A Perfusable Multi-Hydrogel Vasculature On-Chip Engineered by 2-Photon 3D Printing and Scaffold Molding to Improve Microfabrication Fidelity in Hydrogels

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Engineering vasculature networks in physiologically relevant hydrogels represents a challenge in terms of both fabrication, due to the cell–bioink interactions, as well as the subsequent hydrogel-device interfacing. Here, a new cell-friendly fabrication strategy is presented to realize perfusable multi-hydrogel vasculature models supporting co-culture integrated in a microfluidic chip. The system comprises two different hydrogels to specifically support the growth and proliferation of two different cell types selected for the vessel model. First, the channels are printed in a gelatin-based ink by two-photon polymerization (2PP) inside the microfluidic device. Then, a human lung fibroblast-laden fibrin hydrogel is injected to surround the printed network. Finally, human endothelial cells are seeded inside the printed channels. The printing parameters and fibrin composition are optimized to reduce hydrogel swelling and ensure a stable model that can be perfused with cell media. Fabricating the hydrogel structure in two steps ensures that no cells are exposed to cytotoxic fabrication processes, while still obtaining high fidelity printing. In this work, the possibility to guide the endothelial cell invasion through the 3D printed scaffold and perfusion of the co-culture model for 10 days is successfully demonstrated on a custom-made perfusion system.

1. Introduction

In vitro cell-based models are important tools in drug development and medical research. Traditional 2D cell culture using Petri dishes or Transwell inserts does however suffer from a non-physiological environment that may alter the cell behavior and in turn, affect the experimental outcomes. Compared to conventional cell culture, microphysiological systems (MPS), also known as organs-on-chips, provide more precise control over the cell microenvironment, exhibit more biomimetic cell culture conditions, and pave the way to a better understanding of biological mechanisms including barrier function, tumor development, and drug transport.

A key to developing in vitro 3D cell models is the capacity to replicate the extracellular matrix (ECM) of the native tissue with respect to mechanical and chemical properties. Hydrogels, which are polymer-based structures containing a large fraction of water, offer a valuable tool to recreate human physiology in ways not possible with traditional cell culture substrates. However, these hydrogels still exhibit volume changes when equilibrating with a cell culture medium, which can then hamper microstructure printing fidelity. One of the current challenges in 3D cell culture is to build vascular systems into the 3D culture scaffolds to ensure nutrient exchange and waste removal, and therefore prevent cell necrosis in the bulk of the material.

A widely used approach to generate perfusable vascular networks relies on the biological process of angiogenesis within the microfluidic device. The process mimics in vivo architecture and dimensions, but the poor control on the vessel density and geometry hinders comparative studies. To achieve better control, an engineering approach to fabricate perfusable vascular networks is taken. This can include the casting of hydrogel precursor solution around sacrificial structures and needles, or forming vessels via the so-called viscous fingering method. Although the simplicity of these techniques is attractive, the freedom in design and spatial positioning as well as the required resolution for the reproduction of in vivo tissue architecture features is limited.
Physiologically relevant 3D vasculature networks have been obtained by combining casting with more sophisticated 3D printed sacrificial templates.[26,27] Alternatively, multi-material extrusion through co-axial needles can be used to create hollow structures[28] with the possibility to generate co-cultures.[29] However, such techniques are unable to provide a resolution $<$100 μm, and exhibit poor compatibility with on-chip fabrication as they require rather complex manipulations to integrate the hydrogel scaffold.[30]

A replica vasculature network model can be created on-chip by UV-assisted stereolithography 3D printing that provides a resolution of $\approx$ 5 μm and cell-friendly crosslinking chemistry.[31–35] Despite the technology being suitable for on-chip fabrication, the reduced ability to print hollow structures on-chip with an axial resolution $<$100 μm prevents the technology from being used for MPS.[36] Whilst the recently developed volumetric 3D printing presents unprecedented printing speed and surface quality,[37–39] it still displays a resolution and minimal feature size more suitable for hollow structures $>$400 μm.[40,41] 4D printing shows promise in addressing the resolution limits by creating a self-folding tubular structure caused by inhomogeneous swelling of a planar hydrogel scaffold.[42,43] However, the technique poses challenges in creating ramified hollow structures and further developments are required to achieve sub-100 μm resolution.[44]

Two-photon polymerization technology has recently emerged as a high-fidelity 3D printing technique capable of generating hollow structures by confining crosslinking to the laser voxel via a non-linear absorption process.[45–50] The achievable submicron feature size and resolution are suitable for cell-scale studies including scaffold porosity,[51,52] mechanical gradient effects[53], and cell-substrate surface interaction.[54,55] Despite the recent development of novel water-soluble and biocompatible chemistry, the technology however still faces a limited portfolio of biocompatible materials, and the photo-initiator radical generation is a hazard for cells.[56,57] In addition, the lengthy fabrication requires the sample to be placed in an incubator-like environment during the full printing process to preserve cell viability.[58–60] with the drawback of cell sedimentation.[61] Finally, the presence of cells in the bioink negatively affects polymer chain crosslinking due to light scattering and undesired interactions with the precursor components, thus compromising the printing fidelity.[62–64]

To circumvent the photo-initiator toxicity, 2-photon ablation and cavitation have been investigated for generating channel networks in collagen hydrogels.[65–68] Such techniques provide an alternative process that still matches the resolution of 2PP with the advantage of being less harmful to the pre-encapsulated cells due to the smaller processing area.[69] However, the cell concentration and hydrogel turbidity interfere with the printing quality and the issue with a long process time still remains.[70]

In our work, we propose a new strategy to form high-fidelity printing of complex vascular channels inside natural cell-laden polymers without causing cell damage during fabrication. This approach builds upon a two-step process that first sees the fabrication of a hydrogel channel network by 2PP directly inside a microfluidic device. After generating the vasculature backbone, another cell-laden hydrogel can be injected to surround the printed hydrogel channels to form a vascularized system. The proposed technique is capable of creating a multi-hydrogel construct to accommodate different cell needs in the case of a co-culture system. All steps are performed directly inside a PDMS/glass microfluidic device, ensuring a user-friendly process and the possibility to directly connect the vascular network with external pumps for cell media perfusion. Here, 2PP-structured gelatin-based hydrogels with feature sizes down to 10 μm displayed a stable scaffold and enabled co-culture of human umbilical vein endothelial cell (HUVEC)/fibroblast-laden fibrin over 10 days in a customized perfusion system.

