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# Supporting Information

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## Multiphoton-guided Creation of Complex Organ-specific Microvasculature

*Samuel G. Rayner[†], Caitlin C. Howard[†], Christian J. Mandrycky[†], Stefan Stamenkovic, Jonathan Himmelfarb, Andy Y. Shih, Ying Zheng[*]*

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**Microscope system and settings***:* A 2.5 W titanium-sapphire laser (Mai Tai HP, Newport) attached to a commercially available microscope (FV1000, Olympus) and focused through a 25x 1.05 NA objective (XLPlan N, Olympus) was used for all multiphoton ablation experiments. Microscope intensity was measured at a wavelength of 800 nm on two separate occasions using a thermal power meter (Coherent). The maximum power exiting the objective was determined to be 244 mW at full laser intensity. A dwell time of 2 µs was used for all ablation experiments.

**Ablation process:** The workflow described below involves four discrete steps: model generation, model slicing, model conversion to microscopy format, and two-photon ablation. First, models were either generated in CAD software (Fusion 360, Autodesk) or derived from two-photon microscopic imaging of the mouse cerebral cortex. These files were converted to a 3D mesh format (.stl) and imported into a commercially available slicing software (Netfabb, Autodesk). Whole 3D models were sliced into a series of 1-µm-thick layers and converted into vector graphics files (.svg) which contain the bounding points of the model geometry. A custom script was written using R software[1] to then convert the vector graphics files into a format accepted by the Fluoview software (Olympus) used to drive an Olympus FV1000 Multi-photon microscope. The built-in multi-area time lapse (MATL) feature was used as the basis for bringing custom data into the microscope environment. In normal use the MATL function enables users to program acquisition parameters including laser settings, position settings, regions of interest, and other imaging information from within the Fluoview workspace. This information is stored in a Microsoft Access Database and is readily modified programmatically. Using R, the XY coordinates defining polygonal regions of interest were

imported from vector graphics files into the Access database along with ablation parameters including slice thickness, laser power, and pixel dwell time. Through this workflow approximately 3000 custom polygons can be automatically programmed for ablation in a single run on the microscope. For complex structures that require more polygons, the ablation can be split among multiple files and then run in sequence to produce the final structure. Once created, these customized files can be opened within the Fluoview software and run as during traditional image acquisition.

**Microfabrication of materials**: To create stamps for the lithographic patterning of biologic hydrogels described below, geometries were designed using CAD software (Layout Editor, juspertor GmbH) and patterned silicon wafers created within the Washington Nanofabrication Facility through two separate processes. The “parallel channel” geometry (Figure 1a and d, Figure 2 a,b, and d) consists of two parallel channels with diameters of 200 µm, spaced 450 µm apart with independent inlets and outlets. This was fabricated as previously described.[2] Briefly, a negative chrome photomask was created from the CAD file using a Heidelberg DWL 66+ laser writer, and developed in AZ340 (EMD) followed by chromium etchant. A silicon wafer was spin-coated with SU-8 photoresist (Microchem) at a height of 200 µm and soft-baked. The photomask was placed above the wafer, and the wafer exposed to UV light ($\lambda$ = 365 nm). The wafer was then post-baked and developed with SU-8 developer (Microchem). The “color wheel” geometry consists of six “spoke” channels extending from a central circular lumen, interrupted by six 100 µm-long discontinuities (Figure 2c; Figure S6). The “spokes” are 300 µm wide, and the central circular lumen 200 µm wide. The silicon master mold for this geometry was created using deep reactive ion etching. AZ9260 positive photoresist (Microchem) was spun onto the surface of a silicon wafer to a depth of 6 µm. The color wheel design was then directly written into the photoresist using a Heidelberg DWL 66+ laser writer. Resist was developed using AZ400K developer (EMD) and exposed areas etched to a depth of 130 µm using a SPTS Rapier. Resist was then stripped using photoresist remover (EKC) followed by cleaning within a plasma chamber (AutoGlow). For

both geometries, wafers were subsequently silanized using trichloro(3,3,3-trifluoropropyl)silane (Sigma #MKBG3805V). Polydimethylsiloxane (PDMS, Dow Corning #0007902190) was mixed with crosslinker at a 10:1 ratio, poured over the wafer, and cured overnight at 65°C. PDMS was then peeled from the wafers, creating stamps with raised features in the desired geometries.

