Generating vascular structures in a 3D micro-environment

Chris Blaauwgeers (s2507390)
Chair of committee: Prof.Dr.Ir. Séverine Le Gac
Daily supervisor: Utku Devamoğlu MSc.
External committee member: Dr. Tim Segers
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Abstract

Using a realistic and representative situation of physiological functions is necessary for all drug and treatment research. Recreating a realistic vascular structure is a necessary step in enabling this research for both fibrotic diseases such as systemic sclerosis and different types of cancer. Such vascular environments are needed to relieve from animal testing and offer a suitable in vitro alternative to in vivo experiments. In this study, vascular endothelial growth factor (VEGF) induced formation of blood vessels via both angiogenesis and vasculogenesis is studied in varying environments. This is done over a seven-day period in a 3D cell culture based on a PDMS chip housed hydrogel matrix. Using a pre-seeded endothelial cell lumen, different configurations of VEGF supplementation, fibroblast inclusion and chip design are tested to find the adequate setup for vessel formation.

Angiogenic vessel sprouting seems most prevalent when endothelial cells are exposed to a VEGF gradient versus direct supplementation. Introduction of fibroblasts did, however, not return the expected improvement in vessel length. Vasculogenesis seems to appear between clusters of endothelial cells in a co-culture with fibroblasts.

1 Introduction

The formation of new blood vessels happens in different proportions throughout the body. Smaller blood vessels and capillaries are mainly formed via two different processes; vasculogenesis and angiogenesis [1]. Vasculogenesis refers to the formation of new blood vessels through the proliferation and migration of cells in an area lacking vasculature. This only happens in the embryonic phase, with the exception of post-natal vasculogenic processes [2]. A more common way of blood vessel formation is angiogenesis. In angiogenesis, new blood vessels sprout from existing ones via initial formation of tip- and stalk cells [3]. Angiogenesis is induced by the binding of vascular endothelial growth factor (VEGF) to the VEGF receptor on the cell, which starts a downstream cascade of intracellular signalling resulting in reorganisation.

Normally, the purpose of neovasculogenesis is to change the environment to supply nutrients and oxygen to the cells in the adjacent tissue, which is essential in order for them to remain viable. This process, however, does not only occur in healthy tissue; due to their high metabolism and excessive growth, tumours also induce and need angiogenesis in order to receive sufficient nutrients and oxygen [4]. Angiogenesis is also strongly linked to fibrotic diseases, one of which is systemic sclerosis (SSc) [5]. In short, in systemic sclerosis, autoimmune responses damage the existing vascular structures, which is followed by interstitial fibroblast activation due to chronic inflammation [6]. This leads to fibrosis and VEGF secretion by fibroblasts which, as mentioned before, induces angiogenic sprouting and vasculogenesis [7]. These vessels do not have regular morphology, they are leaky and irregular [8].

Since effective targeted treatment is currently not yet available for fibrotic diseases such as SSc, new drug research is in high demand. To allow for meaningful research, a representative situation must be used. As of yet, no models exist that accurately mimic fibrotic diseases. In order to mimic the environment as well as possible, different approaches can be chosen. The simplest setup is a 2D environment. Simplicity is its biggest advantage, however, due to lack of contact with extracellular matrix and other cells, an artificial 2D environment is not sufficient for formation of complex systems such as blood vessels, which naturally have 3D structures [9]. Another option is animal models. Having a complete organism, and thus plenty of extracellular matrix contact, does allow for more complex systems and studies. Due to interspecies differences, these do not always yield

reliable and translatable results. Although results in animal testing may sometimes look promising, the transition to humans is not always representative [10]. In recent history, 3D models have shown to be more promising. 3D-structures, where cells are cultured within a hydrogel, are one type of these models. Combining this with a microfluidic environments allows for complex, yet easily workable models [9]. These organ-on-a-chip (OOC) models are currently advancing into usable constructs for drug- and human developmental research. Due to the representative and useful environments they create, OOC-models form a promising alternative to prior in vitro an in vivo models. Zooming in on vessel formation, latest studies have shown the possibility of vessel formation from endothelial cells. Next to this, addition of fibroblasts in different varieties may have positive impact on these processes [11]. Large, integrated, self-grown, networks have not yet been achieved and are still far away from current technology.

The model that will be created in this study, will attempt to recreate lifelike vascular structures as true to nature as possible. In order to do so, a polydimethylsiloxane (PDMS) device will be used. This chip consists of two side channels connected to a central chamber housing cells seeded in a hydrogel. Differing between experimental situations, vasculogenesis will be induced in this central chamber and angiogenesis from the side channels into the central chamber. The different experimental setups will be further discussed in the Materials & methods section (2). The vascular structures that are formed will be measured and evaluated on the basis of their morphology. [12]

2 Materials & methods

In this study, different situations are considered aiming to induce angiogenesis and/or vasculogenesis. The environment used is a PDMS chip with two side channels lined with endothelial cells and/or fibroblasts, simulating a blood vessel, adjacent to one central chamber filled with a hydrogel. The variation between the situations lies in the number of side channels that is lined with endothelial cells and which of these sides is stimulated with VEGF of 42 kDa, the cell-culture present in the central chamber, and the design of the chip.

The supplier of each of the used compounds and materials, is found in table 1.