2. Results and Discussion

An ideal bio-ink should provide appropriate biocompatibility for the cultured cells to ensure the formation of functional tissues. Encapsulating the cells in the hydrogel precursor solution makes this process more challenging as it requires a biocompatible fabrication process. It also has a negative impact on the crosslinking mechanisms, which reduces printing quality.[53] At the same time, hydrogels used as 3D cell culture scaffolds must also exhibit sufficient mechanical stability to support cell media perfusion and withstand the fabrication process.[11,70] These requirements combined make the synthesis of suitable hydrogel polymers and the development of fabrication techniques challenging.[61,71]

To overcome these problems, we combined high-resolution 2PP printing of a biocompatible ink with a second hydrogel surrounding the structures inside a microfluidic chip. This takes advantage of combining two different hydrogels, each compatible with one cell type (Figure 1). Since previous studies have demonstrated that gelatin-derived hydrogels are a suitable substrate for HUVEC culture, a 2PP gelatin-based hydrogel was chosen for the high-resolution printing of the vasculature backbone.[49,72,73] The chosen 2PP hydrogel displays mechanical properties between 0.5–2 kPa (data provided by the supplier), comparable with the stiffness range for HUVEC culture as shown in previous studies.[66,68,74] Fibrin was used for the encapsulation of fibroblasts as it has previously been demonstrated to support the proliferation of human lung fibroblasts (HLFs).[75,76]

The presented fabrication strategy and chip design have been developed to address four main challenges in high-resolution 3D bioprinting: cytotoxicity, media perfusion, lengthy fabrication, and printing fidelity. When conventional high-resolution 3D printing is used for cell-laden hydrogels, two main negative effects occur: radical formation during light-based printing, and high shear stress in the case of extrusion systems.[16,69] This 2-step strategy prevents cell damage from these sources as cells are not included during the actual printing process.

To address the challenge of media perfusion, the hydrogel structure was fabricated inside a microfluidic platform. This offers a reliable hydrogel-device interface for cell seeding and cell media perfusion while still ensuring high-resolution imaging.[77] Printing of the vasculature backbone without encapsulated cells allowed high printing fidelity as cell scattering could not affect the fabrication process.[63] In addition, printing the hydrogel channels with wall thicknesses down to 40 μm reduced the influence of gelatin-derived hydrogel swelling on the printing quality, while also reducing the printing time of the whole scaffold.[78]
Figure 1. Schematic representation of the cell-friendly fabrication strategy for perfusable co-culture of HUVECs and fibroblasts. a) The empty microfluidic chip and the magnification in the coverslip holes. b) Injection of the 2PP ink and printing of the hydrogel channel. c) Injection of fibroblast-laden fibrin surrounding the hydrogel vasculature and d) seeding of HUVECs inside the 2PP hydrogel channel connected for cell media perfusion.

2.1. Printing Optimization and Channel Formation

2.1.1. Printing Parameter Screening to Identify the Minimal Hydrogel Swelling

The most critical aspect when printing high-resolution structures is to ensure the highest printing fidelity. Despite hydrogels being 3D crosslinked polymer structures, the matrix is still susceptible to volume change over at least 24 h following printing as the matrix equilibrates in aqueous solutions. We therefore investigated the effect of printing parameters on hydrogel swelling in cell media with the aim of minimizing swelling. A parameter screening, including printing speed (200–1000 mm s⁻¹ at 200 mm s⁻¹ intervals) and slicing layer (1–9 μm at 2 μm intervals) was performed with 0.5 μm constant hatching. The slicing parameters “top-down” and “woodpile mode” were chosen over “one-direction printing” and “bottom-up mode”, respectively, since these have been reported to provide better printing fidelity in previous studies. After printing, all structures were incubated in cell media for 24 h to reach equilibrium.

No structures were retained on the surface for the 9 μm slicing layer, and a printing speed of 1000 mm s⁻¹ resulted in heavily distorted structures due to the non-synchronization between the laser on/off switching and the galvanic scanning mirror. The pillars with a 7 μm slicing layer exhibited partially printed structures and were not always retained on the glass surface, while the 1 μm slicing layer resulted in distorted structures that displayed rounded corners and a significantly deformed shape when compared to the other slicing layers (Video S1, Supporting Information). Since the stability of the DTT crosslinker reduces as the temperature increases, we speculate that the 1 μm slicing layer results in too much energy being supplied per unit volume, causing an excessive localized increase in temperature. This hypothesis is supported by observing the increase in deformation and swelling as the printing speed decreases, which resulted in an even higher power localization, and in turn, increased temperature. The inactivation of DTT causes a depletion of the crosslinker, which in turn affects the crosslinker-to-functional group ratio and therefore the hydrogel crosslinking. Consequently, only samples sliced with 3 and 5 μm intervals and scanning speeds ranging between 200 and 800 mm s⁻¹ were further analyzed to measure the top surface area (Figure S1, Supporting Information) and calculate the linear swelling (Figure 2b) with statistical analysis (Table S1, Supporting Information).

The results for the 5 μm slicing indicated a constant increase in swelling toward higher printing speeds as the energy dose decreased with the increase in scanning speed. The swelling observed in the 3 μm slicing was generally smaller than the 5 μm slicing, but differed in relation to the printing speed. For the
3 μm slicing samples, a decrease in swelling for printing speed between 200 and 600 mm s⁻¹ and an increase in swelling from 600 mm s⁻¹ was observed. From this study, we concluded that printer settings of 600 mm s⁻¹ and 3 μm slicing were optimal to minimize hydrogel swelling and these settings were used throughout the manuscript.

To ensure the observed size difference between the intended design and obtained structure was caused by hydrogel swelling and not poor printing capability of the printer itself, we measured the dimension of a hydrogel layer in contact with glass, that is, at a position where it is unable to change dimensions due to surface constraints. An average deviation from the CAD value of only 4% was measured, indicating that hydrogel swelling is the main cause of size deviations in the printed structures reported above.

2.1.2. The Double Challenge in Bioprinting: High Cell Viability with Good Printing Fidelity

A system supporting cells co-cultured in a hydrogel scaffold with an integrated vascular network needs to support both the culture of endothelial cells on the channel lumen’s surface and the cells in the scaffold bulk. Consequently, we investigated the ability of the 2PP ink to support 2D culture of HUVECs on the surface of the printed scaffold (Figure 3a-d), and 3D culture of HLFs encapsulated in the hydrogel matrix (Figure 3d–f).

The biocompatibility of the 2PP hydrogel for 2D culture was investigated by culturing HUVECs on the surface of 2PP-printed hydrogel disks. HUVECs cultured on a glass surface coated with an adhesion-promoting solution (Speed Coating, PELOBiotech) was used as control. For both conditions, high cell viability was observed after 10 days. The control experiment displayed a higher viability of ≈95% against the 85% shown for the hydrogel scaffold (Figure 3d).

