ADVANCED MATERIALS

Supporting Information

for *Adv. Mater.,* DOI: 10.1002/adma.202102661

3D Bioprinting of Engineered Tissue Flaps with Hierarchical Vessel Networks (VesselNet) for Direct Host-To-Implant Perfusion

*Ariel A. Szklanny, Majd Machour, Idan Redenski, Václav Chochola, Idit Goldfracht, Ben Kaplan, Mark Epshtein, Haneen Simaan Yameen, Uri Merdler, Adam Feinberg, Dror Seliktar, Netanel Korin, Josef Jaroš, and Shulamit Levenberg**

Supporting Information

Title: Three-dimensional bioprinted prevascularized constructs for direct host-to-implant perfusion

*Ariel A. Szklanny, Majd Machour, Idan Redensky, Václac Chochola, Idit Goldfracht, Ben Kaplan, Mark Epshtein, Haneen Simaan Yameen, Uri Merdler, Adam Feinberg, Dror Seliktar, Netanel Korin, Josef Jaroš, Shulamit Levenberg**

Material and methods

Hydrogel mechanical properties assessment: The rhCollMA hydrogel mechanical properties were investigated by oscillatory rheology, using an AR-G2 rheometer (TA Instruments, USA) equipped with a 20 mm parallel plate geometry for the loss and storage moduli measurment, and a 40 mm quartz plate geometry for the stress-strain experiment and to obtain the linear viscoelastic regime (LVR). The LVR parameters were obtained by performing an amplitude sweep test from 0.025-1000% at an oscillation frequency of 1 Hz. Storage (G') and loss (G'') moduli curves were obtained performing time sweep measurements with 0.2ml of the pre-hydrogel solution containing 0.1% w/v LAP photoinitiator. The samples were injected into the rheometer and cross-linked in-situ by irradiating with 3 mW/cm^2 of 405 nm visible light after 30' of the test commencement. The stress–strain curve was constructed by compression test, in which crosslinked gel discs with a diameter of 8 mm and a thickness of 2 mm were loaded into the rheometer and were compressed to approximately 75% of its initial thickness. The parameters of the axial force and gap were used to calculate the stress.

Angiogenic cytokine array: After printing vascular constructs, medium was collected from the culture wells at days 1, 3 and 7. Cytokine quantification was performed using Human

Angiogenesis Array GS1 (RayBiotech). A cytokine heat map was generated using Prism9 (GraphPad).

VascFold geometry preparation and mesh: The VascFold was scanned using a high-resolution μCT scanner (Skyscan 1276, Bruker) from which an STL file was produced. The raw model was then imported into MeshLab 2016.12 and smoothed using a Laplacian Smoothing filter with surface preserve option enabled and holes closed to produce a watertight model of the scaffold (Supplementary Figure S19A). To produce the fluid volume model needed for the simulations the scaffold model was importer into SolidWorks® 2019 (Dassault Systèmes SE) and subtracted from a rectangular solid volume. Finally, the ends of the model were truncated to produce a planar inlet and outlet surfaces (Supplementary Figure S19B). The fluid volume model was meshed using tetrahedral elements in Ansys GAMBIT (Ansys Inc. and converged by doubling the number of elements in each iteration producing a final 27M element model.

Wall shear stress simulation: The simulation was done in Ansys Fluent 15® to solve the momentum and continuity of equations a viscous flow setting was used with a pressure-based solver ('SIMPLE') and with a 10^{-6} convergence criterion on all metrics. The flow medium was water at room temperature properties, the inlet was defined as mass flow inlet at $8.33 \cdot 10^{-7}$ kg/sec (0.05 ml/min) and the outlet a zero-gage pressure outlet, a no-slip condition was enforced on all the walls.

In-vitro flow perfusion of endothelialized VascFolds: A bioreactor mold was designed using a negative mold approach. The mold was printed with a Prusa MK3S and BVOH as a

filament. The mold was filled with polydimethylsiloxane (PDMS), cured at room temperature overnight, and the BVOH was then washed away in DDW. The resulting PDMS bioreactor (Figure S20) was assembled with silicone tubings and a 25 G needle to accommodate the endothelialized scaffold, and the assembly was then autoclaved. In a biological hood, the VascFold was connected to the needle within the chamber, the bioreactor was filled with 5 ml of EC medium, and the tubing was connected to a peristaltic pump (EBERS) inside an incubator under sterile conditions. The scaffold was perfused for three days with a flow rate of 50 µl/min. After three days, the scaffolds were retrieved, fixed, and stained for the relevant markers.

Albumin diffusion assessment: Scaffolds were endothelialized and cultured for 2 days before the albumin diffusion test. FITC-conjugated bovine serum albumin (FITC-BSA, Sigma-Aldrich) was diluted in PBS with a concentration of 1 mg/ml. The solution was perfused into endothelialized and acellular scaffolds with a rate of 50 µl/min and imaged every 5 minutes using a LSM 700 confocal microscope (Zeiss). The confocal images were analyzed using FIJI (ImageJ) and the diffusion graph was made using Prism9.

Scanning electron microscopy imaging: To prepare the hydrogels for imaging, 50 µl plugs of crosslinked rhCollMA were flash-frozen using liquid nitrogen and then lyophilized overnight. Acellular scaffolds and hydrogel droplets were coated with a gold-palladium mixture using a Polaron gold coater and imaged using a using a Quanta 200 microscope (FEI).

Laser speckle contrast imaging: Two weeks after implantation procedures, animals were anesthetized with 3% isoflurane inhalation, arranged in a prone position with the soles of the hindlimbs visible, and imaged using an FLPI-2 laser speckle contrast imager (Moor Instruments Inc.). Three consecutive exposures lasted 10 s each, at 752 × 580-pixel resolution. For each graft, the imaging process yielded both a color image and a corresponding perfusion-flux image. Images were exported and analyzed using the moorFLPI-2 Measurement V1.0 © software. To establish blood flow for each rat, several ROIs (n>3) were selected at the same location on the treated and control limbs. The blood flow of each ROI of the treated limb was then normalized to its control ROI counterpart. Values for the whole limb were obtained by averaging the flow values of each ROI. ROIs were consistently selected for all rats at approximately identincal locations; small differences in location arised from differences in the positioning of the rat during imaging.

MicroCT angiography of implanted engineered tissue flaps: Two weeks after implantation, rats were euthanized using CO_2 inhalation and transcardially perfused with 60 ml of warm heparinized saline. Then, 15 ml of freshly prepared silicone rubber contrast agent (Microfil, FlowTech Inc.) was infused. Microfil solution was allowed to polymerize at 4 °C overnight, after which the implants were harvested and immersed for 20 min in 4% PFA solution. Explants containing the vascular tree cast were scanned using a Skyscan 1276 microCT scanner. The following scanning parameters were used: source voltage of 55 kV, source current of 72 µA, applied filter of aluminum 0.25 mm using a 0.2 degrees rotation step. Image acquisition was performed with a scaled pixel size of 10 µm.

Lectin injection for host vasculature perfusion assessment: Two weeks after implantation, rats received two 10 minutes apart tail vein injection of fluorescent lectin solution (Vector labs, DL-1208) which binds specifically to endothelial cells of nonprimates). 30 minutes after injection, rats were euthanized by CO_2 inhalation, and implants were recovered for cryosectioning and further section analysis.

Propagation of hiPSCs and Directed Cardiac Monolayer Differentiation: hiPSC expressing GCaMP (kindly gifted by Prof. Bruce Conklin, Gladstone Institute, USA) were used in this study. They were cultured on Matrigel covered plates with mTeSR-1 medium (StemCell Technologies). hiPSC were passaged by dissociation with 0.5 mM ethylenediaminetetraacetic acid (EDTA, Gibco) every 4–5 days. Cardiomyocyte differentiation was based on a previously described protocol.[2] Briefly, when cells reached 80-90% confluence (day 1 of differentiation), the culture-medium was replaced to a differentiation medium containing RPMI-1640, 2% B27 supplement minus insulin (ThermoFisher Scientific), 1% penicillin/streptomycin, supplemented with 6 µM CHIR99021 (Stemgent) for two days. Medium was then changed to RPMI/B27 supplemented with 2µM Wnt-C59 (Selleck Chemicals) for another two days. From day 5 onward, cells were cultured with RPMI/B27 medium alone. The resulting beating monolayers (10-14 days) were enzymatically dissociated using TrypLE express (Gibco) into small clusters or single cardiomyocytes. For bioprinting experiments, 10^7 cardiomyocytes/ml were suspended in 5 mg/ml bioink and printed as described above.

Optical Imaging GCaMP-Expressing hiPSC-CMs: To measure the fluorescence intensity of the GCaMP expressing cells within the printed tissue, the line-scan mode of a Zeiss

LSM700 laser-scanning confocal microscope (Zeiss) was used. 1µM isoproterenol (Sigma-Aldrich) was dissolved in water and added to the tissue solution. Recording of the calcium transients was performed 10 min after the addition of the pharmacological agent. The GCaMP fluorescent recordings were analyzed to characterize the properties of the optical calcium transients using the Clampfit10.7 program (Molecular Devices). The evaluated parameters include the beating rate and the decay time of the calcium transient (defined as the time interval from the peak of the calcium signal to the point where calcium level is reduced to 10% of the peak amplitude).

Patient-specific vascular construct fabrication: A coronary artery solid object was obtained from the NIH 3D Print Exchange project.[3] A sacrificial mold was created in Solidworks 2019, sliced using Prusa Slic3r, and printed on a Prusa i3 MK3S using a BVOH filament. The mold was designed to have inlets to load the polymer solution at different points, to ensure a homogeneous material distribution within. The mold was filled with a 7% PLLA-PLGA in dioxane solution, frozen at -80ºC, and lyophilized overnight. The mold was washed out in warm water, and the resulting scaffold was trimmed to remove the columns formed in the inlets. The hydrogel consisted of 40% w/v Pluronic F-127 (Sigma-Aldrich) in DDW and 5 drops of red food coloring, mixed overnight at 4ºC. The hydrogel print model was designed in TSIM (AdvancedSolutions) and printed with the Pluronic bioink using a BioAssemblyBot 3D bioprinter (AdvancedSolutions).

Immunofluorescent staining and imaging of in vitro and ex vivo samples: After one week of *in vitro* culturing, constructs were fixed using 4% paraformaldehyde for 20 minutes, followed by 3x PBS washes, 5 minutes each. Cells permeabilization was done using 0.3%

Tryton-X for 15 minutes followed by 3x PBS washes. The constructs were blocked with 5% BSA (MilliPore) solution for 1 hour at room temperature and stained with anti-human αSMA (1:50, Dako) primary antibodies, anti-human CD31 (1:100, Sigma-Aldrich), and anti-human laminin (1:200, Abcam) at 4ºC overnight. Constructs were then washed thrice with PBS, and the staining was completed using goat-α mouse Cy3 (1:400, Jackson Immunoresearch Laboratory) and donkey α-mouse Alexa-Fluor 647 (1:400, Thermo- Fisher Scientific). The live or fixed samples were imaged using a confocal microscope LSM 700. For endothelium imaging, the samples were cultured for 2 days, the primary antibody was anti-VE-Cad (1:100, Invitrogen), and the secondary antibody was goat-α rabbit Alexa-Fluor 488 (1:400, Thermo-Fisher Scientific). The rest of the process was performed as explained above.

Two weeks after implantation, the explanted tissues were washed with saline solution and fixed using 4% paraformaldehyde for 30 minutes, followed by 3 PBS washes. The sample was then kept in 30% w/v sucrose solution at 4ºC overnight. Then, the tissues were embedded in optimal cutting temperature compound (TissueTek) and frozen at -20ºC for cryosectioning, (10 µm and 20 µm thickness). The sections were permeabilized using 0.5% Tween, washed 3 times with PBS, blocked with 5% BSA solution, and incubated at 4ºC overnight with anti-rat CD31 (1:100, BD-Science), anti-laminin (1:200, Abcam) and anti-rat αSMA (1:100, Abcam). The sections were washed thrice in PBS and incubated for 3 hours at room temperature with a secondary antibody in PBS solution consisting of goat-α mouse Cy3, donkey α-mouse Alexa-Fluor 647, and DAPI. Slides were imaged using Axio Observer Z1 (Zeiss) and LSM 700 microscopes.