Table 1: Suppliers of the compounds and materials used during the experiments.

Compound	Supplier
HUVECS	Angio Protemie
NHDFs	Lonza Biologics
Dextran	SigmaAldrich
PDA	SigmaAldrich
Fibrinogen	SigmaAldrich
Thrombin	SigmaAldrich
Collagen	FUJIFILM Wako Chemicals
ECM	ScienCell Research Laboratories
FBM	Lonza Biologics
VEGF	Thermo Fisher Scientific
Primary and secondary antibodies	Thermo Fisher Scientific
Phalloidin	Thermo Fisher Scientific
DAPI	Thermo Fisher Scientific
Trypsin	Thermo Fisher Scientific

The goal is to find the conditions where either angiogenic sprouting or vasculogenesis are most abundant. To assess result, the amount of new vessels, their perfusability and morphology is characterised and compared between the different situations.

The setup used for the experiments is divided in the following segments: device design, cell culture, angiogenic sprouting experiments vasculogenic formation experiments, and immunostaining. The device design and cell culture were in preparation of the experiments. The angiogenic sprouting and vasculogenic formation describe the design of the different types of experiments and their goals. The immunostaining is done as a way of assessing results of said experiments.

2.1 Device design

As a start, a PDMS chip is made using soft lithography. To allow for curing of the the PDMS, it is first mixed with a curing agent in a 10:1 weight ratio. Bubbles are removed from the chip in a vacuum chamber and cured overnight in an oven at $65°C$. After formation, the chip is bonded to a glass slide using a plasma surface treatment. To decontaminate, the chips are treated with UV ($\lambda = 254nm$) for 60 minutes before before being used. In situation 1 and 2, the middle chamber has a so-called "diamond shape" (see image 1). In situation 3, the middle chamber is connected to the side channels via a phase-guide. The phase guide confines the hydrogel in the central chamber. This increases the surface-area of contact which is expected to increase angiogenesis [13] (see image 2).

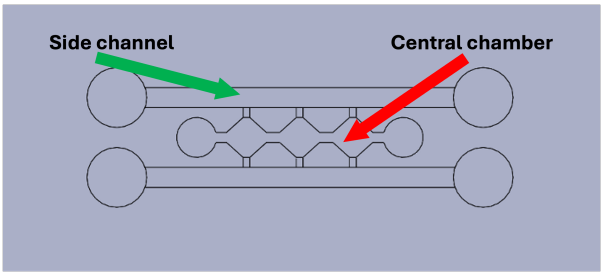


Figure 1: Diamond chip design by Utku Devamoğlu. The green arrow highlights the side channel, the red channel highlights the central chamber.

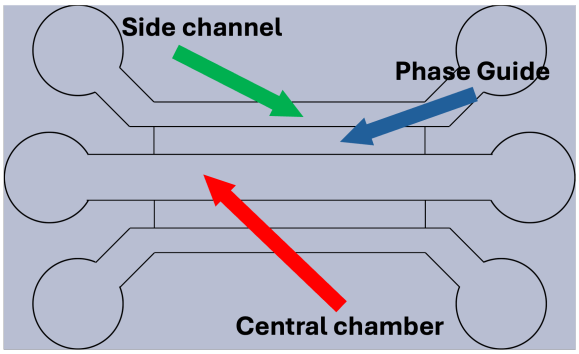


Figure 2: Phase guide chip design by Utku Devamoğlu. The green arrow highlights the side channel, the red channel highlights the central chamber, the blue arrow highlights the phase guide.

The diamond chip has side channels with a length of $11.2mm$ and channel and chamber height of $0.3mm$. The phase guide chip has a channel length of $3mm$ and a channel and chamber height of $0.3mm$. The rest of the dimensions of the chips can be found in the appendix.
Before the cells are seeded, the middle chamber is coated for 60 minutes with a solution of 2 mg/mL polydopamine in Tris HCl buffer. This is removed and after washing with phosphate-buffered saline (PBS), the gel is inserted. The hydrogel consists of 3 mg/mL fibrinogen and 2.5 U/mL thrombin in PBS. After this the side channels are coated with a solution of $0.1mg/mL$ collagen in PBS. After inserting this, the chips are flipped every 15 minutes to ensure a full coating on all sides of the channels. The coating is removed from the channels, after which they are washed with PBS and are ready for cell loading. [14]

2.2 Chip characterisation

While the moulds of the chips are printed with certain theoretical dimensions, the dimensions of the chips themselves might be slightly different due to a non-perfect print resolution, deformation of the mould, caused by post-treatment, or shrinkage of the chip. To assess the actual dimensions, images of the top, or cut open side view, can be taken with a microscope. These images are use to measure the dimensions of the chip using imageJ software.
In the design of the phase-guide chips, the ideal ratio of the guide to open space is examined beforehand. The ideal ratio is where the open space is as large as possible, to allow for a larger contact area, while the contents of the chamber and channels do not spill into each other.