In addition to biocompatibility, proliferation was also investigated to evaluate the ability of cells to populate the scaffold. Steady growth of the HUVECs was seen for the first 7 days (Figure 3c). The cells on the hydrogel substrate showed a slower proliferation on day 1 but this did not result in a lower proliferation over the full 10-day-period. No hydrogel detachment or hydrogel bending was observed on day 10 and a z-stack video of the cells on the 2PP hydrogel inside the device is shown (Video S2, Supporting Information). The microfluidic chip enabled hydrogel disk printing as well as cell culture, staining, and cell imaging. The miniaturization of the wells on-chip offered a valid screening platform for 2PP inks as conventional well plates present too big surfaces for 2-photon 3D printing (Figure 3b).

The possibility of encapsulating cells in the 2PP ink was evaluated with HLFs. A fibroblasts-laden 2PP ink was printed in cubic samples (500 × 500 × 250 μm³) using the optimized printing parameters identified in Section 2.1.1. Fibroblast-laden fibrin hydrogels served as a control for the biocompatibility assay since fibrin is commonly used for fibroblast culture.[81,82] No significant difference in fibroblast viability was observed between the two different hydrogels, with both showing values ≈ 90% (Figure 3d).

However, a cell effect was observed on the printing quality of the cell-laden structure, with the 2PP ink exhibiting areas with lower hydrogel signal surrounding the cells (Figure 3e,f; Video S3, Supporting Information). The lower hydrogel signal was associated with reduced scaffold crosslinking due to the cells acting as a source of light scattering and termination of the free radicals.[83,84] This has previously been reported as a major issue with 2PP of cell-laden hydrogels, in which the cells affect printing fidelity by creating non-uniform crosslinking and areas in the structure with weak mechanical properties.[62,64]

As a possible solution, crosslinking of the affected area would benefit from a higher energy dosage. However, higher energies would negatively influence the cell viability and also drift away from the optimized printing parameters, shown in Section 2.1.1, and cause a higher degree of swelling. In addition, excessive energy dosages are not suitable in the case of sub 100 μm hollow structures, since over-polymerization can occur, leading
Figure 3. Biocompatibility of 2PP hydrogel for 2D and 3D cultures. a) Schematic representation of the 2PP hydrogel scaffold fabrication for seeding of HUVECs. b) Optical micrograph of the device with cells growing on the hydrogel, blue: 2PP ink, green: GFP-HUVECs, red: Cell nuclei. c) Comparison between the cell number over time when cultured on the 2PP hydrogel scaffold and a glass surface coated with speed coating. Error bars from standard deviation (n = 9). d) Viability for 2D and 3D culture of HUVECs and fibroblasts with the 2PP hydrogel after 24 h, respectively (n = 5). e) Optical micrograph showing the top view and side cross-sections of a fibroblast-laden hydrogel, blue: 2PP ink, green: Fibroblasts. f) Signal intensity along the horizontal cross-section (red rectangle in image d)) for the hydrogel (blue) and the cell (green).
to oclusions of channels and chambers.\cite{53} By combining 2PP and micromolding, we address the printing limitations caused by the presence of cells.

Despite fabrication being performed outside an incubator, the encapsulated fibroblast displayed a high viability. In addition, sedimentation of cells at the bottom of the microfluidic platform was observed during 3D printing, causing a non-uniform cell distribution in the printed structure. The conventional strategies of reducing sedimentation by either adding a viscosity enhancer\cite{85} or applying gentle movement to the resin\cite{86} are incompatible due to their effect on light absorption and printing fidelity, respectively. From this study, we conclude that the 2PP bioink successfully supports 2D culture of HUVECs and that cell encapsulation entails limitations for high-printing fidelity. Hence, we utilized fibrin for the 3D culture gel surrounding the pre-defined vasculature scaffold obtained by 2PP.

2.1.3. The Fabrication Process Supports a Range of Hydrogel Combinations

In photo-crosslinkable bioinks, the interaction between the cells and the hydrogel precursor solution often has a negative impact on biocompatibility and structure fidelity.\cite{62} These aspects combined with the hydrogel’s volume change upon reaching equilibrium with the cell medium still represent outstanding challenges in high-resolution 3D bioprinting.

We propose a 2-step fabrication process to obtain multi-hydrogel structures on-chip that is cell-friendly with high 3D printing fidelity. This strategy avoids the influence of cells on the fabrication process. First, a straight hydrogel channel was printed with a 40 μm-thick wall, with a printing time of only 10 min. The 2PP printed design includes two hydrogel connectors with corresponding holes in the glass (Figure 4a,b). The hydrogel connectors present a wall thickness ≈175 μm to guarantee good adhesion with the glass and compensate for any defects in the hole, such as glass chipping generated during the laser cutting. The 2PP scaffold height and width were smaller than the dimension of the microfluidic channel to enable hydrogel swelling without constraint and to reduce deformations (Figure S2, Supporting Information). Then, a second hydrogel, here fibrin, was manually injected by pipetting the precursor solution into the microfluidic device to fully surround the printed structures. Injecting a second hydrogel to surround the vasculature network provided both a basis for 3D cell culture of a second cell type as well as a mechanical support for the printed hydrogel channel. Here, fibrinogen and thrombin mixtures (2.5, 5, and 10 mg mL$^{-1}$ fibrinogen with 0.5 U mL$^{-1}$ thrombin) were investigated to identify a suitable fibrin precursor composition. The 2.5 mg mL$^{-1}$ fibrinogen hydrogel retracted in areas close to the connectors and along the channel structure after the removal of the non-crosslinked ink inside the channel and initiation of cell media perfusion, (Figure 4c-e), whereas the 5 and 10 mg mL$^{-1}$ fibrinogen hydrogels demonstrated good adhesion to the whole channel structure. Both the 5 and 10 mg mL$^{-1}$ fibrinogen hydrogels resulted in perfusible channels, as confirmed by injecting 4 μm beads in the 2PP hydrogel channels and imaging with a confocal microscope in fluorescence mode (Videos S4a and S4b, Supporting Information). Considering the similar result, the 5 mg mL$^{-1}$ fibrinogen hydrogels were preferred over the 10 mg mL$^{-1}$ fibrinogen since better migration and proliferation of HLFs in this gel concentration has previously been reported.\cite{81}

The second hydrogel can be chosen from a range of suitable hydrogels for 3D cell culture, depending on the application requirements. The versatility of the fabrication strategy was successfully investigated by surrounding the printed vasculature with two more hydrogels: pure collagen (2 mg mL$^{-1}$) and a collagen/fibrin mixture (0.5 mg mL$^{-1}$ collagen with 4.5 mg mL$^{-1}$ fibrin) precursor solution (Figure S3, Supporting Information).

Unlike light-based bioprinting, the proposed two-step strategy enabled the fabrication of a vasculature-like system embedded in different natural hydrogels (fibrin with different fibrinogen concentrations, collagen, and fibrin–collagen) without the need to change the process parameters and without any cell scattering effect.