1. Schleimer, K., Grommes, J., Greiner, A., Jalaie, H., Kalder, J., Langer, S., Koeppel, T. A., Jacobs, M. & Kokozidou, M. Training a sophisticated microsurgical technique: interposition of external jugular vein graft in the common carotid artery in rats. *J. Vis. Exp.* (2012). doi:10.3791/4124

2. Burridge, P. W., Matsa, E., Shukla, P., Lin, Z. C., Churko, J. M., Ebert, A. D., Lan, F., Diecke, S., Huber, B., Mordwinkin, N. M., Plews, J. R., Abilez, O. J., Cui, B., Gold, J. D. & Wu, J. C. Chemically defined generation of human cardiomyocytes. *Nat. Methods* **11,** 855–60 (2014).

3. NIH. Phantom Coronary Artery Models | NIH 3D Print Exchange. *2019* at <https://3dprint.nih.gov/discover/3dpx-012589>

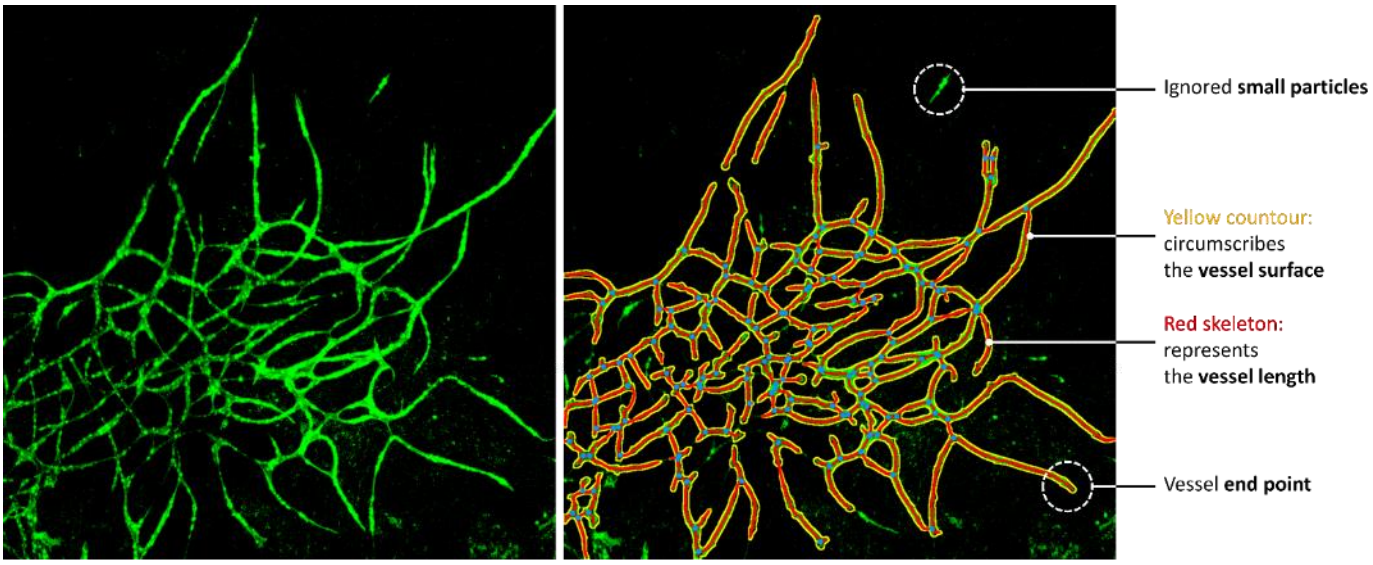


Figure S1 – Quantification explanation of vascular network development parameters.

Left, the original image of the ECs (green) forming the vascular network, obtained by confocal imaging. Right, the resulting image of the analyzed vascular network using the freeware Angiotool. The image shows a visual quantification of i) the vessel area, represented by the area circumscribed by the yellow contour, ii) the vessel length, represented by the red lines following the network skeleton, and the vessel endpoints, represented by the ends of the red skeleton segments, not in contact with another vessel. The elements with no contour are ignored.

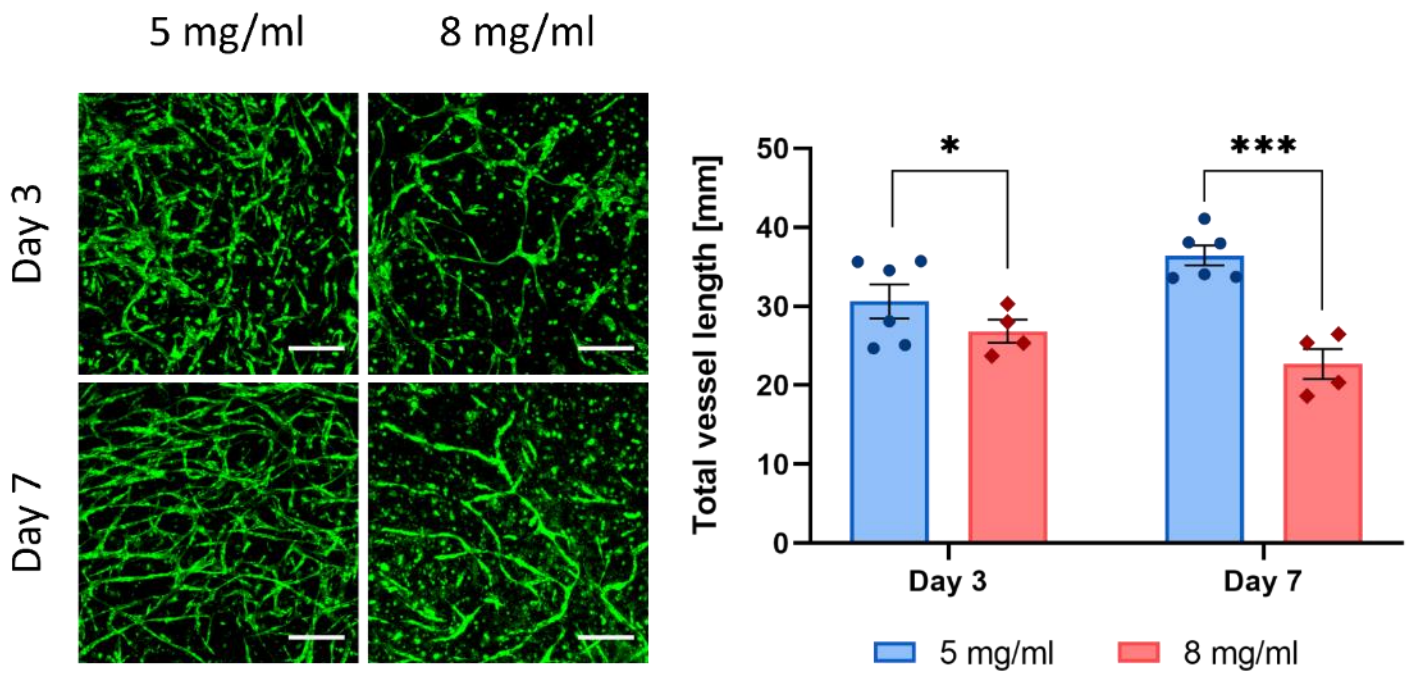


Figure S2. rhCollMA gel concentration effect on vessel network formation. Left, representative images of vessel networks forming in two rhCollMA gel concentrations, 5mg/ml and 8 mg/ml, at days 3 and 7. Right, quantification of total vessel length for the chosen concentrations and time points (n>3; *$p < 0.05$, ***$p < 0.001$). The parameter total vessel length was chosen as a representative parameter of the vessel development (scale bar: 200 µm).

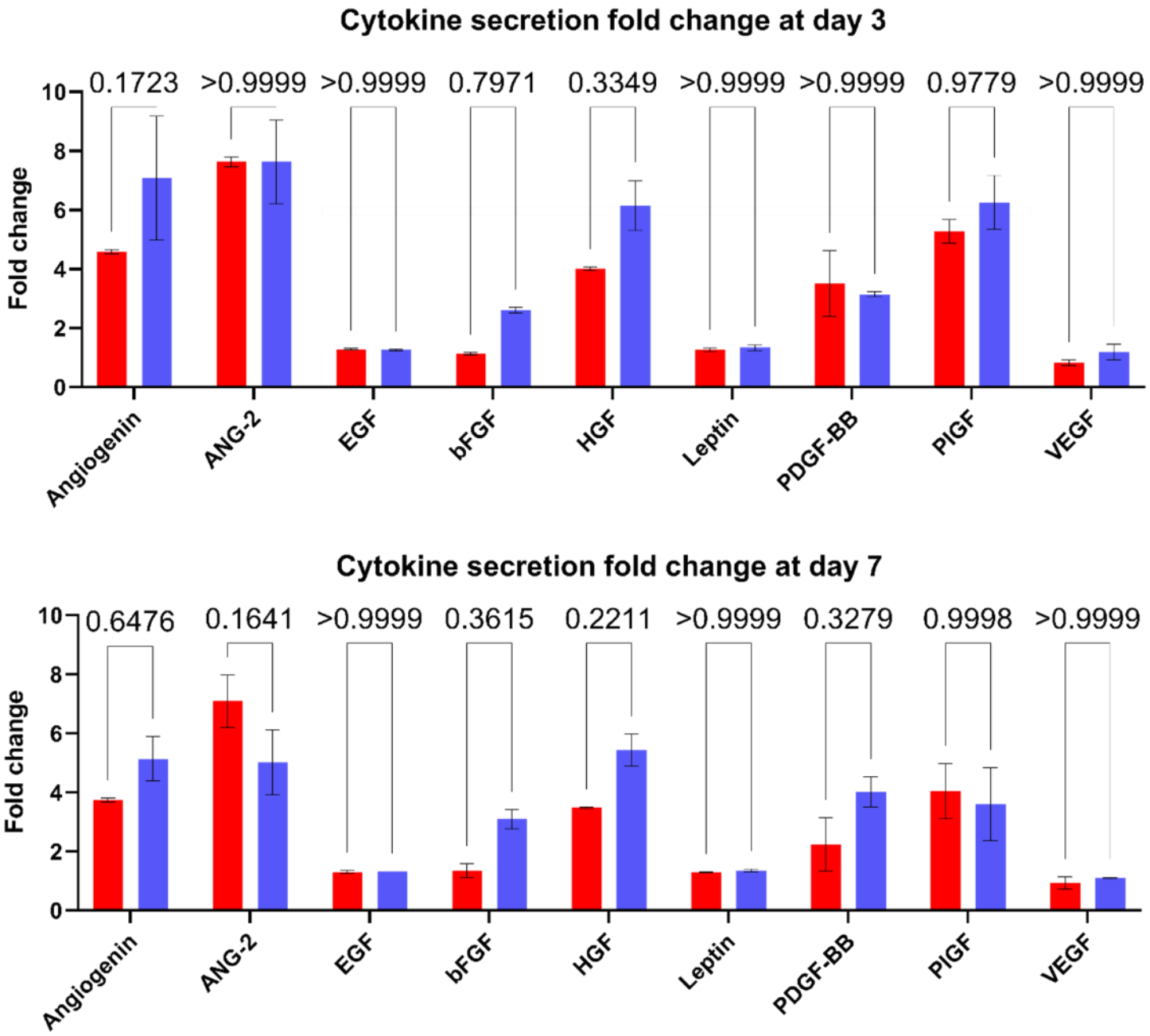


Figure S3 – Angiogenic cytokines secretion comparison between vascularized plugs and printed constructs. The cytokines are expressed as a fold change from day 1 of the same group. The values shown on top of the bars display the p-value between the groups. No

significant differences were found between the two systems for the studied cytokines ($n > 3$).