**Biomaterial preparation and ablation testing**: Three different biomaterials were tested in this study: Type I collagen, fibrin, and a mixture of agarose and gelatin. Type I collagen was extracted from rat tails, suspended in acetic acid (0.1%) at 15 mg $mL^{-1}$, and diluted to 7.5 mg/ml at physiologic pH in buffer just prior to use, as previously described.[3–5] Collagen was injected into the platform being used and allowed to gel at 37°C for thirty minutes. A decellularized kidney extracellular matrix (kECM) and collagen blend was created as previously described.[6] Here, we used a blend containing 1 mg/ml kECM and 6 mg/ml collagen. To make fibrin gel, lyophilized fibrinogen (Sigma #F3879) was suspended as a stock solution (15 mg/ml) in phosphate buffered saline (PBS), and added to a suspension of thrombin (Sigma #T7009) diluted in a buffered salt solution (endothelial basal media, Lonza #CC-3121), to a final fibrinogen concentration of 10 mg/ml and thrombin concentration of 1 U/ml. Immediately following the addition of thrombin, the mixture was injected into the desired device and placed in an incubator at 37°C for thirty minutes. In the third biomaterial condition, Agarose and Gelatin (Sigma) were separately dissolved via gentle heating into 4% solutions in PBS and combined in equal volumes just prior to use. This mixture was allowed to gel at 4°C for thirty minutes prior to ablation.

To test the ablation of different biomaterials (Figure 1a), a 1 mm-tall rectangular well was placed on top of a glass slide (Corning) and filled with biomaterial. A PDMS stamp for the parallel channel geometry was immediately placed on top and removed following gelation. Rectangular trenches were ablated across the bottoms of the two channels at different laser powers and channels perfused with fluorescent beads via gravity. To test the resolution

achievable with the above settings, lines of decreasing width were ablated into collagen and imaged using collagen second-harmonic generation (Figure S1). At a resolution of 2048 x 2048 pixels per field of view, 1.0 µm linewidth corresponded with a width of ~4 voxels, suggesting the theoretical limit of ablation at these settings is well under 1 µm.

**Acellular device fabrication and bead perfusion:** Ablation of the model alveolus (Figure 1c), helical structure (Figure S2), "basket-weave" structure (Figure S3) and a non-vascularized model glomerulus for bead perfusion (Figure 2a; Movie S1) was performed entirely via multiphoton ablation between an inlet and outlet created in 7.5 mg/ml collagen suspended within a plastic housing. To accomplish this, a 5 mm deep well was created in a poly(methyl methacrylate) housing using a computer numerical control mill (TAIG Micro Mill). Four 1.8 mm diameter holes were drilled through the top of the device and into the cavity, with holes spaced a minimum distance of 1.8 mm from one another. The poly(methyl methacrylate) shell was bonded to a glass coverslip using PDMS, metal dowel pins (diameter = 1.5mm, McMaster-Carr) placed through the 4 holes, and 7.5 mg/ml collagen injected through a separate injection port and allowed to gel. Pins were removed, forming four wells within the collagen to serve as inlets/outlets for later perfusion. Desired geometries were created equidistant from these inlets/outlets, and connections then ablated from the inlets/outlets to these ablated structures. In the case of the alveolus and glomerulus, two sets of inlets and outlets were fabricated and used, with one set for perfusion into the alveolar or Bowman's space, respectively, and the other for perfusion through the vascular compartment. Inlets were filled with fluorescent beads with a diameter of 100 nm (FluoSpheres, ThermoFisher #F8797), perfused via gravity (~ 1 cm$H_2O$ pressure drop), rinsed with PBS, and imaging done with the above multiphoton microscope for beads remaining on the wall and outlining the structures of interest.

