinting set up

Two coplanar glass slides are arranged in close proximity (separated a distance of 1 mm) to each other. The upper glass slide, also called the donor slide, is covered with a 60 nm energy-absorbing gold layer using a plasma-enhanced sputter deposition Emscope SC500. A layer of the cell-containing suspension to be transferred is coated on top of the gold layer (10). The lower glass corresponds to the collector slide and is covered with a layer of collagen type I solution (BD Biosciences, Bedford, MA), which cushions the cell impact, providing a moist environment for the cells during the printing procedure. Laser pulses are focused through the upper glass slide onto the gold layer and a high pressure is generated resulting in a jet formation that propels the cell-containing suspension towards the collector slide (26). Fig. 1A shows a scheme of the description given above.

2.3 Preparation of the cell-containing suspension on the donor slide

Cultured cells were detached by the trypsin treatment, resuspended in cell medium, and counted to determine the cell concentration. Cells were then centrifuged and the supernatant was removed. The cell pellet was resuspended in serum-free medium at a concentration of 100 million cells/ml. The obtained suspension is pipetted on the gold-coated donor slide. Cell suspension is distributed on the gold surface to form a homogeneous layer. The cell-coated donor slide is fixed in a metal frame and mounted upside down in the LAB workstation.

2.4 Preparation of the hydrogel layer on the collector slide

The collector slide was sterilized with ethanol. It was coated with ~ 200-µm-thickness collagen type I solution (final concentration: 2 mg/mL; BD Biosciences). After gelation for 1h at 37ºC, the coated collector slide was positioned into the workstation facing the cell-coated donor slide in close proximity. The collagen solution was prepared as described by the manufacturer's procedure.

2.5 Patterning of the HMBSCs and the HUVECs

A specific pattern of dotted lines was established for all the printing procedures. The distance between the dots of cells of the same line was fixed at 250 µm; and between dots of two consecutive lines, distances of 500 µm and 1000 µm were used. Fig. 1B shows a scheme of the description given above.

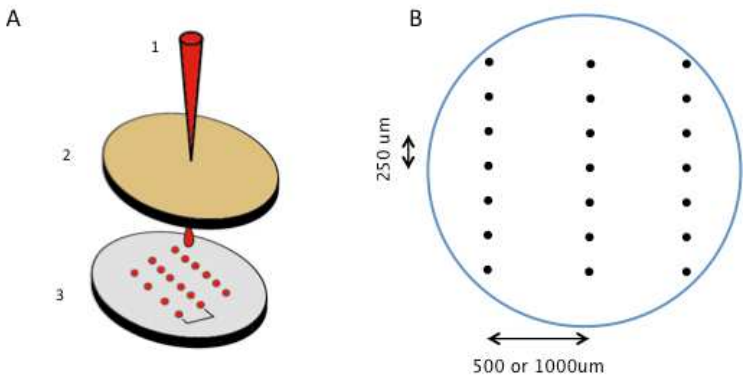


Figure 1. Scheme concerning the LAB setup and the cell patterns
(A) Schema of the LAB setup: 1) laser beam, 2) donor gold-coated slide to generate the jet formation (the cell-containing solution is facing #3), and 3) receptor collagen-coated slide. The red dots in #3 represent the cells that were deposited after all the procedure. (Convention: 1 red dot = n amount of cells depending on the parameters selected and the concentration of the cell-containing solution). **(B)** Pattern used for all the experiments: 250 µm between dots of the same segment, and 500 µm or 1000 µm between two consecutive segments.

2.6 Immunofluorescent staining

The HUVECs Td-Tomato alone and cocultured with HBMSCs in the collagen 3D matrix for 48 hours were fixed with 4% (w/v) PFA (paraformaldehyde) at 4ºC for 20 minutes and permeabilized. Then they were incubated for 1 hour in PBS 0.1 M pH 7.4 (Gibco) containing 1% BSA (w/v) before incubation with primary antibodies: a polyclonal rabbit