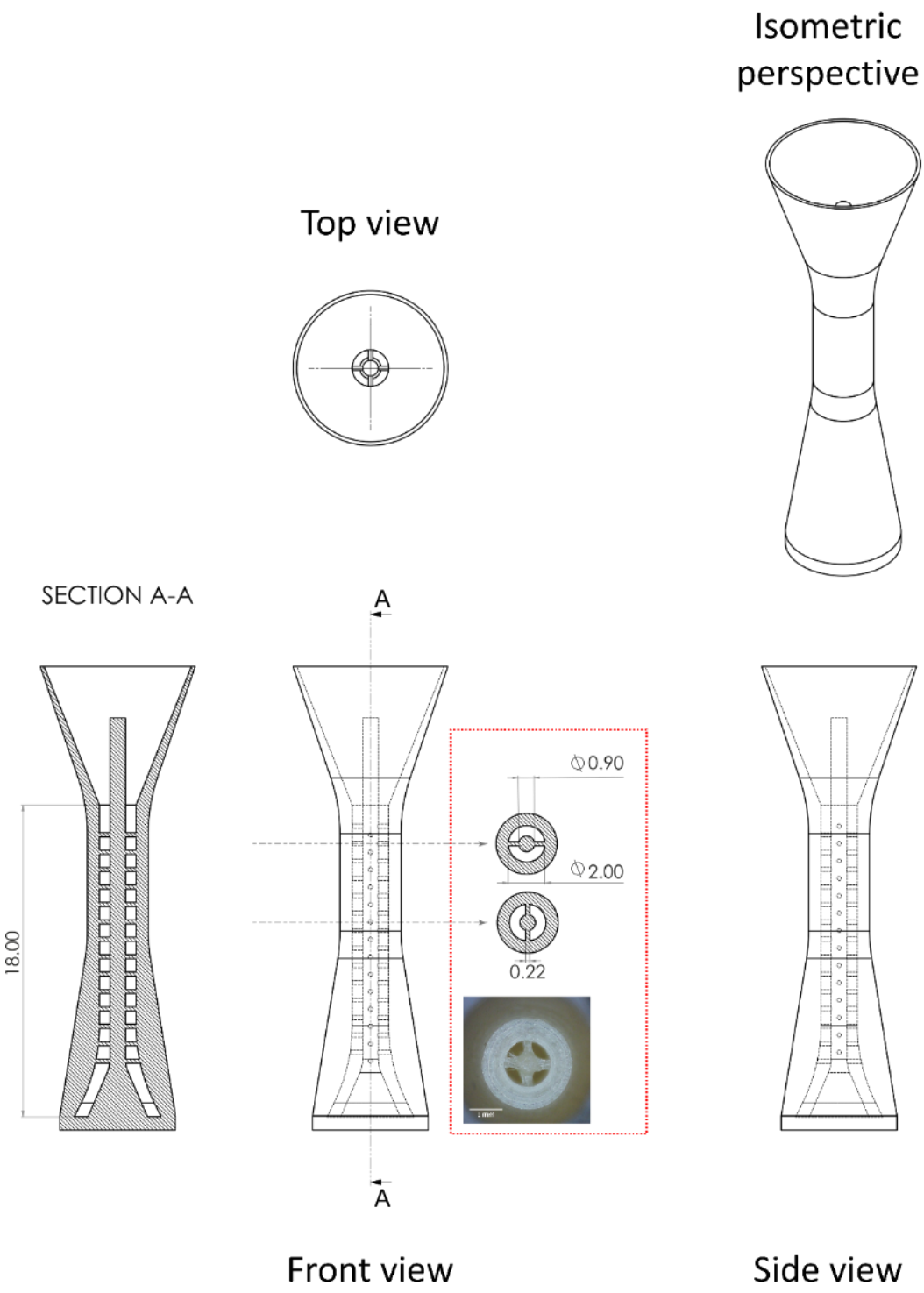


Figure S4 – Mold computer-aided design (CAD) for 3D printing. Orthographic projection and isometric perspective of the VascFold mold. Section A-A shows a cut view of the axial

center plane. The dashed red rectangle shows two sections of the lateral rods to create the fenestrations and an actual photograph of a mold transversally cut in which the lateral rods are visible.

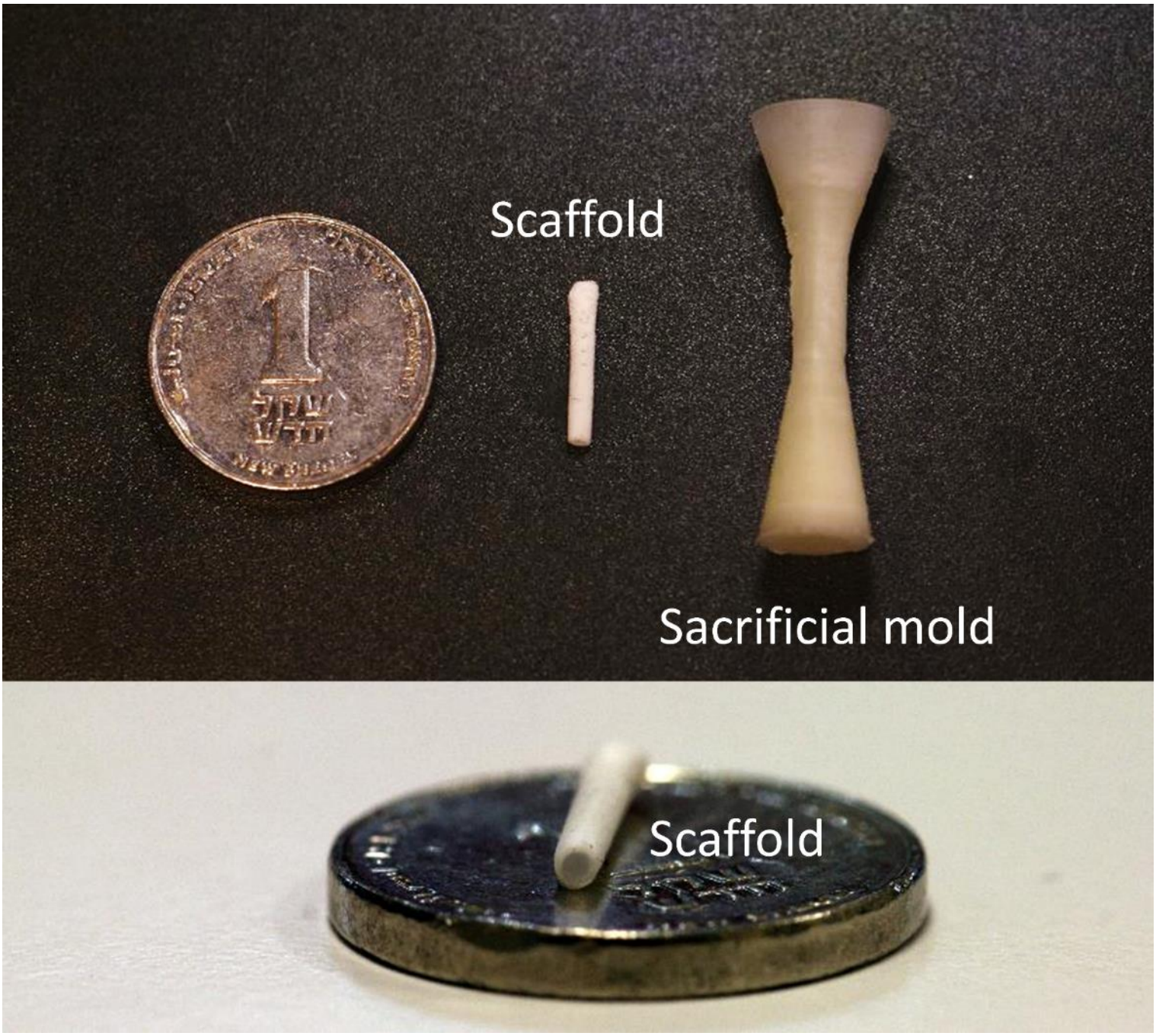


Figure S5 – Scaffold and mold photographs. Top, finished scaffold, and sacrificial mold next to a 1 NIS coin (17 mm diameter) for size comparison. Bottom, a photograph of a scaffold showing the central lumen on a 1 NIS coin for size comparison.

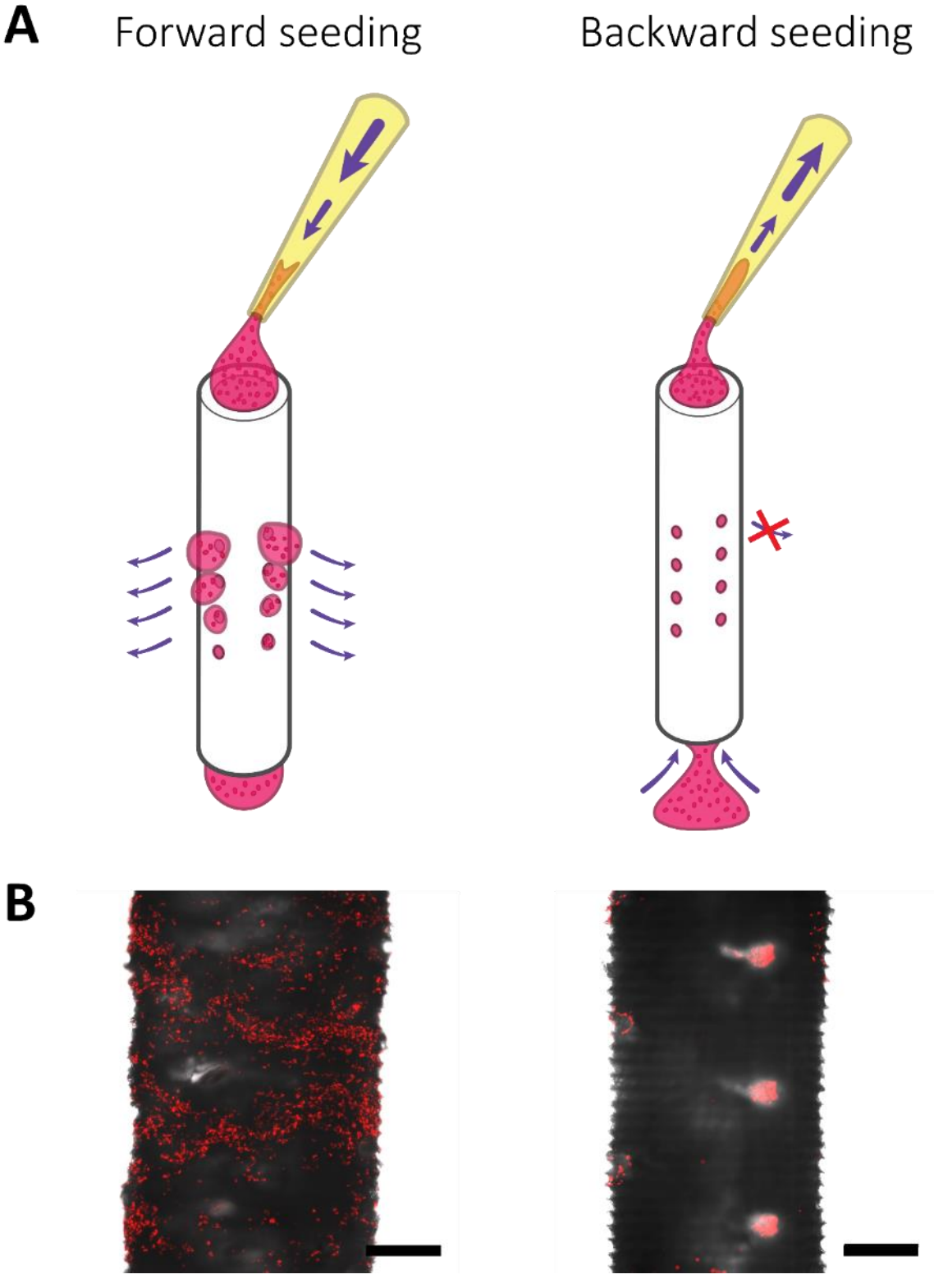

A
Forward seeding
Backward seeding
B

Figure S6 – Forward vs. backward endothelium seeding. A) Representative diagram of ECs seeded in the VascFold lumen by forward seeding (positive pressure, left) or backward seeding (negative pressure, right). Forward seeding is achieved by flowing an ECs suspension through one of the VascFold's ends. Backward seeding is performed by placing one end of the scaffold on top of an ECs suspension droplet and applying negative pressure on the other end, forcing the suspension to flow upwards. B) Confocal images of ECs (red) seeded by forward or backward seeding in a VascFold. ECs seeded using positive pressure escape through the scaffold through perforations to the outer wall, while ECs seeded using negative pressure remain inside the lumen and do not leak outwards (scale bar: 500 µm).