2.3 Cell culture

For all experiments, the cell types used were Red fluorescent protein expressing human umbilical vein endothelial cells (RFP-HUVEC) and normal human derived fibroblasts (NHDF). They were both cultured in their own medium, endothelial cell medium (ECM) and fibroblast growth medium (FBM) respectively. The HUVECS have a passage number 4, 5, and 6 for situations 1, 2, and 3 respectively. The NHDFs have passage number of 11 and 12 for situations 2 and 3 respectively. Detailed makeup of the media are listed in the appendix. Both cell types are passaged twice every seven days, this can however differ if the cells are confluent either sooner or later. Next to this, the medium is changed an additional one time between passages. The cells are seeded on coated plates. The plates are coated with a 0.1% collagen-I solution in PBS. This coating is incubated on the plate for one hour before usage. To passage the cells, they are first detached using trypsin, which is later deactivated by adding four times the volume of trypsin in medium. They are then transferred to a tube and centrifuged at 240 G for 4 minutes. After this, the supernatant is removed, the cells are homogeneously distributed in new medium and seeded back into the new, coated, flask with an additional $8mL$ medium. Both cell-types were grown at 37 °C, 5% CO_2, 20% O_2.

2.4 Angiogenic sprouting experiments

In all the situations used to induce angiogenic sprouting, the hydrogel does not contain any cells initially. This ensures that only angiogenesis is possible and vasculogenesis is ruled out.
The first situation that was set up, has the goal to determine the effect of there being either one or two of the side channels lined with HUVECS and which of those sides is supplemented with VEGF. This means that, in varying combinations, the HUVECS are stimulated either directly or via a gradient by the VEGF. In all situations, the VEGF is supplemented to the ECM at 50 ng/mL.

In situation 1.1, both channels are lined with HUVECS and supplemented with VEGF. In situation 1.2, both channels are lined with HUVECS and one channel is supplemented with VEGF. In situation 1.3, one channel is lined with HUVECS and the opposite side is supplemented with VEGF. In situation 1.4, one channel is lined with HUVECS and both channels are supplemented with VEGF. To line the channels with HUVECS, a suspension of 5 million cells per mL is inserted in the side channel, after which it is flipped every 15 minutes to ensure a confluent lumen. The experiments are conducted for 7 days.
For an overview, see table 2.

Table 2: Situation 1 overview

Situation	Channel lined	VEGF supplementation
1.1	Both channels	Both sides
1.2	Both channels	Left side
1.3	Left channel	Right channel
1.4	Left channel	Both sides

In situation 2.1, one of the channels is lined with HUVECS while the other one is filled NHDFs at 5 million cells per mL. The side of the NHDFs is also the side that is supplemented with VEGF while the side of the HUVECS is supplied with regular ECM. Due to their secretion of pro-angiogenic growth factors, the presence of the fibroblasts should increase angiogenic sprouting [15].
For situation 1 and 2, the diamond chips are used. Finally, in situation 3.1, experiment 2.1 is repeated with a chip presenting a phase guide. To improve the HUVEC lumen in the side channels, the seeding density there is increased to 10 million cells per mL.

2.5 Vasculogenic formation experiments

In order to induce vasculogenesis, HUVECS are seeded into the hydrogel. Appart from that, the hydrogel that is used in these experiments is prepared in the same way as in the previous experiments. They are seeded at a concentration of 3 million cells mL.
In situations 2.2 and 2.3, both channels are lined with HUVECS and supplemented with VEGF. Situation 2.3 has the addition of fibroblasts in the middle chamber, which are in coculture with the HUVECS at 3 million cells per mL for a total of 6 million cells per mL.
In situation 3.2, situation 2.3 is repeated with the only change being the use of the phase guide chips.
A complete overview of all the different situations can be found in the appendix.

2.6 Immunostaining

At room temperature, the samples are fixed with a 4% paraformaldehyde (PFA) solution by incubating for 15 minutes, after which a 0.1% triton solution is added to increase cellular permeability. This is also incubated for 15 minutes. To reduce noise in imaging , a 1% bovine serum albumin (BSA) solution is added to saturate excess protein-binding sites. After this, the primary antibodies are added and incubated overnight at 4°C. The primary antibodies are CD31 Mouse and VE-cadherin Rabbit, both at 1:100 ratio in tris-buffered saline. The next day, secondary antibodies, together with phalloidin, are inserted and incubated for 90 minutes. The secondary antibodies are Alexa-fluor 488 goat anti-mouse and Alexa-fluor 647 goat anti-rabbit, both at 1:200 ratio in Tris-Buffered Saline (TBS). After the incubation, 4',6-diamidino-2-fenylindool (DAPI) is added and incubated for 20 minutes in 1:40 ratio in TBS. Between each step, the samples are washed with PBS.
The chips are now ready for imaging.

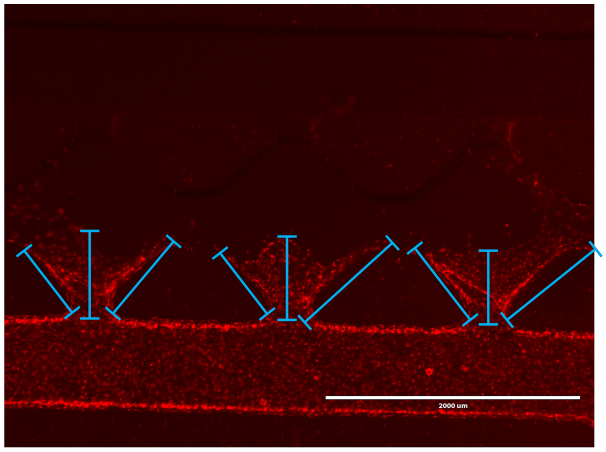
2.7 Imaging

Prior to staining, the chips are examined daily using an EVOS microscope from Thermo Fisher Scientific. Images are taken with different magnifications depending on the situation. After staining, images are taken using a confocal microscope by Zeiss Microscopy.