**Fabrication of the microphysiological system:** To create cellularized 3D structures, as well as the mouse brain microvascular unit, a previously described lithography-based

injection molding technique was used to serve as the starting point for ablation. Briefly, collagen was prepared as above and injected into a PEI/glutaraldehyde-treated poly(methyl methacrylate) housing to form a negative impression of the above “parallel channel” geometry, as imprinted by a PDMS stamp. Stainless steel dowel pins were placed into inlet and outlet holes prior to collagen injection to maintain open ports. Following gelation at 37˚C, the PDMS stamp and dowel pins were removed creating a top housing piece with channels imprinted into collagen. Separately, a bottom housing piece was created by using a flat piece of PDMS to compression mold collagen over a coverslip fixed within a poly(methyl methacrylate) holder. After gelation, the PDMS was removed, revealing a flat collagen bottom. The two plastic housings were then placed together to seal the imprinted channels in the top piece with the flat collagen bottom piece. For acellular studies, PBS was added into the inlet reservoirs. For cellular studies, the appropriate media was added into the inlet reservoirs. For the ablation experiments described below, the microphysiological system was directly mounted under the two-photon microscope and ablation performed through the coverslip on the bottom of the microphysiological system. For upright microscopes, as here, the microphysiological system can be mounted in inverted orientation to allow ablation through the bottom coverslip.

**Staining and imaging of native human tissue:** Human lung and kidney tissue was imaged for purposes of scale/comparison (Figure 1c and 2a). Human kidneys were obtained from a local biorepository (LifeCenter Northwest), and renal cortex samples were cryoembedded and sectioned into 8 µm thick slices using a microtome. Sections were stained with FITC-conjugated sheep anti-von Willebrand Factor (1:100, ab8822, Abcam), Rabbit anti CD-31 (1:30, ab28364, Abcam) and Hoechst (for nuclear staining). Immunofluorescence-labeled kidney sections were then imaged on a Nikon Eclipse Ti2 widefield microscope, and images of glomeruli obtained. Human lung samples were obtained from rejected donor lungs by the National Disease Research Interchange (NDRI) and shipped immediately on ice to the University of Washington. Peripheral lung samples were then fixed in 4% paraformaldehyde

and cut into ~1 mm thick sections. Sections were placed into an optical clearing agent (Rapiclear, Sunjin Lab) and Z-stack images of tissue autofluorescence taken using the above multiphoton microscope, to evaluate the structure of alveoli and surrounding tissue.

**In vivo mouse imaging and vessel tracing:** Chronic cranial window surgery was performed on a 4-month-old C57Bl6 mouse. During surgery, body temperature was maintained at 37˚C with a feedback-regulated heat pad (FHC Inc.). The animal was administered buprenorphine for analgesia prior to surgery at a concentration of 0.1 mg/kg (Patterson Veterinary). After removal of the scalp, an aluminum flange was cemented onto the contralateral hemisphere of the dorsal skull surface to enable head fixation during imaging. A chronic cranial imaging window was generated over the sensorimotor cortex using methods described previously, with slight modifications.[7] Briefly, surgical anesthesia was induced with a cocktail consisting of fentanyl citrate (0.05 mg/kg), midazolam (5 mg/kg) and dexmedetomidine hydrochloride (0.5 mg/kg) (Patterson Veterinary). A dura-intact craniotomy measuring ~4 mm in diameter was created and sealed with a specially-made glass coverslip consisting of a round 4 mm glass coverslip (Warner Instruments #64-0724) glued to a round 5 mm coverslip (Warner Instruments #64-0731) with UV-cured optical glue (Edmund optics #37-322). The coverslip was positioned with the 4 mm side placed directly over the craniotomy, while the 5 mm coverslip laid on the skull surface at the edges of the craniotomy. An instant adhesive (Loctite Instant Adhesive 401) was carefully dispensed along the edge of the 5 mm coverslip to secure it to the skull, taking care not to allow any spill-over onto the brain. Lastly, the area around the cranial window was sealed with dental cement. These procedures were approved by the Institutional Animal Care and Use Committee at the Seattle Children's Research Institute.