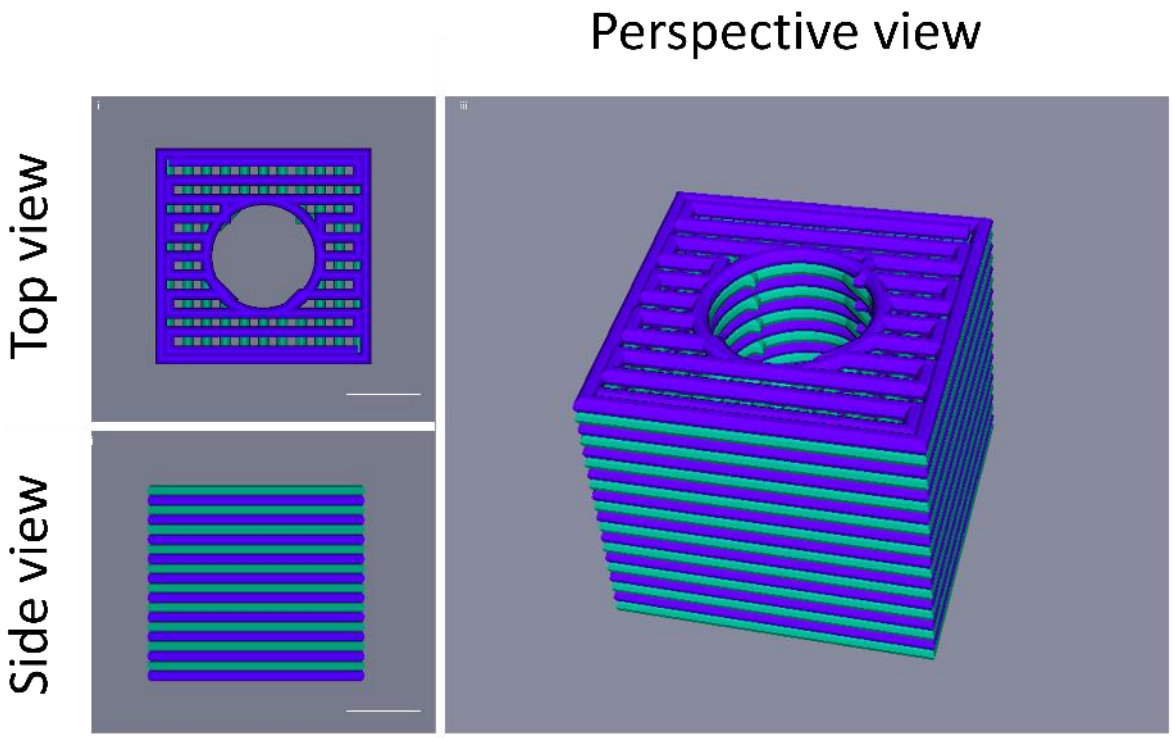


Figure S7 – Computer design for hydrogel bioprinting. Top, side, and perspective views of the computer design that was printed using the rhCollMA bioink. The design measured 6 x 6 x 5 mm (x,y,z), a layer thickness of 150 µm, and the center channel had a 2.5 mm diameter. Alternating layers appear in different colors for an easier understanding of the model. Scale bar: 2 mm.

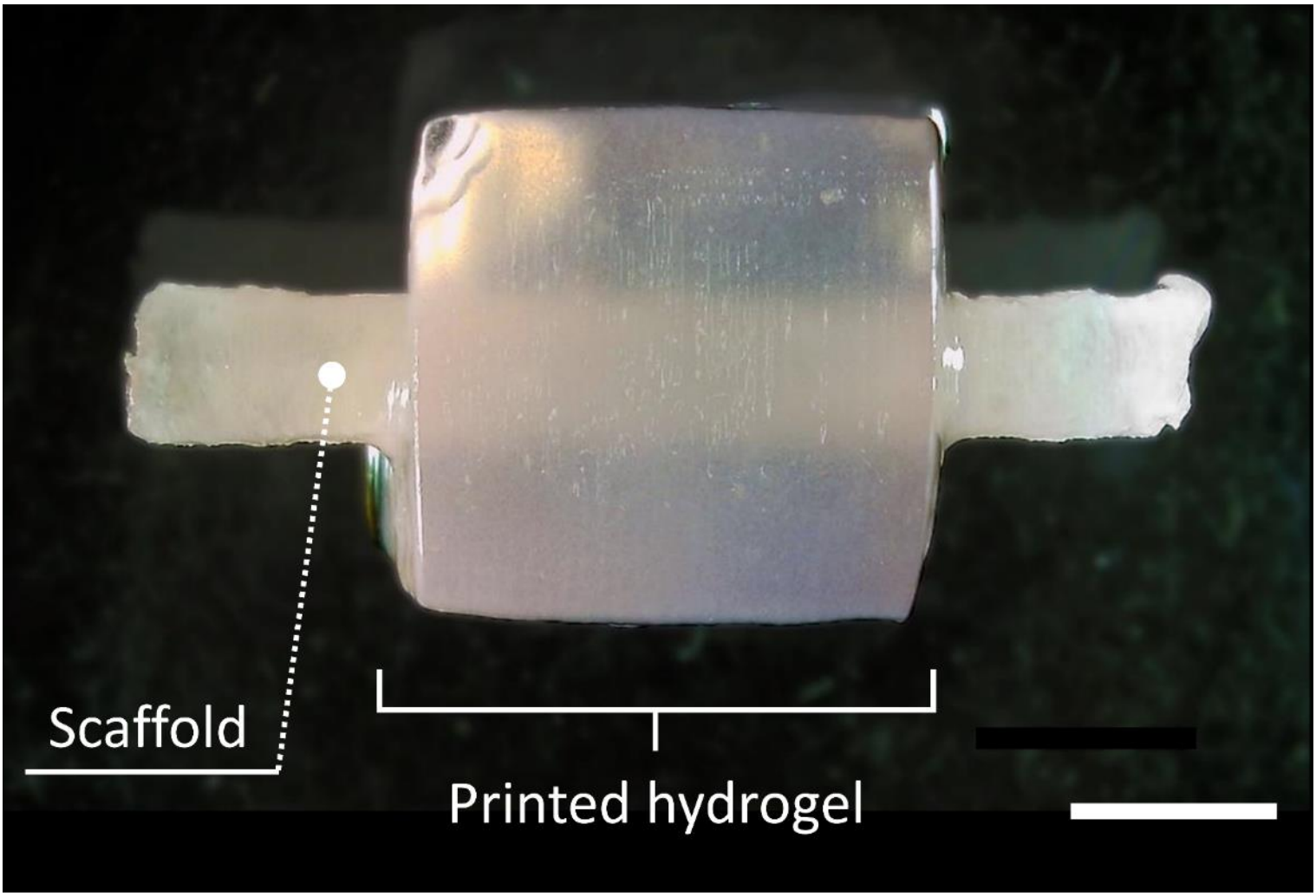
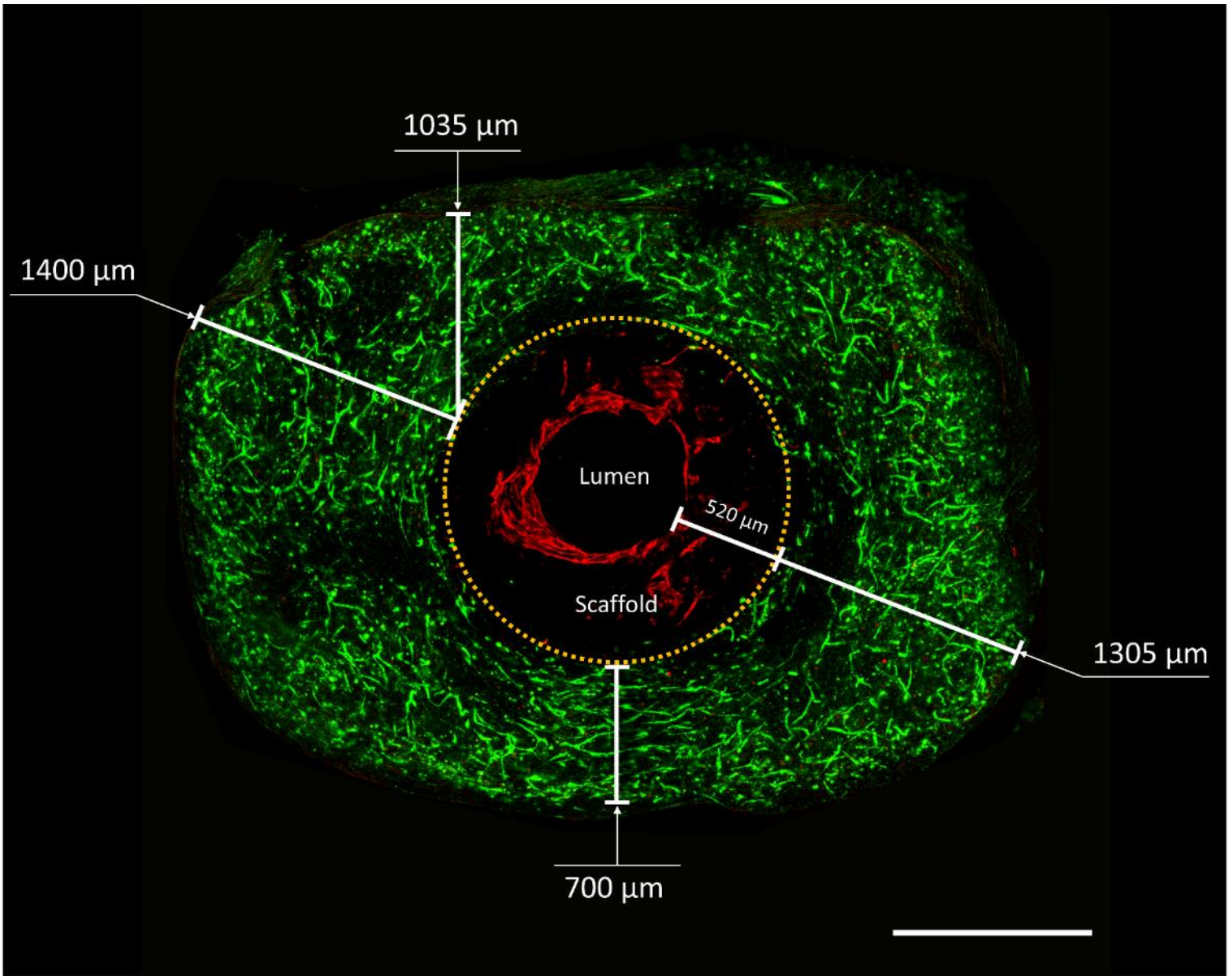


Figure S8 – Assembled scaffold and bioprinted hydrogel. Representative photograph showing a scaffold within a printed construct immediately after assembly. The scaffold can be seen through the slightly transparent printed hydrogel. Scale bar: 2 mm.