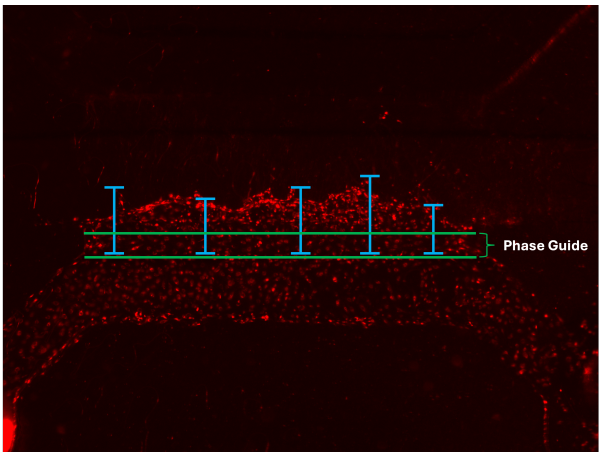
3 Results

3.1 Angiogenic sprouting

To quantify the sprouting, the maximum distance reached by migrating cells has been measured on different points on the chips. These measurement locations for the diamond chips can be seen in image 3a, for the phase guide chips in image 3b. The average of all the points on one chip is taken and plotted per day, per chip.

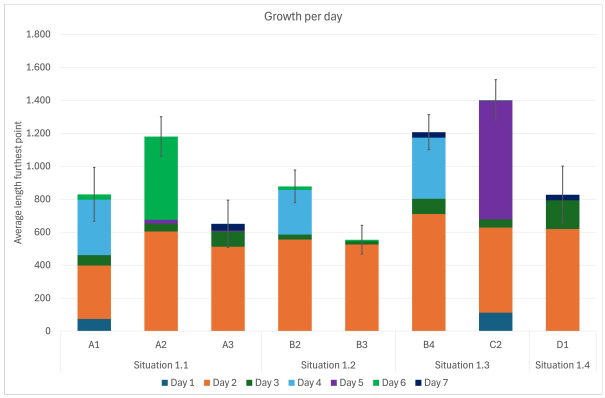


(a) Diamond chip

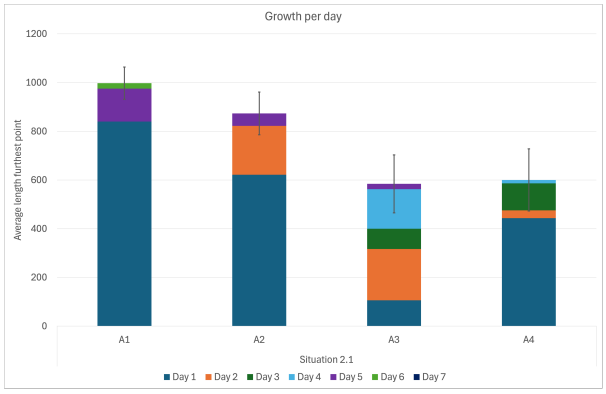


(b) Phase guide chip

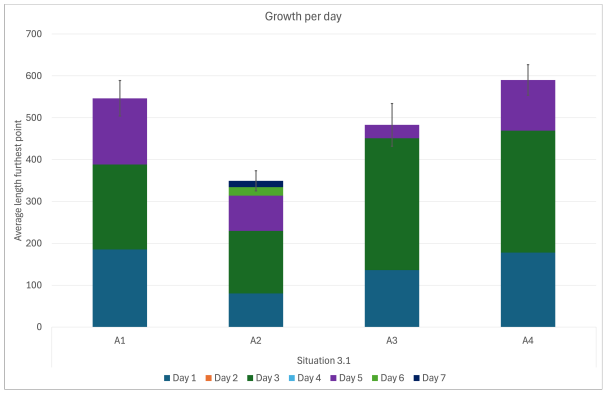
Figure 3: Illustration of an example of the length measurement for each of the chips. Red red dots represent RFP in the cells.



(a) Diamond chip



(b) Diamond chip



(c) Diamond chip

Figure 4: Growth per day of situation 1, 2 and 3. The length is measured in µm. A1, A2 etc. refer to the code of the chip used during the experiments. Each chip has nine measurement locations and each of the depicted bars is an average of these nine measurements.

In figure 4a, 4b and 4c, the growth per day is shown for each chip.The first thing to notice from these graphs, is that most of the growth happens in the first 1-3 days after seeding.
Looking at the growth of situation 1, it can be seen that the highest growth finds itself in situations 1.1 and 1.3. These are the situations with HUVECS as well as VEGF supplementation on both sides and HUVECS on one side with VEGF supplementation on the opposite side respectively. The explanation for this will be found in the discussion (4).