The demonstrated approach of adding a second hydrogel to surround the 2PP fabricated vessel also had additional positive effects. First, it provides mechanical support so that it is sufficient to make the wall of the vessel only 40 μm thick, which avoids structure deformation due to the limited swelling. The thin vessel wall reduces the printing time as the printing volume is confined to the connector and the 40 μm vessel wall. Structures obtained with a 200 μm-thick wall without fibrin support displayed clogging of the channel due to significant swelling of the 2PP hydrogel, (Figure S4, Supporting Information).

Most of the solutes and gas exchanges of the human body happen in the microvasculature,\cite{87} influencing a large number of physiological and pathological processes including angiogenesis,\cite{88} barrier function,\cite{89} inflammation, and thrombosis.\cite{90} Thus, a fabrication strategy that enables the realization of in vitro models with feature sizes from only a few micrometers up to 100 μm could contribute to understanding the mechanisms in key biological events.

Using optimal 2PP conditions, inner diameters ranging from 10 to 60 μm with a constant 40 μm wall thickness were printed to evaluate the ability of the technique to replicate tissue-like feature sizes.

As one of the main challenges of micro-scale hollow hydrogel structures is to preserve the stability of the scaffold,\cite{77,91} the inner diameter and eccentricity of the channels were measured at 24 h and 10 days after printing. The structure with the screened diameters is shown in (Figure 5a–c).

All diameters were successfully printed and no collapse was observed even after 10 days of incubation. However, a change in the inner geometry was noticed over the course of the experiment for all printed channels (Figure 5d). 24 h after printing, the smaller inner diameters (<40 μm) were closer in terms of size and eccentricity to the CAD design. Specifically, the channels with inner diameters ≥40 μm resulted in compression along the y-axis, with an eccentricity between 0.7 and 0.9 (Figure 5d,e). The change in eccentricity was most likely due to pressure applied during the injection of the fibrin hydrogel. The vessels with larger inner diameters proved to be mechanically weaker, resulting in structures more prone to deformation upon injection of the second hydrogel. Between days 1 and 10, an increase in the inner diameter was observed in all evaluated channel structures. We hypothesized that the diameter change was due to the fibrin matrix
Figure 4. Optical images of the perfusable 3D channel were obtained by surrounding the 2PP structure with fibrin precursor solutions with constant thrombin concentration (0.5 U mL$^{-1}$) and different fibrinogen concentrations (2.5, 5, 10 mg mL$^{-1}$). a) PDMS/glass microfluidic device used for structure generation and perfusion. b) Microfluidic chip after 2PP printing (blue channel) and injection of the fibrin. c) Sample obtained with 2.5 mg mL$^{-1}$ fibrinogen and 0.5 U mL$^{-1}$ thrombin. The arrows indicate retraction areas of the fibrin hydrogel after non-crosslinked ink removal. d) Sample obtained with 5 mg mL$^{-1}$ fibrinogen and 0.5 U mL$^{-1}$ thrombin. e) Sample obtained with 10 mg mL$^{-1}$ fibrinogen and 0.5 U mL$^{-1}$ thrombin. Red: fibrin by reflection mode. Blue: 2PP ink. Sample e) shows contaminants located mainly on the top part of the channel that are visible in reflection mode.

rearrangement caused by inelastic deformation during perfusion and washing of the dye over the 10 days.$^{[92]}$ However, no physical damage was detected in the 2PP structures themselves as no leakage of Antonina red was noticed in the channel.

The technique allowed channels with inner diameters down to 10 μm to be fabricated. Diameters smaller than this resulted in clogged channels. In addition, walls thinner than 40 μm caused the collapse of the structure when injecting the second hydrogel. For further biological studies, channels with an inner diameter >30 μm were preferred to avoid too high shear stress for the cultured cells during perfusion and to simplify cell seeding.$^{[93]}$

2.1.4. The 2PP Hydrogel is Permeable to Molecules up to 250 kDa

For 3D hydrogel cell cultures, it is very important that the material supports the diffusion of solutes. These play a key role in several biological functions; regulating viability, migration, and proliferation of the cells, as well as nutrient exchange and waste removal.$^{[34]}$ Specifically, fibroblasts support HUVECs luminal formation by producing cell signaling molecules and ECM components$^{[94]}$ (Table S2, Supporting information). Therefore, it is important that the 2PP-defined hydrogel channel is sufficiently porous to allow for chemical signaling between the co-cultured cells. At the same time, the hydrogel channel wall should not allow the second injected hydrogel precursor component to diffuse into the vessel structures as this will cause clogging of the printed structure. In this study, fluorescence recovery after photobleaching (FRAP) served to investigate the permeability of the 2PP gelatin-based hydrogel on square pillar samples by using FITC-Dextran of different molecular weights as model molecules (Figure 6).

A decrease in diffusivity was observed as the molecular weight increased up 250 kDa. For dextran molecules with a molecular
Figure 5. 2PP printed structure for screening of the inner diameter after 10 days. Blue: 2PP ink. Red: Antonina-dextran. a) Top cross section of the 2PP printed structure. b–c) Cross-section of the channels 30, 20, and 10 μm and 60, 40, and 20 μm, respectively. d) Inner diameter of the printed structure after 1 and 10 days. e) Eccentricity of the printed structure after 1 and 10 days. Error bar from standard deviation (n = 3).

Figure 6. a) Diffusivity of 4, 40, 70, 250, and 500 kDa FITC-dextran molecular weights in the 2PP hydrogel after 24 h. (n = 6). b) Intensity profile of the hydrogel (blue) and 250 kDa FITC-dextran (green) measured in the section highlighted within the red rectangle of image c). c) Confocal section of a hydrogel sample immersed in the 250 kDa FITC-dextran solution. d) Schematic of the used woodpile configuration of the 3D printing by 2PP. The schematic shows two layers of the printing filaments of the printed sample displaying a crosslinking gradient from the core to the edge.
weight of 500 kDa, no diffusion was observed inside the structures (Figure 6a). Consequently, we determined that growth factors produced by the encapsulated cells, at least up to 250 kDa, will be able to diffuse from the cell-laden hydrogel to the endothelial cells during co-culture. In the specific case of collagen and fibrin hydrogels, which have proteins with molecular weights between 300–350 kDa, channel clogging could be expected. Although we cannot determine whether the low cut-off molecular weight for diffusion in the printed hydrogel is <300 kDa or if the cross-linking process is so rapid that the hydrogel precursors do not diffuse across the channel walls, we did not observe any channel clogging in our experimental work. However, as a precaution, this fabrication strategy might not be suitable in cases where the second hydrogel has components in the precursor with molecular weights <250–300 kDa, as these might be able to diffuse through the 2PP channel wall and crosslink inside the hollow structure, thus clogging the channel. Interestingly, we observed that the 3D-printed structures did not display a uniform diffusion of the FITC-dextran molecules across the tested sample (Figure 6b,c). In addition, the highest signal of FITC-dextran molecules was observed in regions showing the lowest hydrogel autofluorescence signal (Figure 6b,c). Since brightfield images confirmed the characteristic fiber-like structure of 2PP printed structures (Figure S5, Supporting Information), the diffusion pattern was correlated to the voxel energy distribution that presents lower energy at the voxel edges, causing an axial crosslinking gradient in the fiber-like structure (Figure 6d). The reduced crosslinking was associated with both higher porosity and diffusion as reported in previous studies.²⁹⁶