Supplementary Figure S9 – Vascularized hydrogel thickness measurements. Confocal image showing the middle section of a cultured construct presenting microvessels (green) in the printed gel and endothelium (red) in the scaffold lumen. The arrows indicate the measured distance marked by thicker white lines with perpendicular endcaps. The dashed yellow circle denotes the scaffold's location. Scale bar: 1 mm.

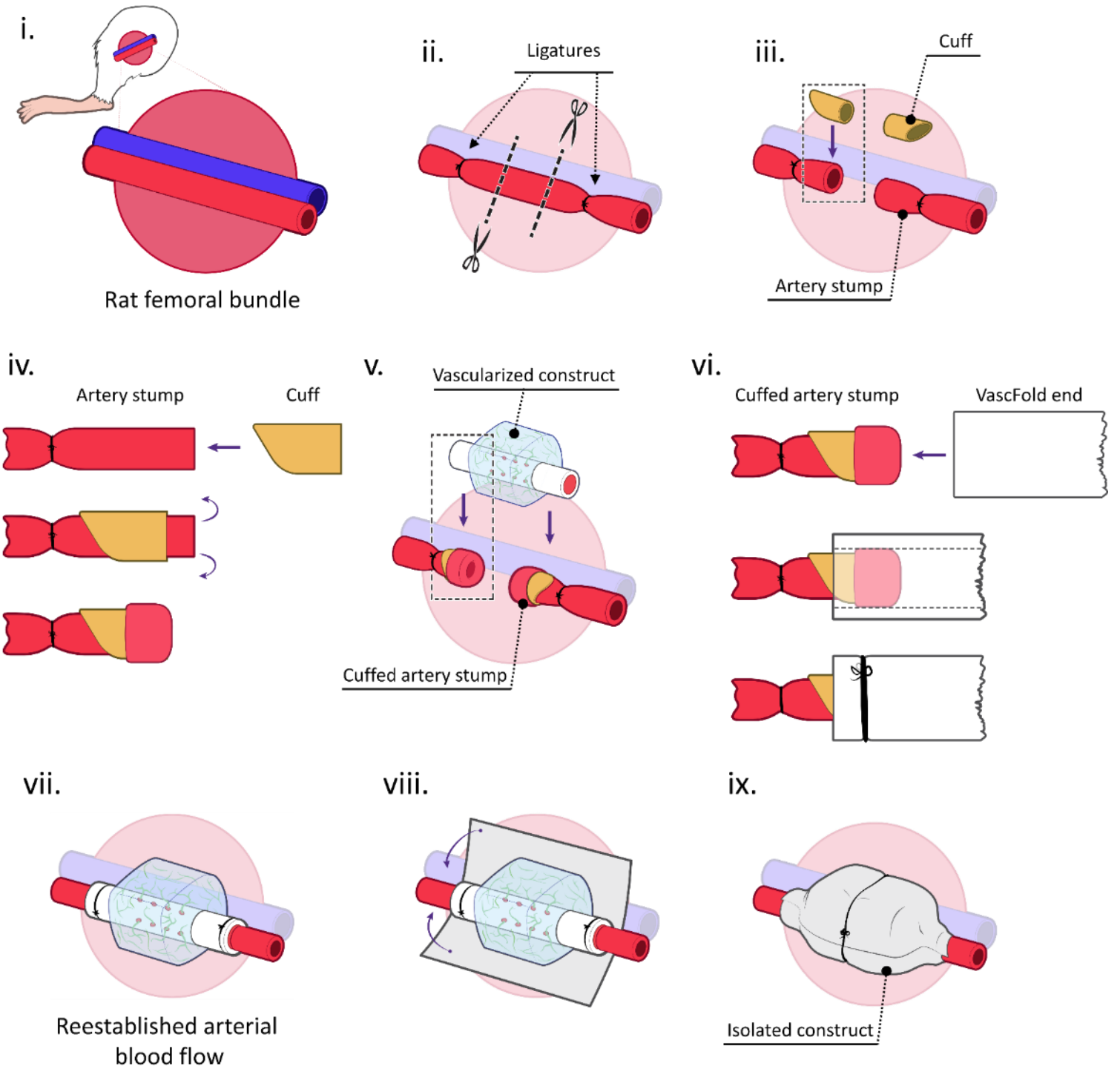
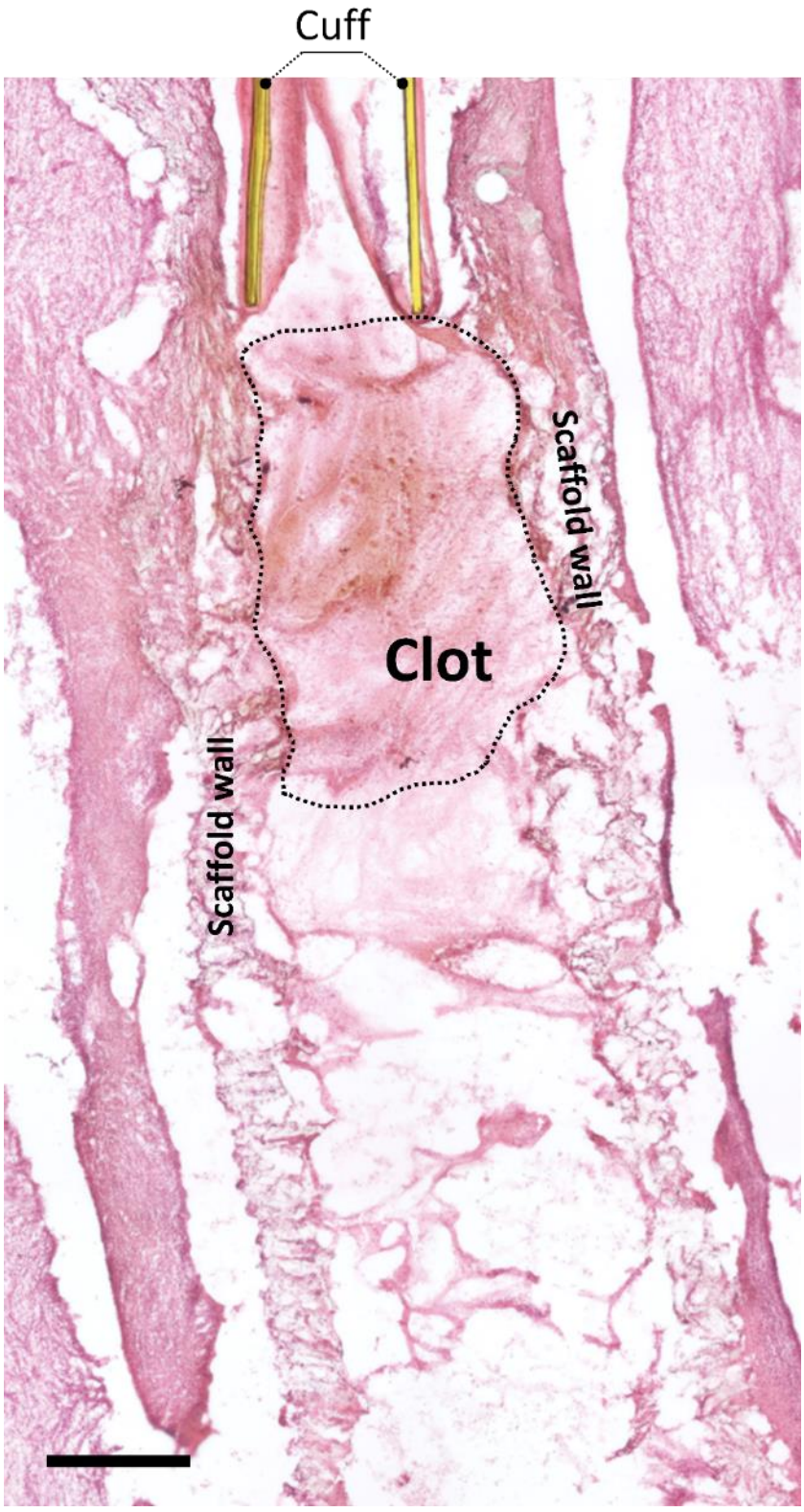


Figure S10 – Schematic explanation of the construct implantation microsurgery. i) The rat femoral bundle is accessed. ii) To stop blood flow to the implantation region, the

femoral artery is ligated and clamped in two places, approximately 1 cm apart. A segment between the stitches is resected, as marked by the dashed lines and scissors. iii) After segment resection, distal and proximal artery stumps are available for cuffing. iv) The artery stump is lodged inside the cuff lumen, and then the artery ends are folded backward. A circumferential stitch is added to secure the cuff and artery in place. v) The cuff stumps are ready to anastomose with the vascularized constructs. vi) The cuffed stumps are inserted into the VascFold ends lumen on both sides (artery-construct anastomosis), and circumferential stitches are added to secure the anastomosis. vii) Once the construct is correctly anastomosed, the clamps and ligatures are removed to reestablish the arterial blood flow circuit. viii) A 0.2 μm polycarbonate filter is placed around the engineered tissue and anastomosis regions to isolate the implanted construct from the surrounding tissues. ix) The filter is closed and secured on top of the construct with a circumferential stitch.


Cuff
Scaffold wall
Clot
Scaffold wall

Supplementary Figure S11 – H&E staining of an explanted clotted scaffold. The implanted scaffold was not endothelialized and was explanted four days after surgery due to the rat host decease. The cuff, scaffold walls, and blood clot (dashed line) can be identified in the picture. Scale bar: 500 μm.

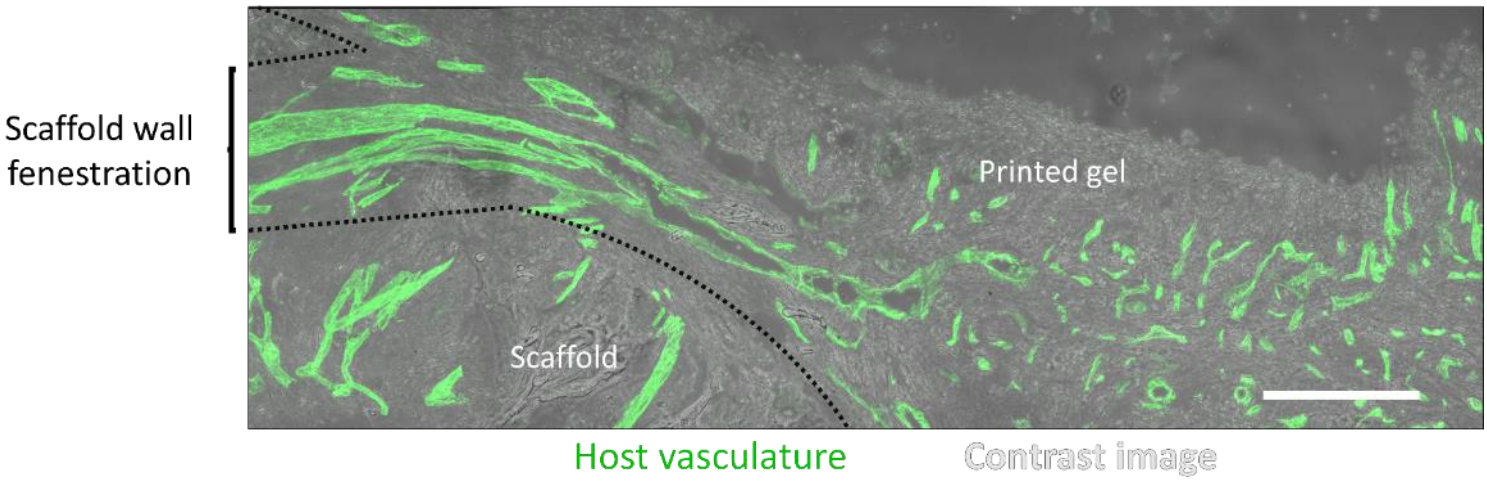


Figure S12 – Host vessels exit the scaffold through the designed fenestrations. Confocal image of a stained cryosection showing a large host vessel (CD31, green) sprouting into the printed hydrogel through the scaffold fenestration. The dashed lines mark the scaffold wall and fenestration. Scale bar: 200 µm.