More observations on situation 1 are found qualitatively in images 5 and 8. Image 5 depicts the the EVOS image of the chips on day 1, 3 and 5, while image 8 depicts the confocal microscopy images of these chips on day 7 stained with DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

DAPI shows the nucleus of the cells, CD31 highlights endothelial cells, VE-cadherin highlights tight-junction proteins and phalloidin shows actin proteins within the cells.
Firstly, the previous premise is supported; Situation 1.1 and 1.3 show the most angiogenic sprouting out of these four setups. Secondly, it is apparent that situation 1.2 and 1.4 not only show angiogenic sprouting, but also give rise to more non-sprouting migration of the cells into the hydrogel. This non-sprouting migration is apparent by the whole mass of cells migrating through the hydrogel, whereas the sprouts form small tips which initially have the width of one or two cells. Despite this migration being partially expected, this is not a desirable observation for creating perfusable vessels. This further supports the usage of the setup in situation 1.1 and 1.3 for the next stages of the experiments.
In image 6, the orthogonal view of one of the chips of situation 1.1 is shown. The orthogonal view is a way of showing 3D images in 2D. On the sides, the different side views are shown for the selected point.
By looking at these orthogonal views, the depth of the cells is visible showing an open space which indicates an open, perfusable vessel.

In situation 2.1, vessels are visible as well. These vessels, however, are not larger than the vessels from situation 1, this is shown in figures 9 and 4b. This can also be said for situation 3, which can also be seen in image 4c.
From the day by day images from situation 2.1 and 3.1 in figures 10 and 11, next to angiogenic sporting, a lot of non-sprouting migration can be seen that trails the initial sprout.

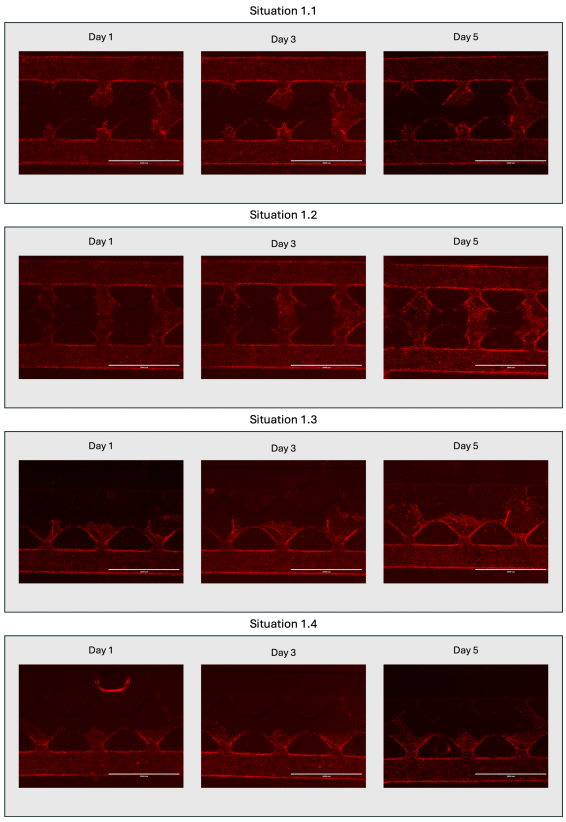


Figure 5: Day by day view of situation 1. Red red dots represent RFP in the cells

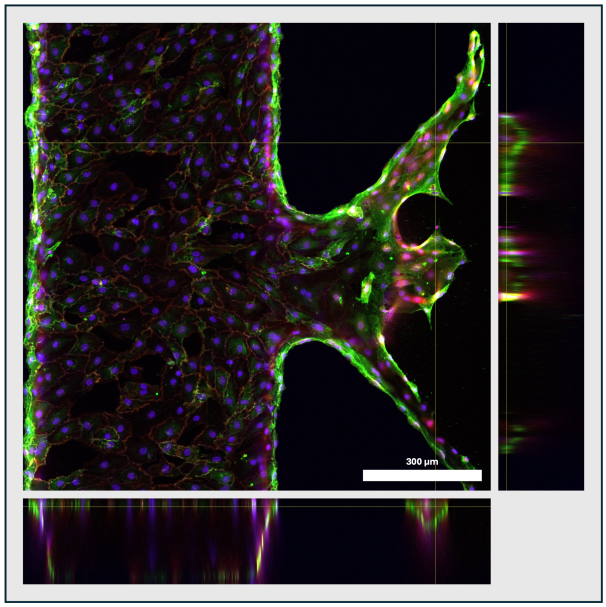


Figure 6: Orthogonal view of situation 1.1. DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

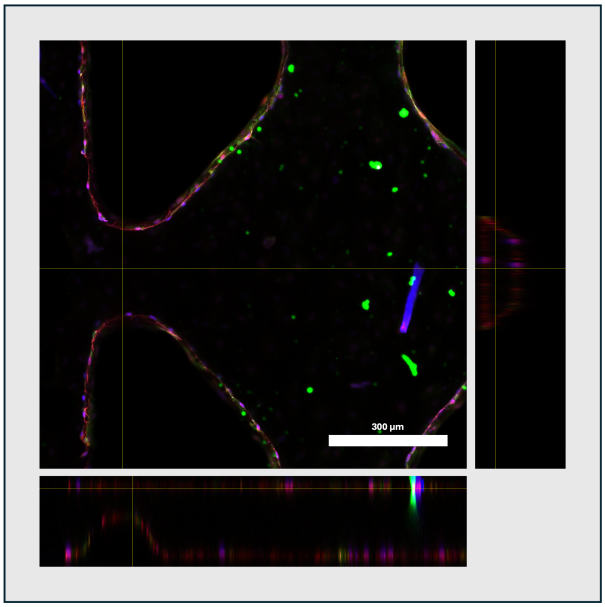


Figure 7: Orthogonal view of situation 2.1
DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