2.1.5. The Fabrication Process Allows for both Straight and Branching Channels Perfusible for Week-Long Co-Cultures

To validate the compatibility of the proposed fabrication strategy for microphysiological studies, a co-culture of HUVECs and HLFs was performed with both a straight channel and a more complex vasculature model, (Figure S6, Supporting Information). The fibroblast cells were chosen as they have been reported as essential in the initial formation and maturation of vasculature systems.²⁹⁵ Here, the HUVECs were seeded in the printed channel after injection of the fibroblast-laden fibrin gel around the 2PP structures. By turning the microfluidic chip upside-down after fibrin injection, fibroblast sedimentation at the bottom of the device could be reduced and 3D cell growth enhanced. Fibroblasts populated the whole fibrin hydrogel and after 4–5 days of culture we could observe a higher HLF density close to the HUVEC-lined microchannel, (Video S5, Supporting Information). From this work, we cannot directly conclude whether this is due to HLFs migrating toward the vessels or a higher proliferation rate of the HLFs in that area. However, both these events are stimulated by a nutrient gradient from the perfused channels, suggesting that the 2PP hydrogel channel walls supported the diffusion of nutrients as anticipated from the FRAP experiments. An optimal nutrient diffusion through the 2PP channel was also hypothesized, as both cell types displayed viability >90% after 7 days of culture (Figure 7f). The calcein staining used for the live assay also displayed a higher fibroblast concentration in proximity to the 2PP connectors and channel (Video S6, Supporting Information).

Of particular interest, we noted that the fibroblast cells were found in the space between the branching sections of the vessel network, demonstrating a uniform spread of the fibrin hydrogel even around this complex structure. Around days 4–7, a retraction of the fibrin gel from the glass substrate was observed. Fibrin retraction has been shown in previous studies.¹⁹⁷ However, the fibrin retraction did not prevent fibroblast growth around the 2PP channel (Video S7, Supporting Information). Fibrin retraction was not observed in the section surrounded by the branched 2PP hydrogel of the structures (Figure 8b) and (Video S8, Supporting Information). We hypothesized that the branched 2PP hydrogel channels present a higher surface area compared to the single channel, thus promoting stability in the fibrin hydrogel. However, confirmation of the hypothesis would require a more detailed future study, outside the scope of this work.

The HUVECs’ initial distribution was higher at the two inlet sides but the formation of a uniform cell layer across the whole channel was observed after 2–6 days of culture under intermittent flow (Figure 7a–c) and (Video S9, Supporting Information).

Confocal microscopy imaging after 10 days of culture showed a confluent HUVEC monolayer in the hydrogel channel (Figures 7d,e and 8b). The nuclei of the HUVECs inside the straight hydrogel channel displayed a preferential orientation along the channel axis, whereas cells cultured on the hydrogel disks and glass of the microfluidic chip did not show preferential alignment (Figure 7g). This compares well with previous studies where the effect of cell confinement on cell orientation has been studied.⁹⁸,⁹⁹ The HUVECs displayed F-actin-rich structures that penetrated the 2PP channel wall. Such structures were associated to filopodia, as involved in cell adhesion to ECM and guidance toward chemoattractants.¹⁰⁰ The presence of filopodia indicated the cells’ ability to penetrate the 2PP hydrogel (Figure 7d,e). A cell filopodia depth orientation was observed from the channel center to the inlets. The higher cell protrusion depth at the inlets could be associated with a longer cell-hydrogel interaction time due to the initial seeding density distribution (Figure 7d). HUVECs cultured in the absence of fibroblasts did not display F-actin protrusion penetration (Figure S7, Supporting Information). The results were in accordance with previous studies investigating the migration and sprouting of HUVEC co-culture with fibroblasts.¹⁰¹

Finally, to demonstrate the formation of complex structures with 2PP and the development of a co-culture platform with direct access of the two different cells to each other, a 2PP hydrogel channel with cone holes along the sides was fabricated. A truncated cone hole shape, with the small side of the cone cavity (10 μm) facing the external hydrogel, was chosen to create a high hydraulic resistance and prevent overflow of the fibrin precursor solution inside the 2PP channel. The aperture did not affect the mechanical stability of the 2PP hydrogel channel and no deformation was observed. The side holes created directional migration paths for the HUVECs toward the cell-laden fibrin, resulting in HUVEC cell spreading on the outer surface of the 2PP hydrogel channel and spontaneous spouting into the fibrin matrix (Figure 9; Video S10, Supporting Information). In the future, we envision that this hybrid engineered-biologically driven system could provide a platform combining guided angiogenesis...
Figure 7. HUVEC growth is monitored inside the hydrogel channel. Green: the HUVEC cytoplasm a) cell distribution after seeding. b) cell distribution after 24 hr of culture. c) Cell distribution after 48 hr of culture. d) After 10 days of culture, the hydrogel channel section (maximum projection of half height channel). The arrows indicate HUVEC filopodia. Yellow: nucleus, white: F-actin. e) cross-section of the hydrogel channel with HUVECs. Yellow: nucleus, white: F-actin The arrows indicate HUVEC filopodia. f) Live-dead analysis for HUVECs and fibroblasts after 7 days of culture. Error bar from standard deviation (n = 3). g) Nucleus orientation distribution for HUVECs growth on the 2PP hydrogel disk, glass, and the hydrogel channel (n = 150).

and controlled vessel sprouting in a perfusable vessel-on-chip model.

The printed connectors at the 2PP ink/glass interface provided a stable connection between the microfluidic chip and the printed structure allowing perfusion of cell culture media. The shear stress distribution for the chosen flow rate around the two structures was calculated with numerical simulation by COMSOL to ensure the chosen flow rate exerted a shear stress that would not be harmful toward the HUVEC cells, as shown in previous studies [26,74] (Figure S8, Supporting information). The branched structure displayed a less uniform shear stress than the straight channel due to the different structural geometries.

A key advantage of our strategy is that the microfluidic device provides a platform where all necessary steps, 2-photon hydrogel printing, second hydrogel casting, HUVEC seeding, cell media perfusion, fixation, staining, and high-resolution imaging of the cells, can be performed without the need to dismount or make any changes to the platform, which significantly simplifies fabrication and handling of the sample.