In vivo mouse cerebral vascular imaging was conducted using a Bruker Investigator two-photon microscope (Bruker) and an InSight X3 laser source (SpectraPhysics). For imaging, the mouse was kept under light isoflurane (1–1.5%), which was provided through medical air

(20–22% oxygen and 78% nitrogen, moisturized by bubbling through water; AirGas Inc.) during the imaging period.[8] Body temperature was maintained at 37˚C with a feedback-regulated heat pad (FHC Inc.), and blood serum was labeled by retro-orbital vein injection of 0.5 mL of 2 MDa Alexa 680-dextran conjugated “in-house” and prepared at a concentration of 5% (w/v) in sterile saline.[9] High-resolution two-photon imaging of the microvasculature labeled with Alexa 680-dextran was then performed at 1210 nm excitation using a 25-X, 1.05 NA water-immersion objective lens (XLPlan N, Olympus). Image stacks were collected across a 483x483 µm field, starting 50 µm below the pia mater and ending at a cortical depth of 700 µm. During imaging laser power ranged between 20 and 100 mW exiting the microscope objective, with higher powers required for greater cortical depth. Lateral sampling (x, y) was 0.943 µm/pixel, and axial sampling (z) was 1 µm/pixel. Following imaging, vessels were segmented using the Filament Tracer module of Imaris software version 7.7.2 (Bitplane, Oxford Instruments). Vessel segmentation was performed manually using the Auto Depth tracing option. First a penetrating arteriole and an ascending venule in the immediate vicinity were traced, followed by tracing of several different capillary vascular paths connecting the two.

**Fabrication of a perfusable mouse brain microvascular unit**: The above in vivo imaging data was next used to generate a microvascular structure in collagen fully recapitulating native mouse brain microvascular architecture. First, a cuboidal section of mouse imaging data with dimensions ~200 x 300 x 400 µm was isolated using Fiji’s 3D plugin (ImageJ)[10] and exported as a rough surface model to be sliced and used for ablation, as above. Following ablation into collagen, the created structure was visualized via second-harmonic generation imaging using the above FV1000 multiphoton microscope (Figure S4). As a next step, to create a perfusable mouse brain microvascular unit derived from in vivo imaging, we used the above microphysiological system with lithographically determined parallel channel geometry as the starting point for ablation. A single artery, single venule, and several connecting capillaries were traced as above. Capillaries traced using the filament tool in

Imaris software, were represented as 7.5 μm diameter cylinders for the purposes of ablation. These was exported as a mask using ImageJ and ablated into collagen, as above. After ablating the mouse brain microvascular unit between these two channels, connections were ablated to the two channels, forming a system that enabled facile syringe-based perfusion of the vessels with fluorescent beads.

**Cell culture:** For cellularized constructs the following cells and culture conditions were used. Human umbilical vein endothelial cells (HUVECs) were commercially obtained (Lonza #CC-2519), cultured in endothelial growth media (EGM, Lonza) and used in devices from passages 4-6. GFP-HUVECs were created by transducing cells with a GFP-labeled lentiviral vector (PGIPZ nonsilencing shRNA, Dharmacon). Smooth muscle cells containing an mCherry-expressing construct (mCherry-SMCs) were produced by transducing human coronary artery smooth muscle cells (CASMCs; Lonza #CC-2583) with a CMV-mCherry lentivirus produced in-house. mCherry-SMCs were expanded for 1-2 passages in smooth muscle cell growth media 2 (SmGM, Lonza #CC-3181 and #CC-4149) following transduction, prior to being used for experiments. A human stromal cell line (HS27a-mCherry) was generously provided by collaborators in the Torok-Storb lab.[11] These immortalized human marrow stromal cells were cultured in RPMI 1640 (Thermo Fisher #11875093) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco #10082147), 0.4 mg/ml L-glutamine (Sigma-Aldrich # 59202C), 1 mM/L sodium pyruvate, and 100 μg/ml penicillin-streptomycin (Thermo Fisher #15140122).