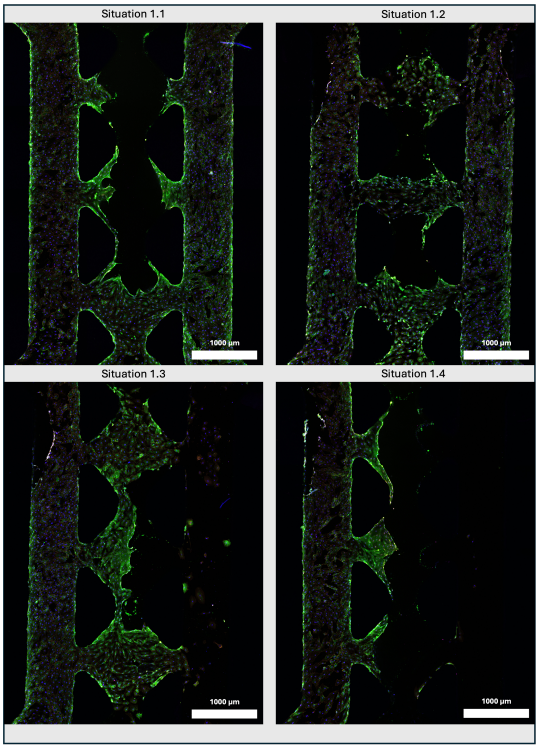


Figure 8: Confocal microscope images of situation 1
DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

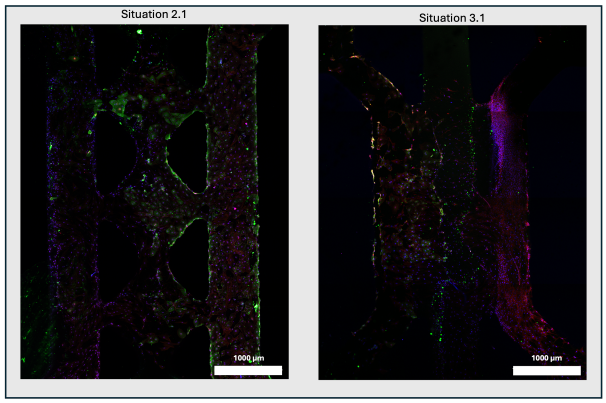


Figure 9: Confocal microscope image of situation 2.1 and 3.1
DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

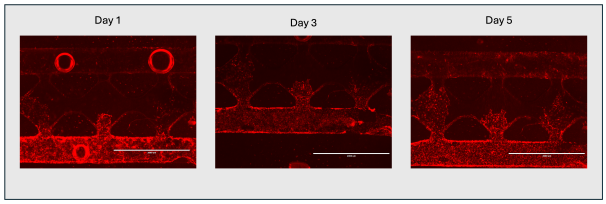


Figure 10: Day by day view of situation 2.1. Red red dots represent RFP in the cells.

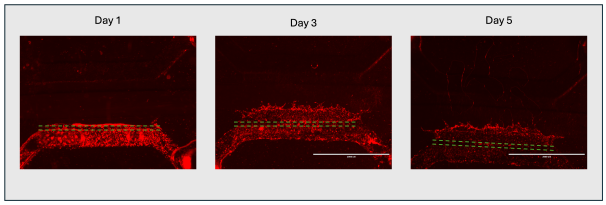


Figure 11: Day by day view of situation 3.1. Red red dots represent RFP in the cells.

3.2 Vasculogenic formation

During the experiments, many of the chips designed for vasculogenic formation failed throughout the culture period. An explanation for this can be found in the discussion (4).
Chips that did not fail are ones from situation 2.3. The day by day examination and confocal microscopy image are shown in figures 12 and 13 respectively. The cell culture in the middle chamber formed vascular structures, as is also visible from the orthogonal view in image 14.

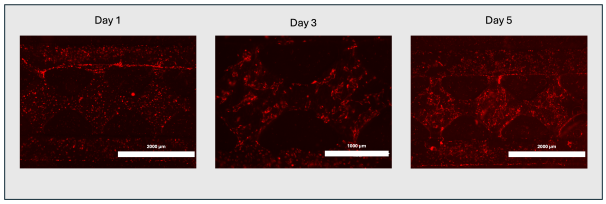


Figure 12: Day by day view of situation 2.3. Red red dots represent RFP in the cells.