anti-human von Willebrand Factor (diluted in PBS 1X with 0.5% (w/v) BSA at 1/1000; Dako, Glostrup, Denmark), or a monoclonal mouse anti-human CD31 Purified (diluted in PBS 1X with 0.5% (w/v) BSA at 1/100; eBioscience, California, USA) for 2 hours at 37ºC. Afterwards, cells were washed with PBS 1X (Gibco) and incubated for 1 hour at 37ºC with Alexa Fluor 488 goat anti-rabbit IgG (diluted at 1/300; Invitrogen, Oregon, USA), and Alexa Fluor 488 rabbit anti-mouse IgG (H+L) (diluted at 1/200, Invitrogen), respectively. Cells were incubated with the nuclear probe DAPI (4', 6'-diamidino-2-phenylindole, Sigma 5 mg/mL, dilution 1/5000) for 20 minutes at room temperature to label the nuclei. For the F-actin, cells were incubated with Alexa Fluor 488 phalloidin (diluted in PBS 1X with 1% (w/v) BSA at 1/40, Invitrogen) for 1 hour at room temperature. Samples were then observed with a fluorescence microscope (Leica DMi 3000 B) equipped with the epifluorescence filters required.

2.7 Cell viability assay

To evaluate viability after 48 hours of culture after printing, cells were stained using the live/dead assay kit (Molecular Probes) according to manufacturer's instructions. Hydrogels were incubated for 15 minutes at 37ºC in medium without red phenol with 2 µM calcein-AM and 4 µM ethidium homodimer (EthD-1). Samples were observed using an epifluorescent microscope.

2.8 Measuring of the post-printing precision

Two conditions were chosen to define the distances between two dots of different segments and between dots of the same segment. The first is a segment separation of 500 µm, and the second of 1000 µm. For both cases the dot's separation was fixed at 250 µm. Three samples (n=3) were selected from each condition and distances were measured using ImageJ software.

3. RESULTS

3.1 Post-printing precision.

The mean distance measured between segments after printing at 500 µm was 520±18 µm and 1007±28 µm when set at 1000 µm. Between dots of 250 µm, the mean was of 258±16 µm. Considering these results it was assumed that the bioprinting technique used had a maximal error of 20 µm until a distance of 1000 µm.

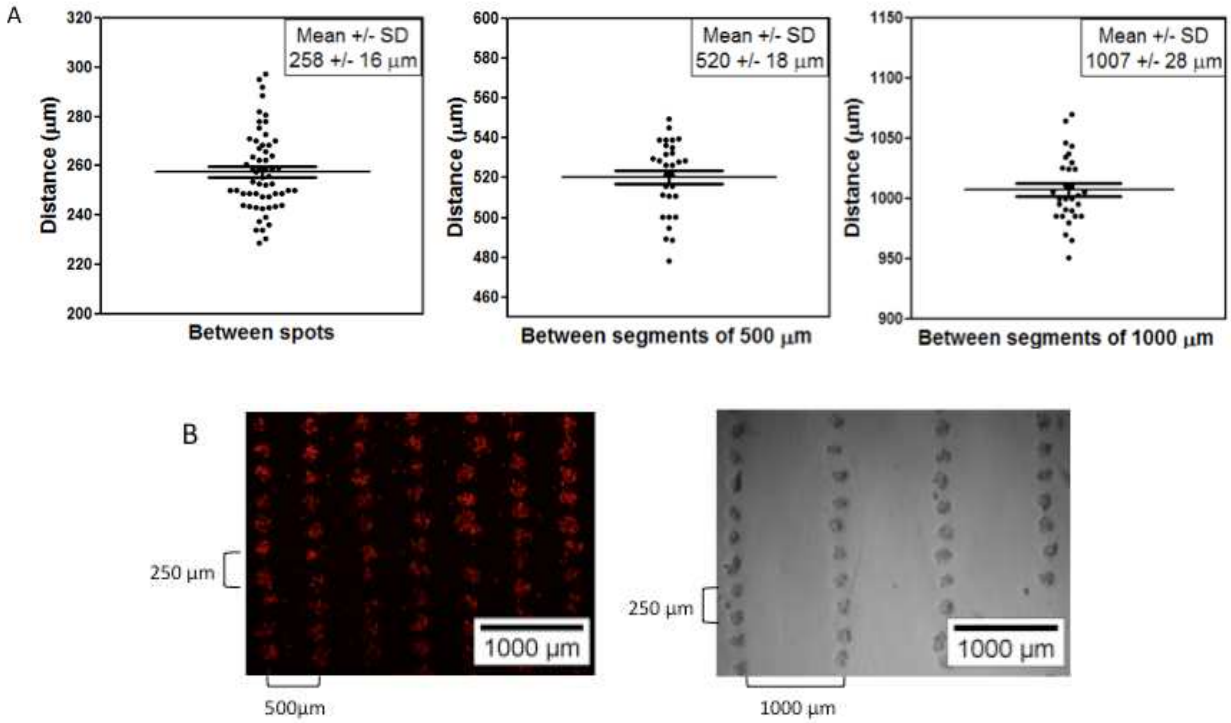


Figure 2. Mean distances between segments and between dots. (A) Mean distances between spots (n=60) and between segments (n=30 for each distance), measured on the collector slide with ImageJ. **(B)** Patterns used along the study. Data are expressed as mean ± SD. SD: standard deviation. One representative experiment is presented.