**Creation, perfusion, and staining of a cellularized model glomerulus**: Microphysiological systems were created incorporating two parallel microchannels with a diameter of 200 μm, as above. HUVEC cells were seeded into the microchannels, with an average volume of 10 μL of cell suspension (density of $10^7$ cells/mL) used. Devices were cultured under gravity-driven flow for 48 hours as previously described.[2,4] After 48 hours, the model glomerulus was ablated between the two microchannels and connections formed from the afferent and

efferent limb of the glomerulus to one of the microchannels. The device was then further cultured under gravity, with a pressure drop maintained at ~1 mmHg across the glomerulus region. Cells were photographed daily as they grew from the microchannels into the ablated glomerular structure, using a Nikon Eclipse Ti2 widefield microscope (Figure S5). After culture for another week, vessels were fixed in paraformaldehyde (4% w/v) for twenty minutes followed by incubation in blocking buffer (2% bovine serum albumin and 0.1% Triton X-100) for one hour. Vessels were stained overnight at 4°C in primary antibody (1:100, rabbit anti-VE-Cadherin, ab33168, Abcam) followed by one hour of staining at room temperature in secondary antibody (1:100 goat anti-rabbit Alexa Fluor 488, A11008; ThermoFisher), Hoechst, and phalloidin Alexa Fluor 568 (1:100, A12380, ThermoFisher). Vessels were then imaged with the above multiphoton microscope using 1 µm Z-slices.

To test blood perfusion in glomerular capillaries, blood was drawn from healthy donors into 3.8% sodium citrate tubes and centrifuged at 150g for 15 minutes at room temperature. The red blood cell layer was isolated and diluted 1:100 into PBS. Diluted red blood cells were then perfused through a paraformaldehyde-fixed microphysiological system and imaged in real time using a Nikon Ti2 widefield microscope (Figure 2b; Movie S2). For dextran perfusion, 500 kDa FITC-dextran (Sigma #FD500S) was diluted to a concentration of 1 µM in EGM, perfused via gravity (1 cm H20 pressure) into the microphysiological system, and imaged in real time using a Nikon Ti Microscope attached to a Yokogawa W1 spinning disk system. A full Z-stack (10 µm optical sections) was obtained every 15 seconds. Within a single optical section, mean fluorescent intensity over time was averaged across three 20 µm x 50 µm rectangular sections 20 µm away from vessels of interest for both parent channels and a limb of the ablated glomerulus structure, and displayed in Graphpad Prism (Graphpad).

**Creation of a continuous vessel with multiple cellular subpopulations:** The housing plates of the above microphysiological system were modified to incorporate six inlets/outlets for perfusion (rather than 4, as above) to generate vessels with “color wheel” geometry

imprinted into the collagen. Cultured HUVEC cells were passaged and divided into six populations, and labeled with one of six possible combinations of membrane dyes (Figure S7). Labeling was done according to the manufacturer's directions (Sigma Aldrich) and as previously described.[12] Briefly, cells were separated into 6 aliquots of 2 million cells each, processed in manufacturer-supplied buffer, and then added to the same volume of a 2 x $10^{-6}$ M solution of dye, either green (PKH67, Sigma #MINI67), Red (PKH26, Sigma #MINI26), Claret ("Cellvue Claret," Sigma #MINCLARET) or a combination thereof as noted (Figure S7). Cells were allowed to label for 3 minutes at room temperature, washed once with fetal bovine serum and twice with EGM, and then transferred to fresh tubes. The six cellular populations were then seeded into the six separate inlets, respectively, with $10^5$ cells injected per inlet, at a concentration of $10^7$ cells/mL. Cells were cultured for 24 hours, after which all of the 100 µm discontinuities except one were removed via photoablation, leading to a completed circular vessel with one inlet and one outlet. This was then cultured under gravity-driven flow for 48 hours at which time it was fixed and imaged using a Nikon Ti Microscope attached to a Yokogawa W1 spinning disk system.