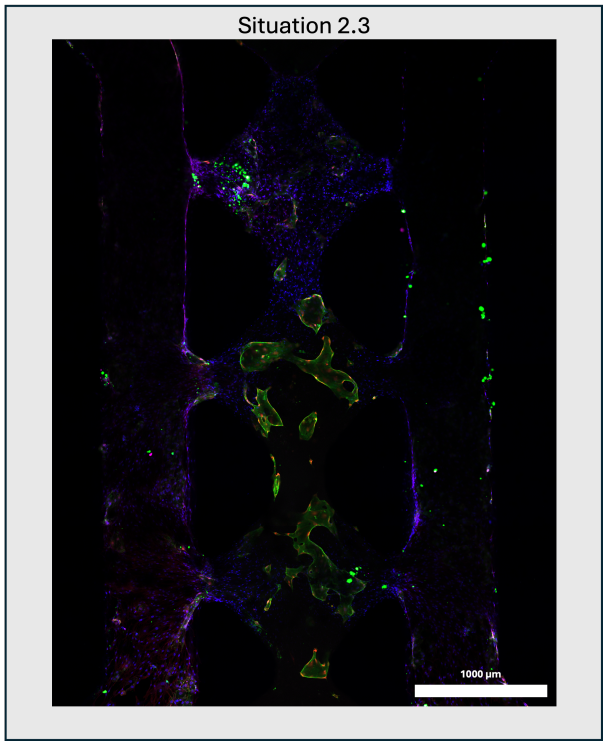


Figure 13: Confocal microscope image of situation 2.3 DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

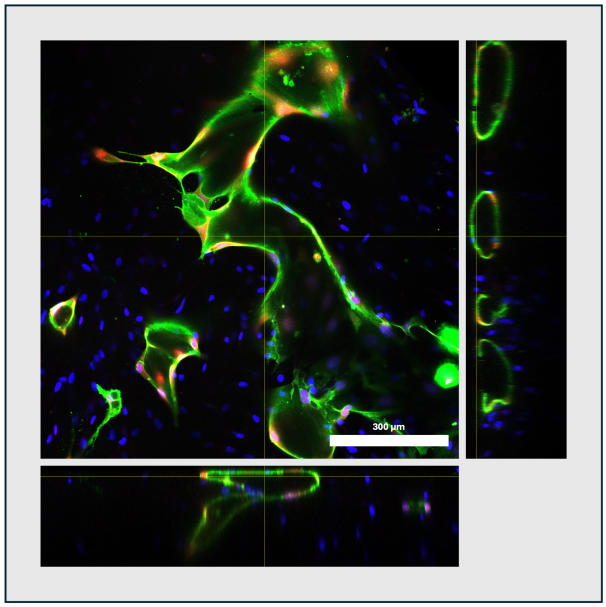


Figure 14: Orthogonal view of situation 2.3 DAPI in blue, CD31 in green, VE-cadherin in pink and phalloidin in red.

4 Discussion

In the results section, it is shown that, to a certain extent, vessel formation has been acquired. However, these vascular structures are still far from ideal.

As discussed previously, the HUVECS reacted better to the gradient of VEGF, than solely to supplementation. This is a result of positive chemotaxis and makes sense when compared to in vivo situations. Secreted growth factors from, for example tumours, promote the development of vessels toward them instead of sprouting away from them.

From situation 2.3, vessel formation within the coculture is visible. The figures show clustering of the HUVEC cells within this coculture, after which these clusters formed, seemingly perfusable, vessels between them. Although this clustering was not a planned occurrence, it seems to have helped induce vasculogenesis. This is discussed further in 4.1.

Another very prevalent phenomenon is, as mentioned before, the non-sprouting migration of cells trailing the initial sprouts. These cells tend to situate themselves on either the top or bottom of the chip, as can be seen clearly from figure 7. Currently, the definitive reason for this is unclear. However, one cause for this, might be degradation of the hydrogel. When the cells lack the hydrogel, they attach to another nearby surface, which in this case is either the bottom glass plate or the PDMS chip. This results in an inability of forming vascular structures, as the cells form a monolayer.

In 3.1, a lack of length increase is mentioned after

addition of fibroblasts and changing chip design to a phase guide based chip. This is not in line with current knowledge and certainty of these results should thus be heavily doubted [15]. As mentioned in the introduction (1), fibroblasts have shown to improve vessel formation. Currently, it is unknown as to why this discrepancy is present.

4.1 Technical discussion

As mentioned in 3.2, a number of chips failed anywhere during the seven day culture period. This also resulted in a lack of resources to perform Dextran perfusion tests. This may have several reasons and shall be described in this section.

The first reason as to why the experiments may fail, is the aforementioned degradation of the hydrogel. HUVECS have been shown to degrade fibrin based hydrogels [16]. Another phenomenon, as described by Morin et al. in [17], is that HUVECS usually need a hydrogel with a lower fibrin content. This would make gel-degradation a non-issue, however, it does lead the non-sprouting migrating cells to lack a hydrogel which is a problem. This lack of hydrogel also causes the environment to lose its structural integrity which may lead to flushing the middle chamber with medium when refreshing the medium.

Another reason for experiments failing lies in the preparation of the devices. The seeding of the cells and implementation of the gel are actions which are very prone to small mistakes. These types of mistakes can easily go unnoticed but spiral into failure further on in the experiment.

5 Conclusion and recommendations

A fibrin-based hydrogel has proven to support both angiogenic sprouting as well as vasculogenesis. Differing from expectations, however, neither addition of fibroblasts nor changing the chip design improved the angiogenic sprouting ability. To improve knowledge of this matter, experiments should be conducted where the difference between sole supplementation and addition of fibroblasts without VEGF supplementation is investigated.