3.2 Cell fate and cell viability after printing.

In order to verify cell viability after printing, a Live/Dead assay was performed. At 48 hours HUVECs do not suffered of drastic damage (Fig. 3A). Posteriorly, each cell type was marked to label the F-actin; which showed the capacity of cells to spread into the

collagen matrix and the possible migration driving the alignment between two adjacent dots of the same segment. As the alignment of HBMSCs was observed 24 hours post-printing, this time point was selected to label the stress fibers (Fig. 3B). In the case of HUVECs, cytoskeleton fibers were marked at 6 hours (Fig. 3C).

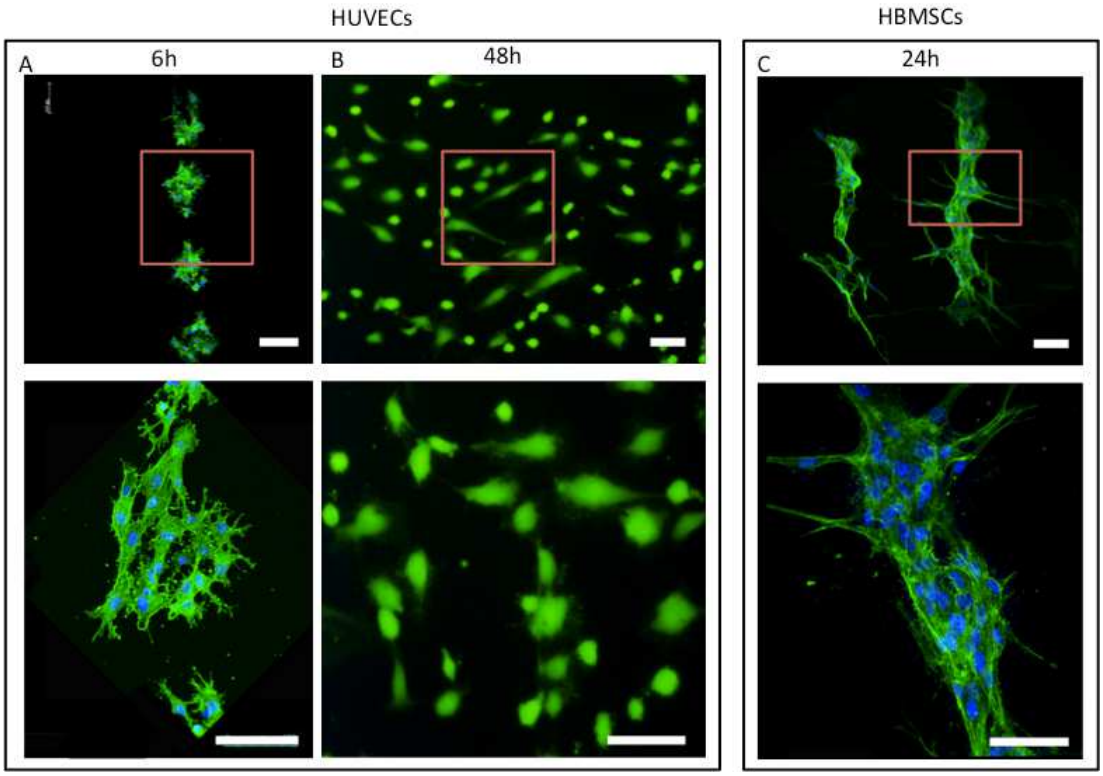


Figure 3. Live/dead assay and immunofluorescence staining of printed HUVECs and HBMSCs. (A) Actin in green and nuclei in blue of HUVECs 6 hours post-printing. **(B)** Live/dead test of printed HUVECs at 48 hours: living cells are labeled in green and death cells in red. **(C)** Actin and nuclei of HBMSCs 24 hours after printing. Images were representative of n=3 experiments. Distance between segments: 500 µm. Scale bar: 100 µm.