**Fabrication of hierarchical vessels**: To form a hierarchical alveolar-capillary-venule unit, a microphysiological system incorporating parallel channel geometry was fabricated. Fibronectin (50 µg/ml, Sigma #F0895) dissolved in SmGM was perfused through the parallel channels for one hour to aid in cellular adhesion. Next, parallel channels were seeded with 10 µl of mCherry-SMCs at a concentration of $10^7$/mL. Devices were cultured under intermittent gravity-driven perfusion of SmGM for 48 hours, after which time a serum-free media (1:1 DMEM/F12 Ham, ThermoFisher #11320033) was used instead, to encourage quiescence.[13] After 24 more hours of culture, media was changed to a DMEM/F12 Ham mixture containing 50 µg/ml fibronectin, which was perfused through both channels for one hour. Next, a microvascular "grid" was ablated between the two parallel channels, containing lumens of 100 µm in diameter (Figure 2d). Immediately after ablation, 10 µl of a $10^7$ concentration of GFP-HUVECs were introduced into both inlets on the left side of the vessel,

seeding endothelial cells through the empty grid and on top of the mCherry-SMCs in both parallel channels. The devices were cultured for 72 hours in a 1:1 mixture of endothelial growth media and smooth muscle cell growth medium, fixed in 4% paraformaldehyde, and imaged using a Nikon Ti Microscope attached to a Yokogawa W1 spinning disk system.

**Vascularization of pre-cellularized hydrogels**: To determine the viability and behavior of cells embedded in bulk hydrogels following ablation and vascularization, a microphysiological system incorporating 200 µm diameter parallel channel geometry was fabricated using a 7.5 mg/ml collagen hydrogel with $5*10^5$ cells/mL HS-27A-mCherry cells incorporated into the bulk matrix of the gel. Channels were filled with EGM (200 µL/well) prior to ablation to prevent the hydrogel from drying out. A 60 µm diameter spiral structure was then ablated between the larger parallel channels. To create a pressure differential across the spiral structure to allow for seeding of endothelial cells, 100 µL of EGM was removed from the inlet and outlet of the bottom parallel channel. The devices were then seeded with HUVEC cells by adding 10 µL of a $7*10^6$ cells/mL cell suspension to the inlet of the top channel, causing cells to seed along the entire top channel, across the spiral region, and into the outlet of the bottom channel. Devices were left to allow cell attachment at 37°C for 30 minutes, then the bottom channel was seeded by adding 10 µL of the cell suspension to the inlet of the bottom channel. Devices were cultured for 72 hours in EGM, fixed using 4% paraformaldehyde, and imaged using the above multiphoton microscope.

**Ablation and cell seeding within human-derived extracellular matrices**: To evaluate the compatibility of ablation with complex human-derived matrices, a microphysiological system incorporating 200 µm diameter parallel channel geometry was fabricated using a blend of 15 mg/mL rat tail-derived type I collagen and 6.5 mg/mL human kidney decellularized ECM (kECM), derived in house as previously described.[6] The blend was diluted to 6 mg/mL type I collagen and 1 mg/mL kECM at physiological pH in buffer immediately prior to use and channels were seeded with HUVEC cells by adding 10 µL of a $7*10^6$ cells/mL cell suspension

to the inlets of each channel**.** Devices were cultured for 48 hours in EGM to ensure confluency before ablation. A 50 µm diameter grid was ablated between the two larger channels and the devices were cultured for 72 hours in EGM, fixed using 4% paraformaldehyde, and imaged using the above multiphoton microscope.