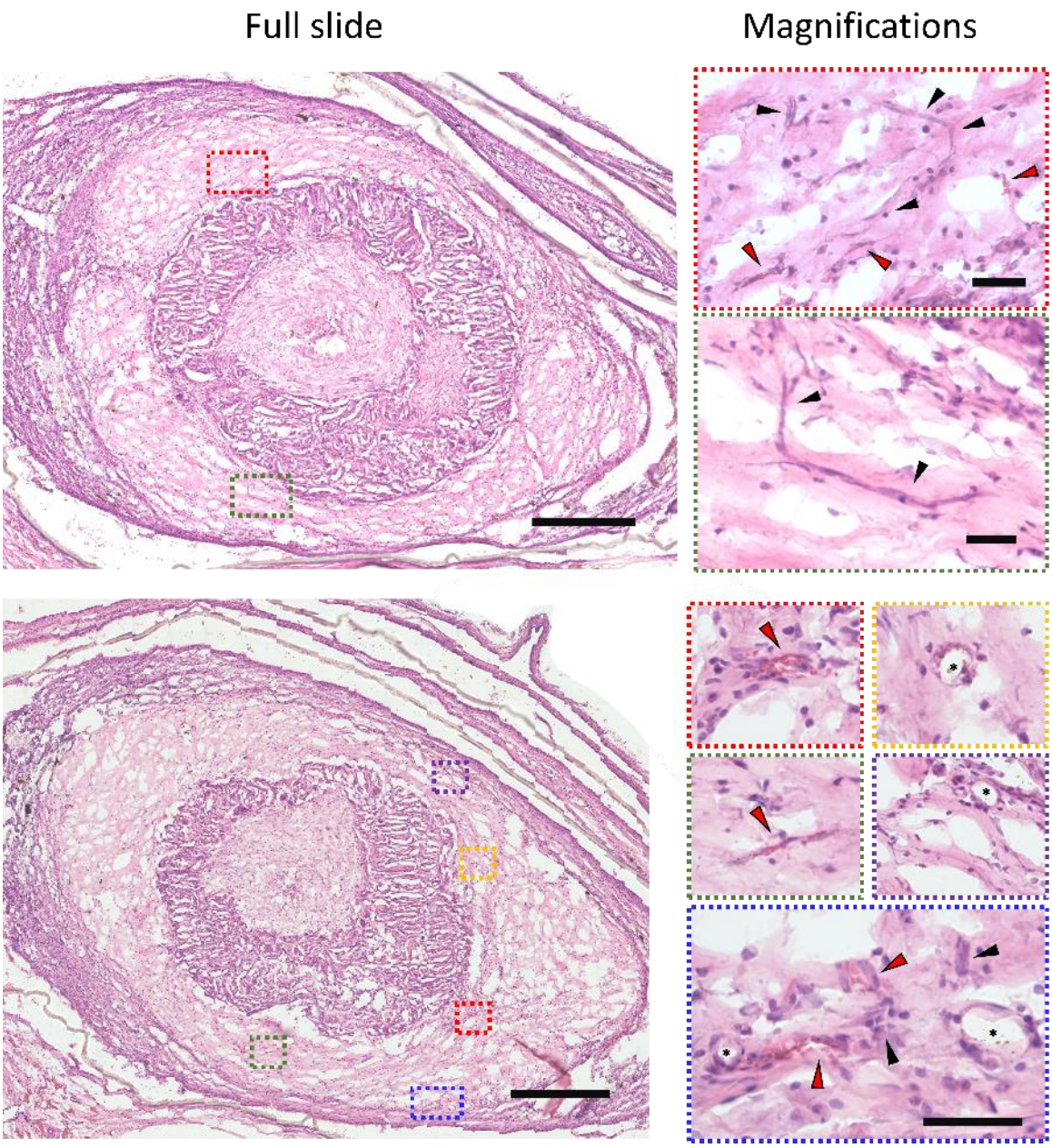


Figure S13 – Cryosection of explanted tissue after 14 days in vivo. H&E images of cryosections showing full transversal cut planes (left, scale bar: 500 µm) and magnified regions (right, scale bar: 50 µm) evidencing the presence of vascular structures in the explanted tissues. The colored dashed rectangles on the left mark the location of the magnifications on the right. Black arrowheads mark vessel-like structures; red arrowheads

mark vessel-like structures with red blood cells; asterisks mark transversal cuts of vascular structures in which the lumen is visible.

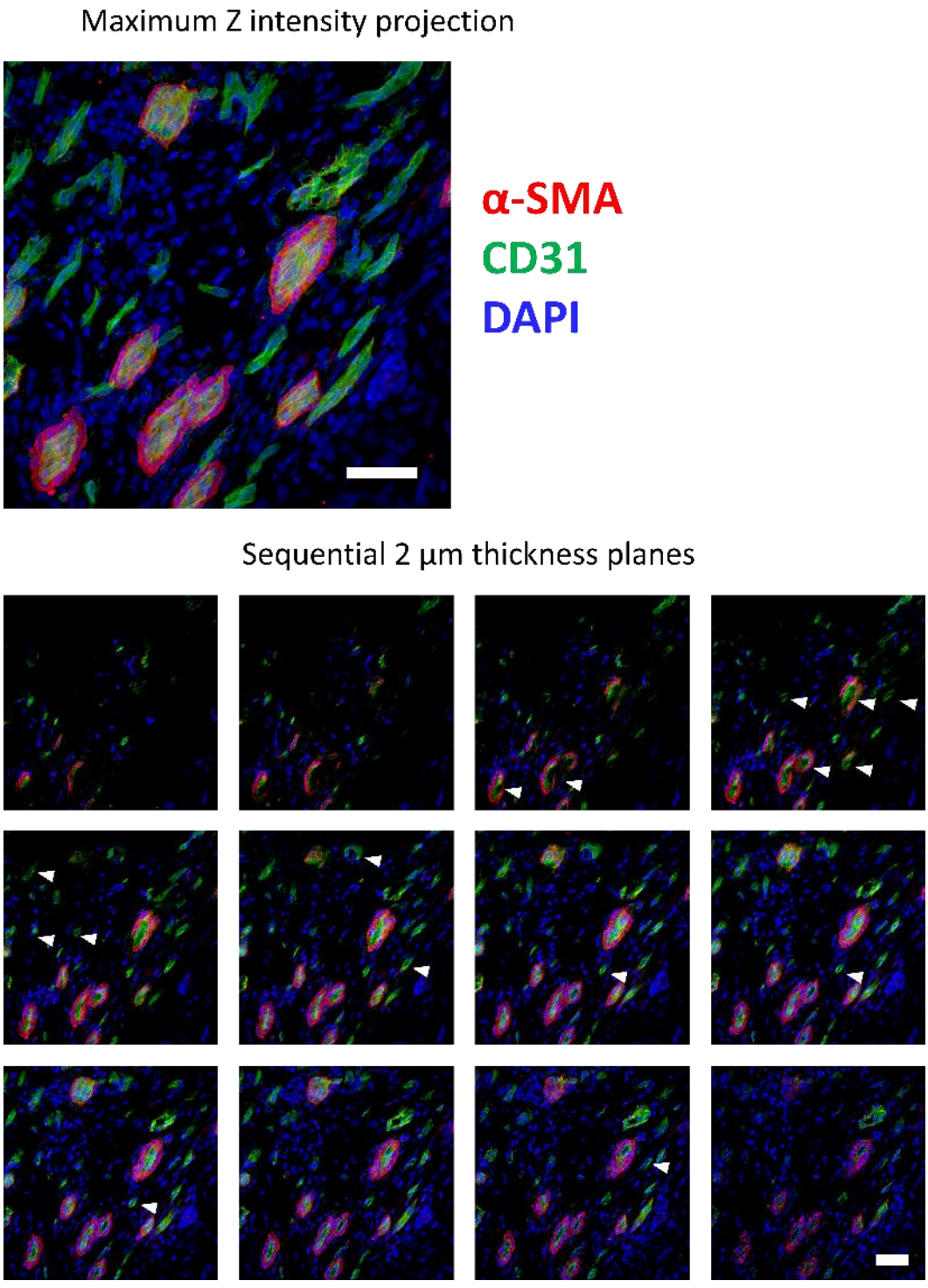


Figure S14 – Vascular structures in explanted tissues present a tubular structure. Top, maximum Z intensity projection of vascularized explanted tissue. The vessels are identified by CD31 (green), the +αSMA-SC are identified by +αSMA (red), and the nuclei are

identified by DAPI (blue). Bottom, the single-plane images conforming to the top image are displayed in a sequential organization to evidence lumen in the vascular structures, indicated by the white arrowheads (scale bar: 50 μm).

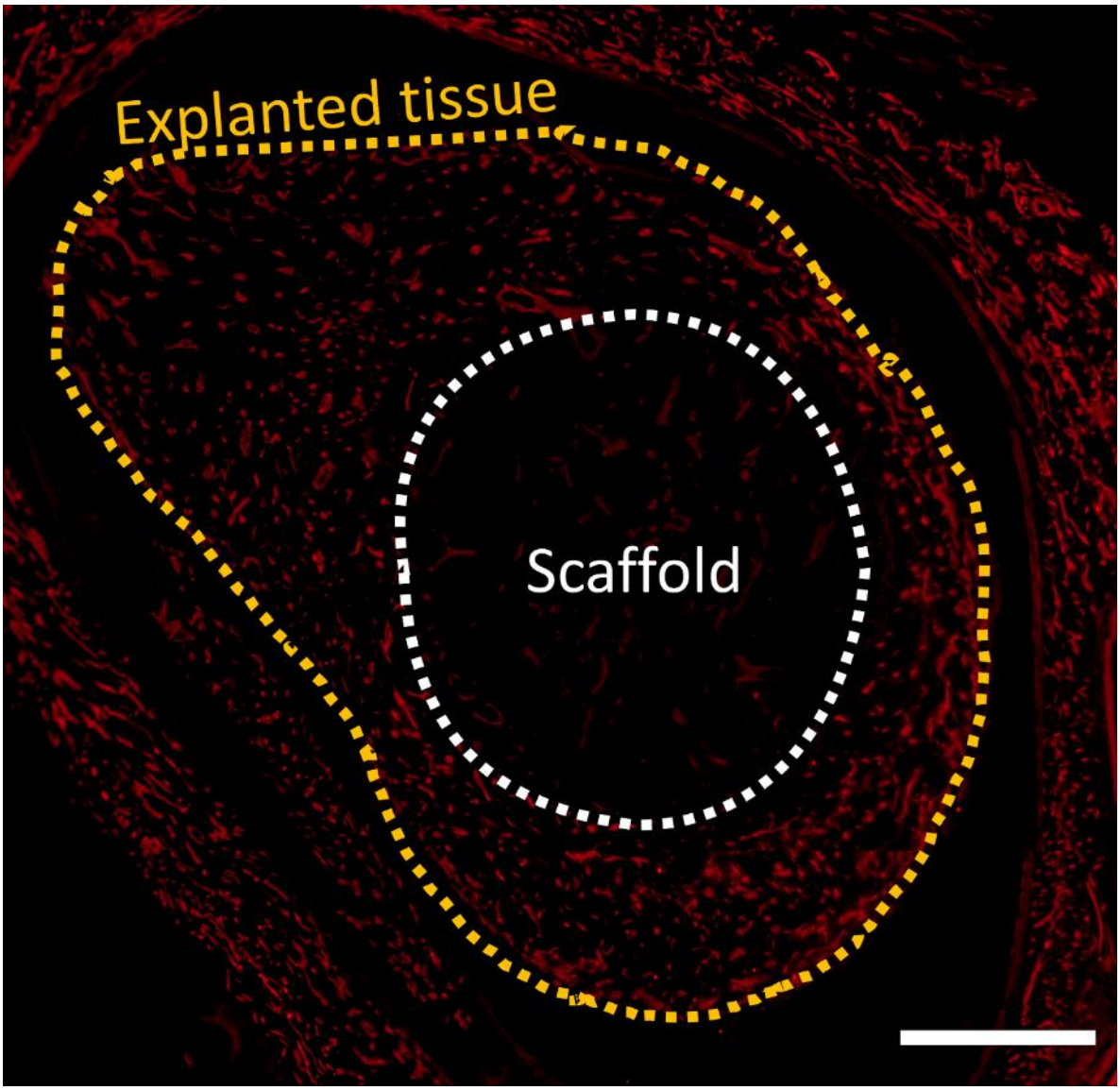


Figure S15 – Explanted tissues present comprehensive vascular coverage of the whole engineered construct volume. Representative image of vessels perfused with lectin (red) covering the surface delimited by the external scaffold boundary (dashed white line) and the 0.2 µm membrane bounding the explanted tissue (yellow dashed line). Scale bar: 1 mm.