3.3 Cell organization towards the alignment.

Both HUVECs and HBMSCs were first printed separately on a collagen layer, searching for the optimal conditions required in order to favor the cell alignment between spots of the same segment rather than between neighbor segments. HUVECs Td-Tomato were printed at 500 µm and 1000 µm between segments to study the difference in behavior over time. At 24 hours, cells printed at 500 µm formed a homogeneous layer; while at 1000 µm there was an apparent conservation of the pattern with a reduction of ~500 µm

in distance between two segments and a delay in the formation of the uniform layer (Fig. 4).

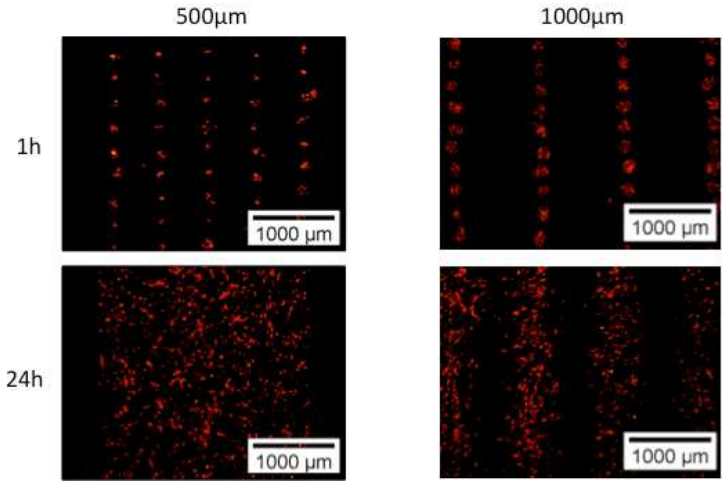


Figure 4. Cell behavior after bioprinting. Comparison of the pattern conservation of printed HUVECs Td-Tomato at 500 µm and 1000 µm between segments, and 250µm between dots. Images were representative of n=5 experiments.

Using an epifluorescent microscope, a follow-up was developed for each dissociated counterpart and in coculture at 500 µm of segment separation and 250 µm of spot separation. HBMSCs showed an alignment 24 hours post-printing due probably to cell migration all along the 250 µm. At 48 hours, no specific pattern was observed due to migration into the collagen matrix and possible proliferation between two dots of different segments. During the first 24 hours, HUVECs showed a beginning of cell alignment between 6 hours post-printing and later on cells started to migrate everywhere. A lose of the pattern was observed along the 6 and 24 hours of culture, but a formation of a homogeneous layer was obtained from the 24 to the 48 hours of culture.

Interestingly, when cells were printed in coculture (HUVECs Td-Tomato and HBMSCs, ratio 1:1) a complete aligned structure was observed at 24 hours and even if this organization was not maintained until 48 hours, HUVECs were governed by HBMSCs migration and showed a difference in behavior (See Fig. 5).