The ability to create a perfusable vasculature model, combined with the flexibility to guide cell invasion and interaction while not having constrains with cell encapsulation concentration in the hydrogel, opens up new possibilities for generating tissue models with physiological cell densities that...
3. Conclusion

In this work, we present a new fabrication strategy for making a dual-hydrogel microvasculature model by combining the precision of 2PP and the cell compatibility of hydrogel casting. In the presented two-step process, a vasculature network is first defined using 2PP inside a microfluidic device. Second, another cell-laden hydrogel is injected around the 2PP-defined structures. The vasculature is finalized by seeding endothelial cells inside the channel network. The study showed that this set-up supports 3D culture of fibroblasts for up to 10 days in a perfusable vascularized hydrogel. The presented strategy not only preserves the cell viability >90% of the 3D culture but also enables the generation of channels with feature sizes down to 10 μm, thus replicating the capillary size of human tissue. The versatility of 2PP allowed the creation of side apertures in the 2PP channel to promote HUVEC migration and sprouting, leading to a defined angiogenesis density for more comparative studies in the future.

By combining the reliable perfusion of the system with a new high-resolution fabrication strategy, there is a unique opportunity to create innovative in vitro models of vascularized multi-hydrogel co-culture microphysiological systems.

4. Experimental Section

Preparation of Multi-Hydrogel Structure. A commercially available 2PP ink (U200, Bioinx) was used to print the sub-100 μm diameter channels.
The 2PP ink was obtained by mixing 7.5 μl of the crosslinker solution (Dithiothreitol, provided as a ready-to-use solution by the supplier), 42.5 μl of PBS (Sigma–Aldrich, concentration 1X) and 50 μl of the 2PP ink stock solution heated to 37 °C (provided as a ready-to-use solution by the supplier) as instructed by the supplier. The hydrogel precursor solution was vortexed and centrifuged (100 × g) to remove air bubbles and collect possible fibers or debris at the bottom of the Eppendorf tube. Subsequently, the solution was injected inside the microfluidic platform through one inlet of the fluidic device using a pipette. After the precursor solution injection, the inlets and outlets were sealed with 3-mm punched PDMS disks (Super clear silicone sheet 0.5 μm, Silex Silicones). The hydrogel components were mixed and injected in a sterile environment and all prints were performed within 3 h after mixing the hydrogel precursor components, as instructed by the supplier.

The obtained 3D printed structures were surrounded by three different hydrogels: fibrin (Sigma–Aldrich), collagen (rat tail type 1 collagen, Gibco) and a fibrin–collagen mixture to display the versatility of the proposed approach. Fibrin and collagen were chosen since they have both been reported as suitable for 3D cell culture.[11] For fibrin, a fibrinogen solution was mixed with thrombin (Sigma–Aldrich) in PBS to obtain a final concentration of 2.5, 5, and 10 mg mL$^{-1}$ fibrinogen and 0.5 U mL$^{-1}$ thrombin. For collagen, a 3 mg mL$^{-1}$ stock solution was diluted to a 2 mg mL$^{-1}$ solution by adding PBS and 1 M NaOH solution (11 μl of 1 M NaOH solution per 18 μl of 2 mg mL$^{-1}$ collagen solution). For the fibrin–collagen hydrogel, the fibrin and collagen stock solutions were mixed to obtain a 4.5 mg mL$^{-1}$ fibrin and 0.5 mg mL$^{-1}$ collagen final concentration. After mixing, all the hydrogel precursor solutions were immediately injected into the chip and incubated for 3 min to allow full crosslinking before immersing the whole chip in endothelial cell media.

For the 3D bioink biocompatibility assay, the protocol indicated by the supplier was followed for the fibroblast encapsulation. Briefly, 50 μl of the 2PP ink stock solution was heated for 10 min at 37 °C before adding 7.5 μl of the crosslinker solution and 42.5 μl of a PBS solution with fibroblasts to achieve a final 2 × 10^4 cells mL$^{-1}$ concentration. As a control, fibroblasts were also encapsulated in fibrin hydrogels. The fibroblast stock solution was first diluted in a PBS solution containing fibrinogen and thrombin and then mixed with a thrombin solution to achieve the final concentration of 5 mg mL$^{-1}$ fibrinogen, 0.5 U mL$^{-1}$ of thrombin and 2 × 10^6 cells mL$^{-1}$.

The fibroblast-laden fibrin for the multi-hydrogel structures were at a concentration of 7.5 × 10^6 cells mL$^{-1}$, a cell density found to be suitable via pilot experiments to prevent overgrowth of the fibroblasts during the 10 days culture. After the fibrin solution injection, the chip was rotated every 30 s for 3 min to prevent sedimentation of the cells at the bottom of the device. The hydrogel was prevented from drying out by immersing the chip in cell media until further use.

**Cell Culture:** HUVEC-expressing green fluorescent protein (GFP) (Angio-Proteomie, PELOBiotech GmbH) and HUVECs not expressing GFP (ATCC) were cultured in a cell culture medium with an enhanced growth kit provided by the supplier (Cellovations, Endothelial Cell Growth Medium kit enhanced GFP, PELOBiotech). The culture flasks were coated to promote cell adhesion for 1 h (Speed Coating, PELOBiotech) prior to seeding cells that were used from passages 6 to 10.

Human Lung Fibroblasts (HLF) (Cellovations, PELOBiotech) were used between passages 5 to 10 and cultured according to the supplier’s instructions (Cellovations Fibroblast Growth Medium kit enhanced, PELO-Biotech). All cell types were kept in culture in an incubator at 37 °C with 5% CO₂ and saturated humidity. The cell culture medium was changed every 2 days.

**Microfluidic Device Fabrication:** Hydrogel analysis and cell cultures were performed inside a PDMS-glass microfluidic device. The glass and PDMS layers were air-plasma bonded (power: 200 W, time: 5 min, model: Alto, Diener electronic GmbH) and then placed in contact at 100 °C for 2 h to form a strong bond. Five different designs were used for i) evaluating hydrogel swelling, ii) performing FRAP experiments, iii) analyzing hydrogel cytocompatibility for 2D culture, iv) investigating cytocompatibility for 3D culture and v) combining two different hydrogels. The more detailed layer-by-layer structures and 3D schematics of the microfluidic chips are displayed in (Figures S9, S10, Supporting Information).

**Device 1** (hydrogel swelling) consisted of a 250 μm-thick PDMS ring (Super clear silicone sheet 0.25 mm, Silex Silicones) with an inner diameter of 3 mm and outer diameter of 8 mm was bonded by air plasma to a 150-μm cover slip (VWR).

**Device 2** (FRAP experiments and 2PP biocompatibility for encapsulated fibroblasts) consisted of a PDMS master (Figure S11, Supporting Information) was created with a 150 μm SU-8 photore sist laminate (D) Micro-Laminates, Inc.). PDMS prepolymer (Wacker Chemie AG) made with a 9:1 mixture ratio between base and curing agent was cast against the master and degassed for 20 min before curing overnight in an oven at 60 °C. After removing the PDMS structure, inlet and outlet holes were punched using a 1 mm puncher. The bonding surfaces of the PDMS and glass slide were plasma treated in air plasma.