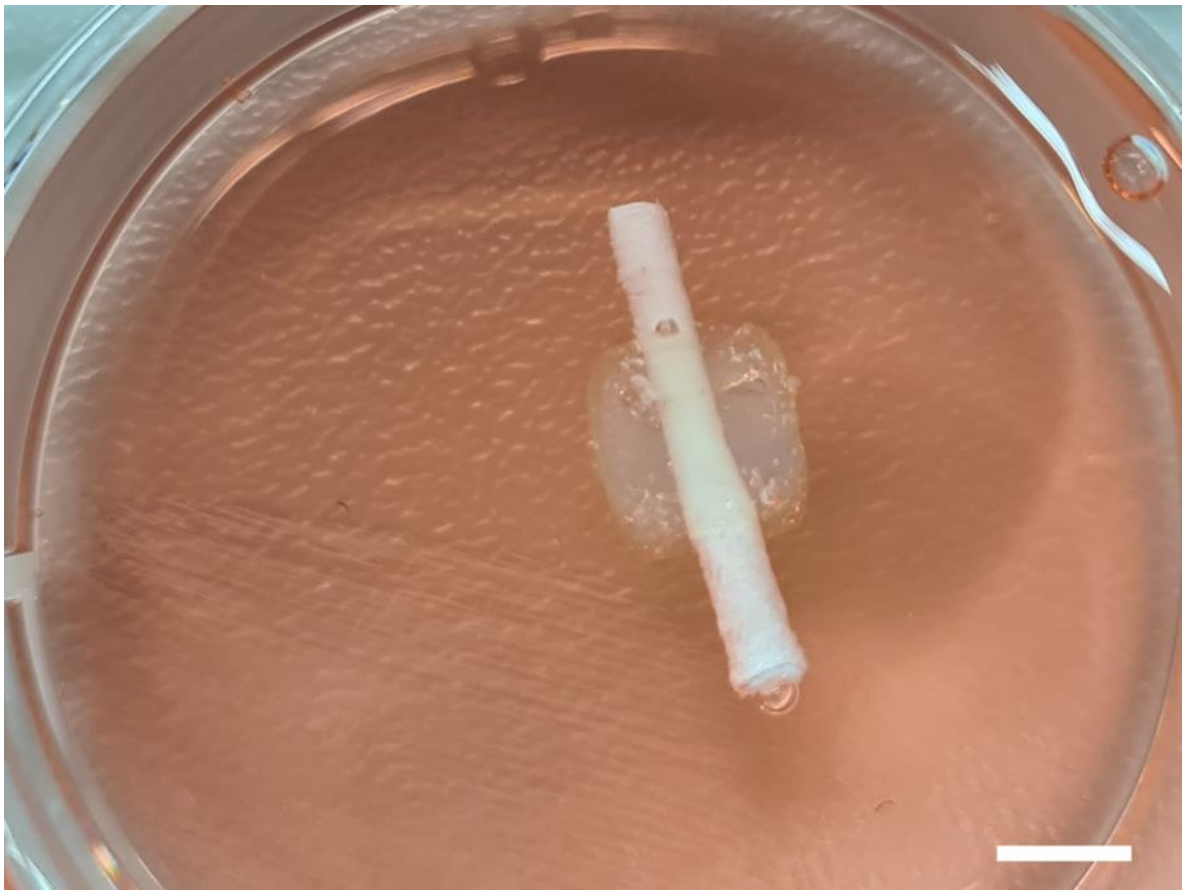


Figure S16 – Assembled vascularized cardiac flap. Photography of the whole vascularized cardiac flap immediately after assembly within a well filled with medium (scale bar: 2 mm).

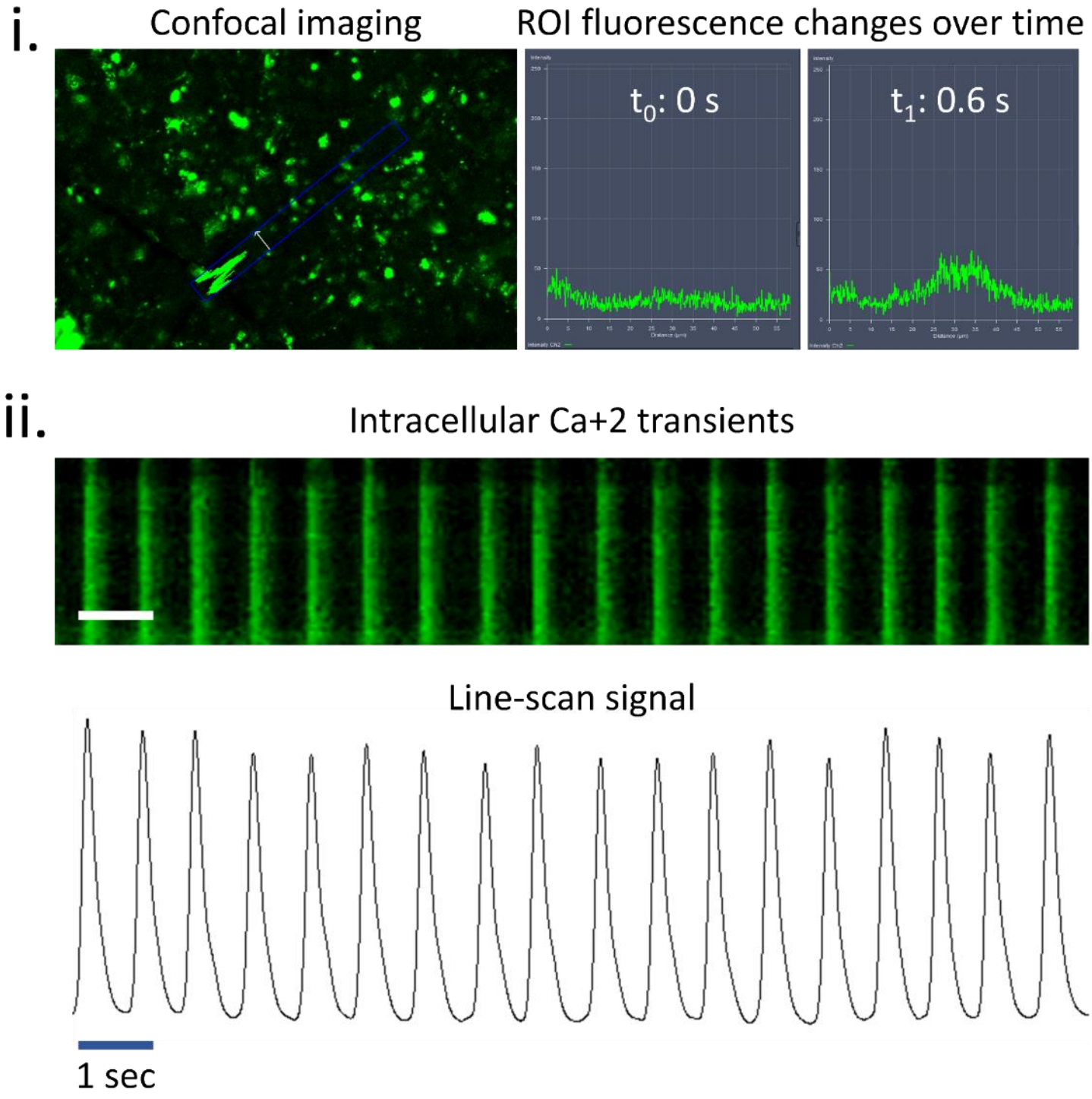
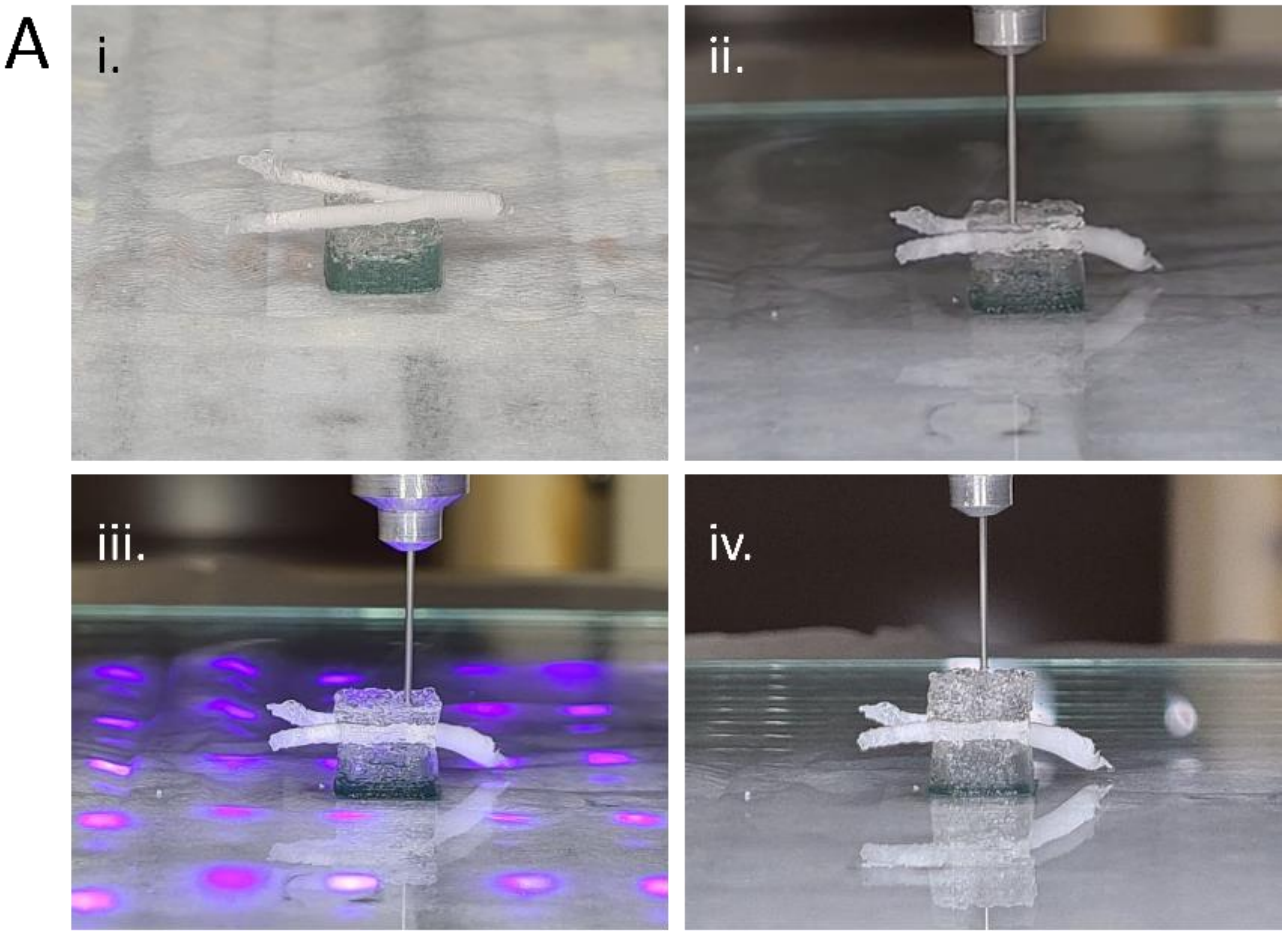


Figure S17 – Cardiomyocyte intracellular Ca^{+2} transient quantification. i) Using confocal imaging, a cardiac cluster is chosen and marked for line-scan imaging, obtaining the fluorescence intensity changes over time at the specific region of interest (ROI). ii) The ROI

transients are imaged for at least 10 seconds, evidencing the iPSC-CMs cardiac cyclic activity. The fluorescent transient images are then translated into line-scan signals, which can be used for quantification and comparing between treatments.