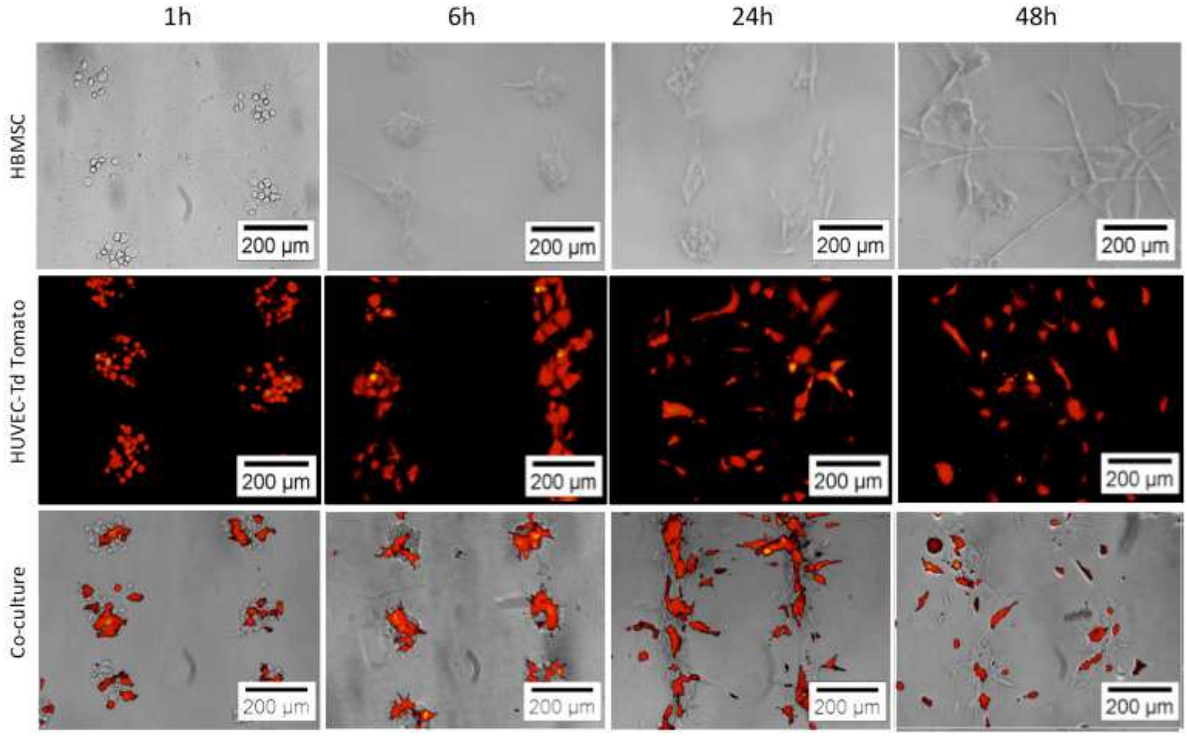


Figure 5. Cell distribution of HBMSCs and HUVECs-TdTomato printed in monoculture vs. coculture. Follow-up over time of HBMSCs (upper line), HUVECs-TdTomato (middle line) and HBMSCs – HUVEC Tomato (lower line) from 1 to 48 hours post-printing, using an inverted microscope (Axiovert). In red: HUVECs, expressing Td-Tomato. Images were representative of n=7 experiments. Distance between segments: 500 µm.

3.4 Characterization of HUVECs post-printing.

To verify if LAB procedure does not damaged the phenotype of the cells; immunostaining with antibodies was developed to characterize HUVECs after printing in the hydrogels. Non-printed cells were used as controls. Cells were stained against von Willebrand Factor (vWF, a protein founded specifically in endothelial cells and that is present in the cytosol (38)) and against CD31 (a platelet endothelial cell adhesion molecule founded mostly in the membrane (39)). The vWF was marked after 48 hours of culture in the control condition, as well as in monoculture and coculture after 48 hours post-printing (Fig. 5A, C). The CD31 was stained after 48 hours of culture in the control cells, and after 6 hours post-printing following the time point at which they showed more proximity between them (Fig. 5B). HUVECs were positive to vWF and CD31, meaning that the printing procedure do not affected the phenotype of the cells (Fig. 5A-C).

Additionally, HUVECs were stained against vWF when printed in coculture with HBMSCs (Fig. 5C).