The next improvement can be made in the time of culturing the chips. Since the growth of the sprouts stalls after approximately day 5, it could be useful to compare the current experiment with a situation where cells are fixated after day 5. Since in these last two days, nearly all migration is non-sprouting, these days worsen the vascular network. Due to this migration, the vessels shorten in length compared to the other cells. By excluding the last two days and fixating after five days, the vascular network can be improved.

Finally, a solution should be found for the migration of the cells toward the top and bottom of the chip. To fully identify this problem, the hydrogel degradation and adhesion should be monitored throughout the experiment in a situation with, and without cells seeded.

Acknowledgement

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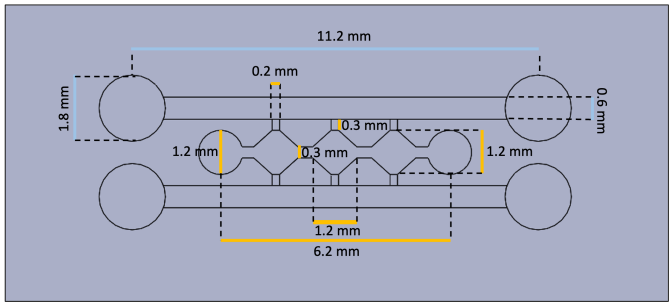
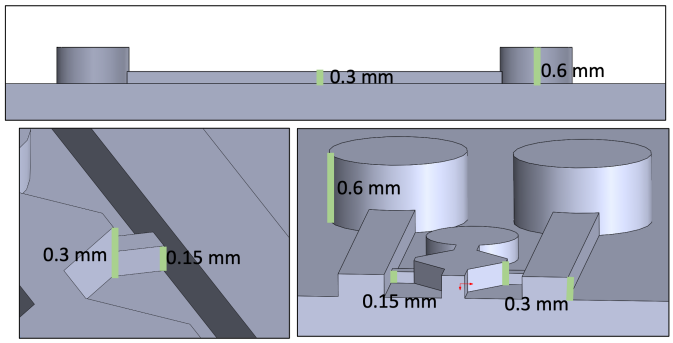
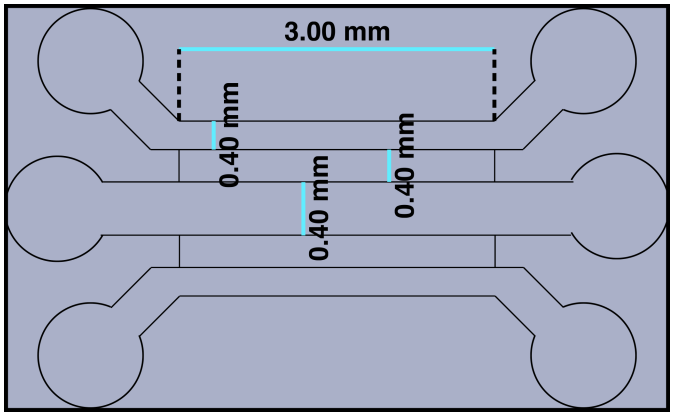
6 Appendix



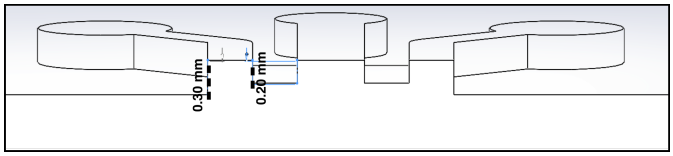
Appendix A : Diamond chip design by Utku Devamoğlu. top view, dimensions included. Two side channels with a diamond shaped central chamber.



Appendix B : Diamond chip design Utku Devamoğlu. Side view, dimensions included.



Appendix C : Phase guide chip design. Top view, dimensions included.



Appendix D : Phase guide chip design by Utku Devamoğlu. Side view

Appendix E : ECM makeup

Compound	**percentage**
Endothelial basal medium (EBM)	93%
Fetal bovine serum (FBS)	5%
Penicillin-Streptomycin (Pen-Strep)	1%
Supplements	1%

Appendix F : FBM makeup

Compund	**percentage**
Fibroblast basal medium	97.7%
Fetal bovine serum (FBS)	2.0%
GlutaMAX	0.1%
Fibroblast growth factor	0.1%
Insulin	0.1%

Appendix G : Complete experimental design

Situation	Middle chamber	Channel 1	Channel 2	VEGF supplementation	shape
1.1	Hydrogel	HUVEC	HUVEC	Both sides	Diamond
1.2	Hydrogel	HUVEC	HUVEC	One side	Diamond
1.3	Hydrogel	HUVEC	Empty	Empty side	Diamond
1.4	Hydrogel	HUVEC	Empty	Both sides	Diamond
2.1	Hydrogel	HUVEC	NHDF	NHDF side	Diamond
2.2	HUVEC's	HUVEC	HUVEC	Both sides	Diamond
2.3	HUVEC + NHDF	HUVEC	HUVEC	Both sides	Diamond
3.1	Hydrogel	HUVEC	NHDF	NHDF side	Phase guide
3.2	HUVEC + NHDF	HUVEC	HUVEC	Both sides	Phase guide