**Device 3** (2PP ink biocompatibility and proliferation assay) consisted of a 250 μm-thick PDMS layer (Super clear silicone sheet 0.25 μm, Silex Silicones) with three 1-mm punched holes sandwiched between the PDMS chip and a cover slip. The device layers were manually aligned to ensure overlapping of the channel and punched holes, followed by air-plasma treatment.

Using Device 4 (biocompatibility for fibroblast encapsulation), for chip fabrication, the same protocol presented for device 2 was followed by a channel design that took advantage of hydrogel pinning to confine the fibrin hydrogel, as previously shown.[102]

In Device 5 (multi-hydrogel constructs), the microfluidic chip consisted of a top channel (2 mm wide, 15 mm long and 0.25 mm high) and two top channels (1 mm wide, 10 mm long and 0.2 mm high). The bottom channel was fabricated by a cutter plotter (Craft ROBO Pro, Graphite Corporation) using PDMS sheets (Super clear silicone sheet 0.25 μm, Silex Silicones) while the two top channels were prepared by replica molding. Five holes were punched in the PDMS layer obtained by replica molding. The central hole served for the seeding of HUVECs while the 4 outer holes were used for the injection of the ink, hydrogel or cell media perfusion. The two microfluidic layers were separated by a 150 μm coverslip (Microscope cover glasses, 24 mm × 24 mm, VWR) that included four holes. Two central holes allowed interconnection of the top and bottom channels, and two outer holes allowed for the injection of the fibroblast encapsulated hydrogel.

The coverslips in contact with the 2PP ink were functionalised with methacrylated silane (Sigma–Aldrich) to improve adhesion of the hydrogel structures.[103] The treatment was initiated by an air-plasma clean (power: 200 W; time: 1 min, Model Atto, Diener electronic, GmbH) followed by immersion at room temperature in 2% w/w of (3-mercaptopropyl) triethoxysilane. 50% w/w ethanol, 47.7% w/w deionized water and 0.3% w/w acetic acid for 20 min. Afterward, the chips were washed three times with deionized water, dried under nitrogen gas and finally placed on a hotplate at 60 °C for 3 h. The functionalized chips were used within 1 month from functionalization and protected from light during storage.

**2PP Set-Up:** All 3D printed structures were imported directly into Think 3D software (UpNano, GmbH) as an STL file generated from Solidworks (Dassault Systèms). The objects were printed with the UpNano One 3D printer (UpNano GmbH) in top–down mode with a 10x objective (0.4 NA, Olympus) by alternating layers scanned in the x and y directions (woodpile mode) to reduce asymmetrical mechanical properties in the structures. All 3D printing parameters were set in the Think3D software. Prints were performed with a laser with a central wavelength of 790 nm and 100 mW power. The refractive index for the 2PP ink hydrogel precursor solution was set as 1.36. All structures were printed with a hydrogel-glass adhesion layer due to the oxygen crosslinking inhibition effect near the PDMS surface.[104,105]

**Hydrogel Swelling:** Swelling of the 2PP ink was assessed using a quantitative method employed in previous studies.[53,106] First, 25 cubes (100 μm × 100 μm × 100 μm) were printed (Figure S12, Supporting Information). For the retained cubes with little distortion, the size of the top structure surface (i.e., the surface furthest away from the hydrogel-glass interface) was measured via confocal microscopy after 24 h incubation in
cell media. Then, from the surface area, the side length of each top surface was calculated. The degree of swelling was determined by comparing the dimensions of the square side to the dimension in the original CAD file, according to the following formula:

\[ \text{Swelling} \% = \frac{S_{\text{cube}} - S_{\text{CAD}}}{S_{\text{CAD}}} \]  

where \( S_{\text{cube}} \) is the top side length of the cube and \( S_{\text{CAD}} \) is the top side length of the CAD file.

Different \( z \) intervals (1–9 \( \mu m \)) and scanning speeds (200–1,000 \( \text{mm s}^{-1} \)) were screened for printing at a constant laser power (100 mW). The space between two adjacent samples was chosen to avoid printed stitching lines from overlapping in the samples. For the sample displaying the least swelling, the hydrogel-layer interface was also measured via confocal microscopy to investigate the printing quality when not affected by hydrogel swelling.

**Single and Multi-Hydrogel Structure Fabrication:** For the multi-hydrogel structures, the 2PP channel and connector wall thicknesses were set to 40 and 175 \( \mu m \), respectively. After printing in device five, the non-crosslinked hydrogel was gently removed with warm media. The fibrin, collagen, and fibrin–collagen precursor solutions were injected around the printed channels by manually pipetting (Figure S13, Supporting Information). The single-hydrogel structure was printed with 200 \( \mu m \)-thick walls for both the channel and the connectors. Overnight incubation in cell media at 37 °C allowed the hydrogel structures to equilibrate before being evaluated.

Fluorescence of the hydrogel allowed to image its structure via a confocal microscope (Leica-SP8, 10x objective and 0.3 NA). Reproducibility of the printed structures was evaluated with \( x \)-\( y \)-confocal images, and the inner diameter of the hydrogel channel was compared with the dimensions in the CAD design. In addition, images were recorded in reflection mode to visualize the fibrin, collagen and fibrin–collagen network surrounding the 2PP structure and evaluate the effect of the non-crosslinked ink removal.

For the investigation of minimum achievable feature sizes, five different hollow structures were printed with inner diameters of either 10, 20, 30, 40, or 60 \( \mu m \) and a constant wall thickness of 40 \( \mu m \). After removal of the non-crosslinked hydrogel ink, a 500 kDa Antonina solution (1 mg mL\(^{-1} \), Sigma–Aldrich) was injected inside the vessel structure at day 1 and 10 to measure the diameter and eccentricity of the channels. The images were taken by confocal microscopy (Leica, SP8, 10x air objective 0.3 NA) with a z-stack of 200 \( \mu m \) and analyzed with CellProfiler software.[107]

**FRAP Experiments:** FITC-dextran (Sigma–Aldrich) with five molecular sizes, 4, 40, 70, 250, or 500 kDa (concentration 50 \( \mu m \), Sigma) were used. The hydrogel precursor solution was manually injected with a pipette into the microfluidic device and 6 structures (300 \( \mu m \) x 300 \( \mu m \) x 150 \( \mu m \)) were printed at the bottom of the channel (Figure S14, Supporting Information). The chip was then immersed in cell media overnight to remove the non-crosslinked ink. Afterward, the hydrogel samples were incubated in FITC-dextran solution for 24 h to reach equilibrium. FRAP was then monitored (recorded time 60 s, frame interval 0.5 s) with an inverted confocal microscope (Leica, SP8, 25x water objective, 0.95 NA). For each FITC-dextran molecular size, six sample recovery sequences were processed with the “FRAP analysis” library from Matlab.[108]