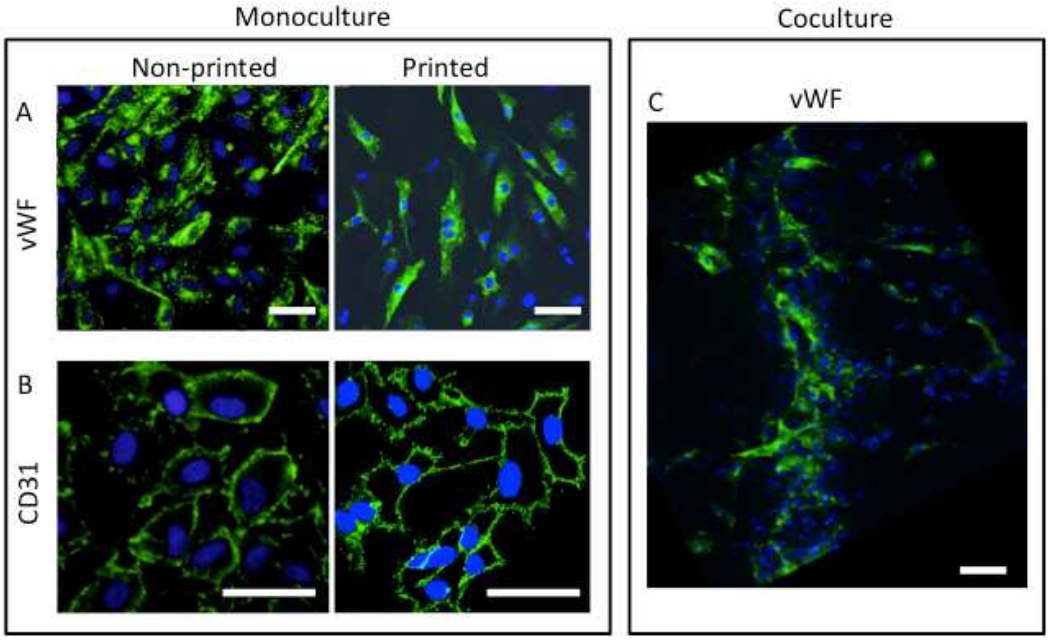


Figure 5. Immunofluorescence images of printed and non-printed HUVECs in monoculture and coculture. Immunofluorescence images of printed and non-printed HUVECs, demonstrating that they preserved their specific endothelial markers. Von Willebrand expression (vWF, green) of non-printed and printed cells after 48 hours **(A)**. CD31 expression (green) of non-printed cells after 48 hours and of printed cells after 6 hours **(B).** In coculture, HUVECs were stained with the vWF 48 hours post-printing, and the nuclei were labeled in both HUVECs and HBMSCs **(C)**. The nuclei (in blue) were also labeled in images **(A-C)**. Images were representative of n=3 experiments. Scale bar: 50 µm

3.5 Position in the z-axis of printed cells

With the use of the Confocal Microscope (Leica®) and reflection mode, samples were measured in the z-axis to investigate the depth at which they were deposited during the printing procedure. Yellow lines show the limits of the different components of the entire sample. Starting from the bottom, the first yellow line appears in the interface between the glass slide and the hydrogel. The second yellow line is founded in the interface between the hydrogel and the air. In this case, cells are exactly in the surface of the hydrogel. Nuclei are labeled in blue and HUVECs are stained for vWF (Fig. 6).

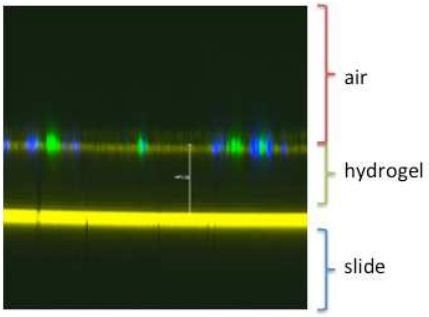


Figure 6. Position in the z-axis of printed cells. HUVECs were stained with the vWF (in green) and nuclei were stained in both HUVECs and HBMSCs (in blue).

4. DISCUSSION

Vascularization of implants is an issue that has to be overcome to ensure cell survival in 3D matrices. Our approach was based on promotion of morphogenesis by controlling the organization of each cell type and prevascularization. We demonstrated in this study that Human bone marrow stem cells (HBMSCs) promote alignment of Human Umbilical Vein Endothelial Cells (HUVECs) after Laser-Assisted Bioprinting into collagen type I.

The laser-assisted bioprinting was used to print cells at a laser pulse repetition rate of 1kHz aiming the obtaining of a good precision (10). Previous study based on a micropatterned hydrogel system demonstrated a high susceptibility of mesenchymal stem cells to chemokine-induced migration on a distance of 500 µm (7). A distance of 500 µm and 1000 µm between two consecutive segments was then chosen to verify the behavior of our cells. A 250-µm distance between dots of the same segment was selected to promote an aligned structure because the migration rate was assumed to be higher at a closer distance.

Graphics of Fig. 2A show the data obtained when distances of 250 µm, 500 µm and 1000 µm were randomly measured to calculate the precision of the LAB technique. The mean and standard deviation were obtained using GraphPad prism® software. Results showed the higher the distance, the higher is the incertitude. Regarding the patterns and distances of 500 µm and 1000 µm that were selected for the experiments, the precision of the laser was calculated and considered with a final error of around 20 µm.

Even if it is not necessarily too high for the spatial organization we gave to the cells, it was expected a lower error.