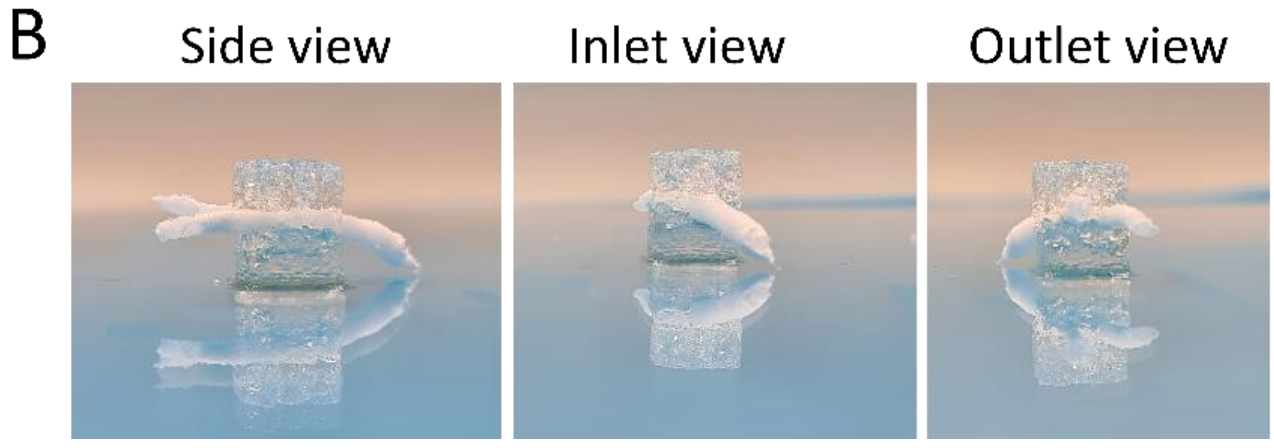


Figure S18 – Printed patient-specific coronary flap using rhCollMA bioink. A) Printing process of the rhCollMA coronary flap: i) After printing the base, the scaffold is allocated

in the printed grooved gel, ii) rhCollMA is printed on top of the VascFold, encasing it within the bioink, iii) During printing, a 405 nm led array located underneath the stage is used to crosslink the printed rhCollMA, and iv) The printing continuous until the desired flap size is achieved. B) Photographs showing different views of the finished rhCollMA patient-specific coronary flap. The experiment was performed using the models used in Figure 7 reduced to 40% of their original size.

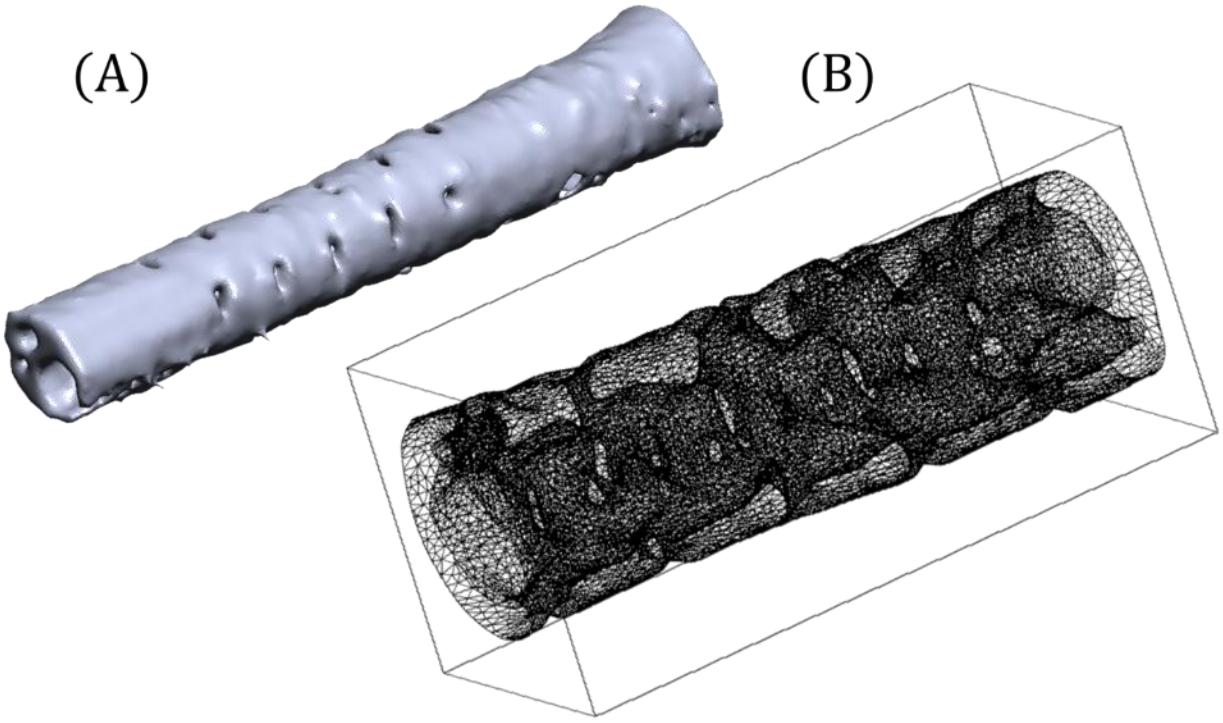


Figure S19 – Computational model of VascFold scan. A) Smoothed and repaired scan of the scaffold B) Final solid model of the fluid volume to be used in the simulations.

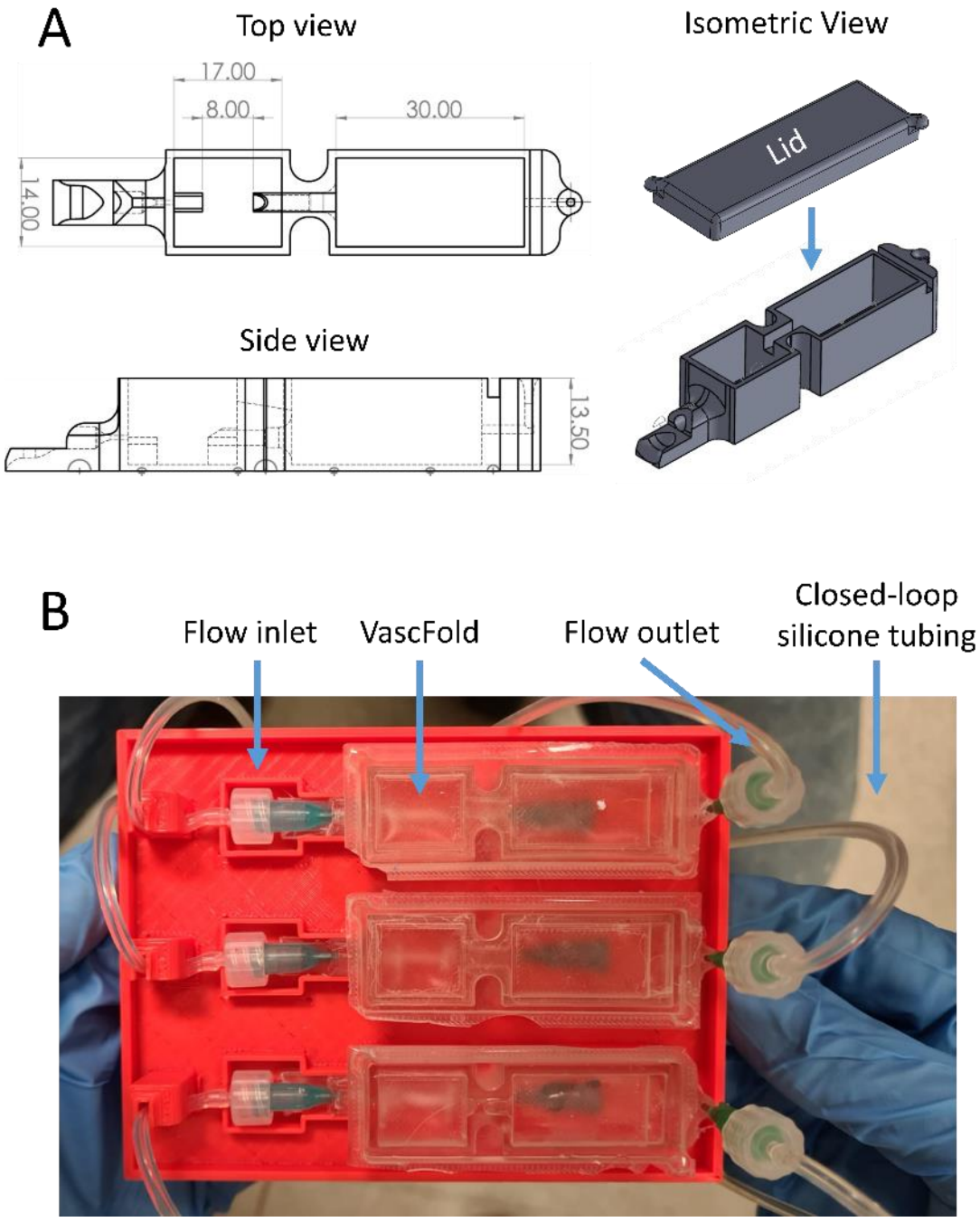


Figure S20 – PDMS bioreactor for perfusion experiment. A) CAD diagram showing the bioreactor design. B) Three assembled bioreactors on a transportation tray (orange). The leftmost needle is inserted into the bioreactor and the endothelialized VascFold in the first chamber. The medium flows through the scaffold and into the second chamber from

which the outlet needle extracts the medium. The outlet and inlet are connected by a silicone tubing connected to a peristaltic pump, creating a closed-loop perfusion system.

Movie S1. Fluorescent-albumin (green) outward diffusion timelapse in scaffolds with and without an engineered endothelium.

Movie S2. Sixteen-hour timelapse of vascular-forming bioprinted hydrogels drastically shrinking around a vessel-like scaffold after assembly.

Movie S3. Contrast real-time imaging of cardiomyocytes spontaneously beating in a large 3D bioprinted rhCollMA construct.

Movie S4. GCaMP-CMs display synchronous Ca^{+2} transients traveling in bioprinted hydrogels.

Movie S5. GCaMP-CMs beat spontaneously in vascularized cardiac tissues.

Movie S6. 3D printing of a patient-specific hierarchical tissue model.