The diffusion pattern observed in the hydrogel structures was analyzed with Fiji by plotting the intensity profile of the hydrogel and FITC-dextran solution.[109]

**2PP Bioink and Cell Interaction:** For 2D culture assessment, a disk with a 1 mm diameter and 100 \( \mu m \) thickness was printed at the bottom of the wells on device three. The non-crosslinked 2PP ink was gently removed by injecting fresh media into the microfluidic chip, and the printed structures kept in an incubator overnight to equilibrate. Non-GFP-expressing HUVECs and GFP-expressing HUVECs were seeded into the microfluidic chip at a concentration of 10⁵ cells cm⁻² for cell viability and proliferation, respectively. Cell viability was measured at 24 h after seeding (\( n = 5 \)). Proliferation was monitored over a period of 10 days with measurements collected at days 1, 3, 7, and 10 (\( n = 9 \)). As control, the same microfluidic chip design was treated with an adhesion-promoting solution recommended by the cell provider (SpeedCoating, PELOBiotech) and cells were cultured on this surface. For 3D culture biocompatibility, the fibroblast-laden 2PP ink (2 x 10⁵ cells mL⁻¹) was injected in device two to fabricate cube structures (500 x 500 x 250 \( \mu m ^ { 3 } \)) with the optimized printing parameters. After printing, the non-crosslinked hydrogel was removed by injecting warm media. For the control experiment, the fibroblast-laden fibrin precursor solution was injected into the central channel of device four and kept in the incubator for 3 min before adding cell media on the side channels. Cell viability of HUVECs and fibroblasts was tested 24 h after hydrogel crosslinking by performing a live/dead assay (Thermo Fisher Scientific) with 0.75 \( \mu m \) \( \text{L}^{-1} \) propidium iodide and 2 \( \mu m \) \( \text{L}^{-1} \) calcein AM, with concentrations from the stock solution provided by the supplier. The samples were incubated for 15 min after assay solution injection and then washed three times with PBS before imaging by confocal microscopy (Leica SPB 10 x 0.4 NA air objective). Cell proliferation was measured by total nuclei counts and total cell coverage area. On day 10, cells were stained with a live nuclear fluorescent dye (Spy DNA S55, Spirochrome) by direct injection inside the microfluidic chip 1 h before images were captured. The same live/dead protocol was followed for assessing the viability of fibroblast and HUVEC cell viability in the multi-hydrogel structure. Image analysis for both 2D and 3D cultures were performed using CellProfiler.

**Shear Stress Simulation:** The wall shear stress experienced by the cells in the different hydrogel channel designs was determined with COMSOL Multiphysics software (Version 6.0, COMSOL Inc). Hydrogel channel structures were designed in Solidworks software (version 2021). After importing the structure into the COMSOL software, the assumptions used in the models were Newtonian and incompressible fluid with the dynamic viscosity of pure water. In addition, the following settings were used: The no-slip boundary condition ensured zero velocity at the wall, and the atmospheric pressure was set at the outlet. Inlet boundary conditions served to investigate the shear stress for the applied flow. The simulation was performed with the laminar flow module and finer mesh. The flow-induced deformation of the hydrogel channel was assumed to be negligible after no deformation was observed in the channel during perfusion by micro-scope imaging (Lumascop 560, Etaluma).

**Perfusable Multi-Hydrogel Construct for Co-Culture of HUVECs and Human Lung Fibroblasts:** After 2PP hydrogel channel printing, a fibroblast-laden fibrin solution (7.5 x 10⁵ cells mL⁻¹) was manually injected with a 100 \( \mu m \) pipette tip to surround the vessel structures, and incubated overnight. The next day, HUVECs (1.5 x 10⁵ cells mL⁻¹) were seeded inside the printed network by pipette injection. This cell concentration was chosen experimentally to ensure a sufficient number of cells in the hydrogel channel. After 12 h, the channels were connected to a syringe pump (T Sunrise, low pressure module) so that the microfluidic medium could be refreshed with an intermittent flow (flow rate: 0.1 \( \mu m \)⁻¹, shear stress: 0.6 dyne cm⁻², 3 min perfusion every 4 h) for 10 days to promote endothelial monolayer formation. The cell culture was monitored with an incubator microscope (Lumascop 560, Etaluma) and the use of a customized microfluidic carrier allowed user-friendly handling, as shown in previous studies[110,111] (Figure S15, Supporting Information).

At day 10, the samples were fixed with 2% paraformaldehyde for 15 min. (Thermo Fisher Scientific) and stained for F-actin (SPY620 actin, Spirochrome) and nuclei (SPY555 DNA, Spirochrome), before being imaged with a confocal microscope (Leica SP8, 25x water objective, 0.95 NA).

**Cell Nucleus Orientation:** At day 7, the cells cultured on the hydrogel disks (cell seeding and culture protocol are shown in Section 4.3.6) were cultured on the microfluidic chip channels and on the hydrogel channels (cell seeding and culture protocol are shown in Section 4.3.8) were stained (SPY555 DNA, Spirochrome) and imaged by confocal microscopy (Leica SP8). For the hydrogel disks, three samples in different microfluidic chips were imaged. Regarding the microfluidic chip channel, a surface area of 1 mm² was acquired. Finally, for the hydrogel channel, the section between the hydrogel connector was imaged. Then, the nucleus orientation of all the conditions was measured with CellProfiler.

**Statistical Analysis:** For the nuclei orientation, at least 150 nuclei were analysed per condition. As the alignment of the nuclei was always centred
on 0° there was no difference between the means of this study. Instead, they differ because they had different variances. For the nucleus orientation experiment RStudio was therefore statistically tested if there was a difference in variance between the different conditions using an Ansari-Bradley test with a confidence level of 95%.

For hydrogel swelling, there was a difference in means between the conditions and thus a more standard statistical approach was applied. The variable swelling was transformed according to the Box-Cox test to meet the assumption of normality. Differences between factor variables were determined using a Tukey post hoc test with a confidence level of 95%. P-values < 0.05 were considered statistically significant as follows where "denotes p < 0.05, ** denotes p < 0.01 and *** denotes p < 0.001.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author contributions
F.C., L.B., and M.T. conceptualized the study. F.C. and L.B. conceived and optimized the 2-step hydrogel fabrication process and the device designs. F.C., L.B., H.P., and M.T. planned the experiments. F.C. performed the experiments and collected the data. F.C., L.B., H.P., and M.T. supervised the research. M.T. acquired the funding. All authors edited and approved the final manuscript.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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