Two hypotheses were detected as the possible causes that should be checked to assure that the experimental error obtained is not that far from the real one. The first is to measure the donor slide to ensure that the error founded do not increased due to an inability of the software to control exactly the mirror's movement, the linearity of acceleration and the coordination between shutter opening and mirror speed. The second is related to the bio-ink homogeneity and hydrodynamic perturbations linked to cell clumps and bio-ink thickness (40)

As described in previous works, cell viability post LAB depends on laser fluence (41). Correct parameters for printing HUVECs and HBMSCs were determined using live/dead assays. We therefore confirmed that the laser caused no damage to the cells even 48 hours after printing (26). Regarding the actin staining, it was evidenced that both HUVECs and HBMSCs continued having the capacities to spread and survive post-printing. As shown in Fig. 3 HBMSCs were able to migrate towards the alignment after 24 hours of printing. This alignment was possibly favored because these cells have the capacity to form multilayers between them. HUVECs also showed their capacity to spread and form F-actin-rich podosomal organelles in sites of adhesion and that are found all around the spot of cells (42). Neither HBMSCs nor HUVECs suffered a morphological change. The capacity of printed cells to self-organize was demonstrated depending on the location of neighbor cells.

As mentioned before, the closer the distance the higher the migration (7). We decided to verify this fact with the use of LAB technique. It was observed that a distance of 500 μt between segments of printed HUVECs or HBMSCs in monoculture (results not shown) favors the formation of a homogeneous layer after 2 days of culture. Contrarily, when HUVECs were printed at 1000 μ1000when HUVECs werethe uniform layer was delayed assuring that cells migrated along dots of the same segment and then started to reach neighbor segments.

A follow-up until 48 hours of culture was developed when cells were printed at 500 μ because this condition did not favor the cells alignment. When HBMSCs were printed alone, a beginning of spread over the hydrogel was observed at 6 hours. 24 hours after, cells continued spreading and started migrating to nearest cells from the forward and the backward spots, producing the alignment at this time point. Then they started to migrate through neighbor segments. However, when HUVECs Td-Tomato were printed, a beginning of an alignment was evidenced due to cell migration. At 24 hours, cells were dispersing in the collagen matrix. 48 hours after, cells were completely dispersed due to movement. Interestingly, when HUVECs-HBMSCs were printed in coculture, it was observed that HBMSCs started guiding HUVECs at 6 hours after LAB procedure. At 24 hours, an aligned structure was formed and contrarily to what was obtained in monoculture, HUVECs were completely guided by HBMSCs self-organization. Even if cells were not completely aligned after 48 hours of culture, many HUVECs still preferred staying on HBMSCs rather than the collagen. As previous studies, we demonstrated the potential of these cells in coculture to form tubular-like structures *in vitro* (13). We assumed that these results were a consequence of cell migration due to population doubling time of each cell type (~30 hours for HUVECs and 48 hours for HBMSCs) (Data not shown). However, as we do not test these two issues, it cannot be assured that there is only an effect of cell migration producing the self-organization, but also of cell proliferation. To clarify this issue, it may be interesting to develop a migration test or to add an anti-proliferative drug in order to be able to identify the moments at which migration and/or proliferation take place.

Among the common endothelial markers KDR (VEGFR2), CD31 (PECAM and vWF, we chose two of them (CD31 and VWF) to verify their expression in printed and non-printed cells (used as control) (43). It was demonstrated by immunofluorescence that HUVECs conserved these two important specific markers after printing. Controls on printed cells were developed as a way to compare results. These results show that HUVECs maintained their phenotype and functionality as non-printed cells did. This was

important to verify if the effect of organizing and patterning the cells in coculture or monoculture, changed or not the phenotype of HUVECs. It might be interesting to stain HBMSCs against CD44, CD54 and CD90 to characterize them as we did with endothelial cells (44). Expression of Connexin 43 (Cx43, gap junction protein) should be checked to assure cell-cell communication when printing in coculture. This protein is important because the osteo-endothelial communication is essential to establish a network between these two types of cells, favoring the bone development (5). From the qualitative point of view, it will be necessary to implement techniques permitting quantitative data on the respective phenotype. One of these techniques is the *in situ* hybridization and PCR on isolated cells could use to quantify gene expression of printed cells (45).

Finally, the position of cells in the z-axis was measured using a Confocal Microscope. As it was obtained that cells were deposited in the surface of the hydrogel, it will be interesting to print at a higher energy (superior to 27 μ in to capture the cells into the matrix. Entrapping cells to apply the principle of layer-by-layer assembly of complex meso- or macroscale tissue constructs, having at each layer micropatterns of cells, biochemical cues, physical cues, and defined shapes (46).

5. CONCLUSION

This study shows that the Laser-Assisted Bioprinting is a promising tool for the fabrication of tissue-engineered constructs. Printed cells are able to survive and spread within the collagen hydrogels, and are able to maintain their phenotype and functionality. It was demonstrated that mesenchymal stem cells have a great influence on endothelial cells fate in a 3D *in vitro* microenvironment, by guiding their self-organization. To enhance self-organization cells could be printed at different time points, first the mesenchymal stem cells with a specific pattern and then the endothelial cells.

GENERAL CONCLUSION AND PERSPECTIVES

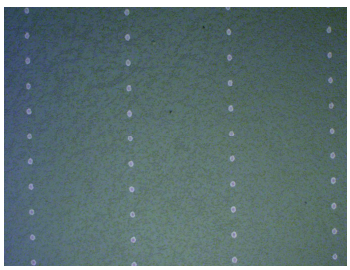
The bioprinting technology allows the organization of cells in coculture and monoculture with a cell-level resolution and a personalized localization. Both HUVECs and HBMSCs showed a beginning of an alignment at 6 and 24 hours, respectively; while in coculture they showed a complete alignment at 24 hours where HBMSCs conduce HUVECs to stay over them. Specific markers of ECs, such as von Willebrand Factor and CD31 were expressed post-printing in mono-cultured and cocultured HUVECs. HBMSCs guide HUVECs organization in a coculture model. As a continuation to this study, it would be interesting to investigate the optimal moment at which HUVECs must be printed over HBMSCs to favor the formation of a tubular-like structure. It is also important to add shear stress to verify the ability of cells to align when they are subjected to a flow. Different patterns should be developed to search the optimal organization between these two types of cells, by printing one segment of one type of cell at different distances from another segment of the other type of cell. This way, we will be able to study at least one of the different mechanisms of cell communication, which is the secretion of diffusible factors (including chemo-attractive factors). Finally, what is expected with this alignment is that they can also be differentiated depending on the mechanical constraints (leading to differentiation in different cell types, depending on the location), and permitting the modular fabrication of constructs that could resemble more to native tissues.

To summarize, the bioprinting is a technique that offers new sources for tissue engineering, with a high potential to be used in clinics, but that has to be more experimented and studied in order to evaluate and investigate all the factors until it could be used in humans. Its methods may offer a strategy to bioengineer replacement using stem cells as a source to regenerate multiple tissues. The future success and broad applicability of bioprinting technologies will benefit from evaluation of cell function during and after bioprinting. Bioprinting methods provide a new possibility for stem cell patterning and differentiation, with precision and control over the cell microenvironment.

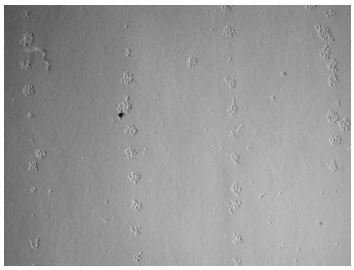
ADDITIONAL CHAPTER: Hypothesis

After obtaining the results about the precision of the prints in the receptor slides, two hypotheses came to possibly explain the reasons why the precision was not the expected one.

1. The first hypothesis consists on inability of the software to control exactly the mirror's movement: as the mirror moves at a non-linear acceleration, this may cause differences in the pattern that are also related to the homogeneity of the ink. The following picture illustrates that patterns in the gold or donor slide are coherent with the ones designed in the computer, leading conclude that results on laser precision may be primarily caused by the second hypothesis.

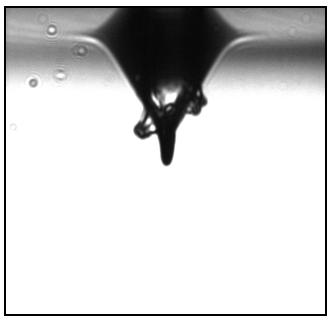


Donor slide with cells



Printed cells in the collector

2. The second hypothesis resides in the ink heterogeneity and hydrodynamic perturbations linked to cell clumps and bio-ink thickness: the following picture was taken with a Time Resolved Imaging microscope and illustrates when a cell was jetted after a laser pulse. When perturbations are created at the formation of the jet, the ink that is going to be deposited in the substrate could be displaced when compared to the organization of the laser shoots on the donor slide. The jets obtained could differ depending on the thickness and viscosity of the bio-ink.



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POSTED COMMUNICATION

Mesenchymal stem cells guide endothelial cells in a model organized by laser-assisted bioprinting.

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