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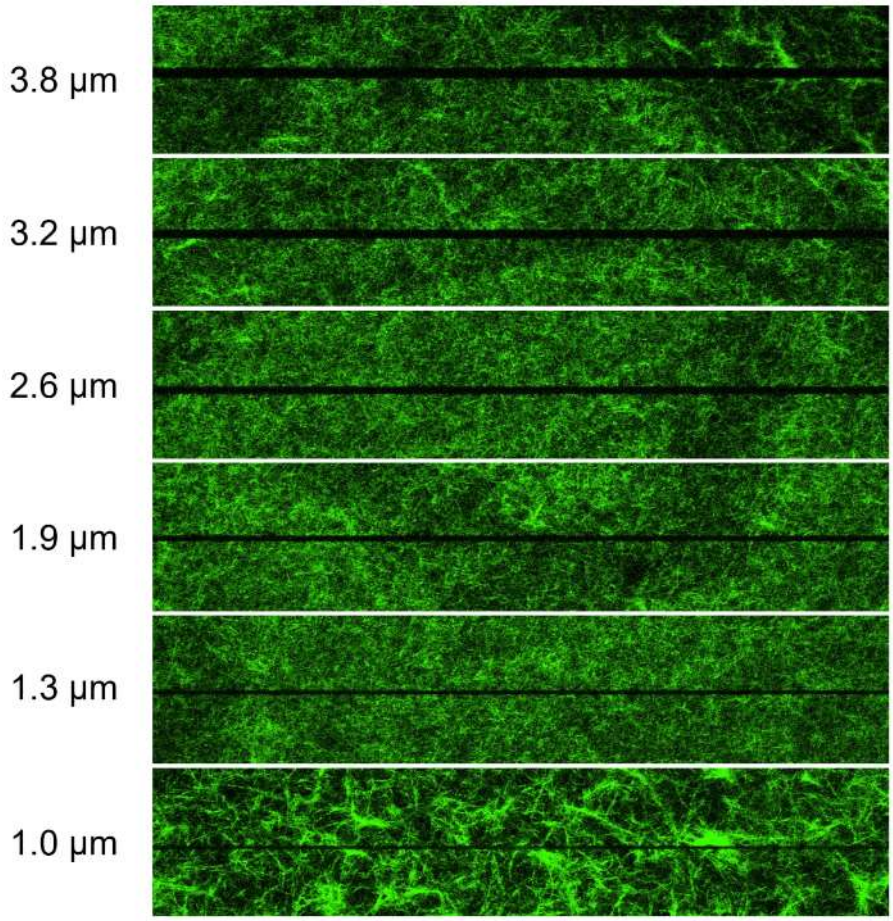


**Figure S1: Ablation resolution testing.** Channels with width ranging from 1.0 µm to 3.8 µm were ablated into collagen using a two-photon microscope, with settings as described in the main text, and visualized using second-harmonic generation imaging. The measured width of ablated channels corresponded precisely with the intended widths shown to the left of the figure.

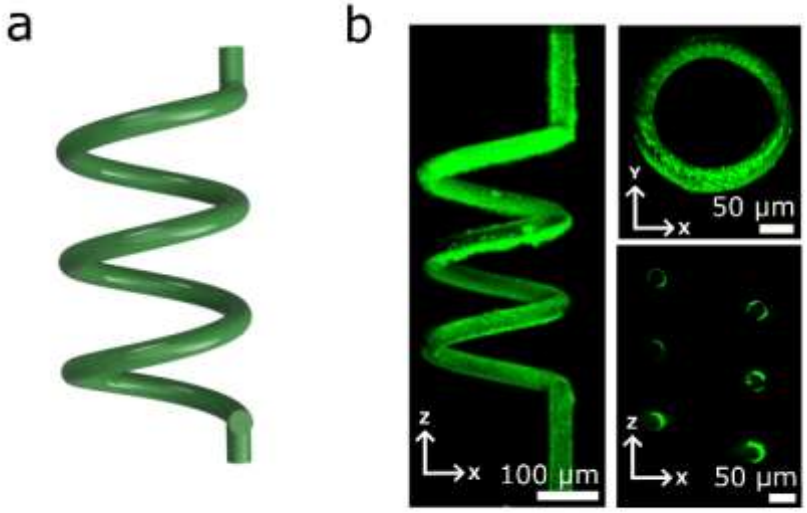


**Figure S2: Design and ablation of a perfusable helical structure. a,** Model CAD structure. **b,** Fluorescent bead perfusion of an ablated helical structure. XY projection (right, top) shows smooth circular curvature and an XZ optical section (right, bottom) demonstrates open, circular lumens.

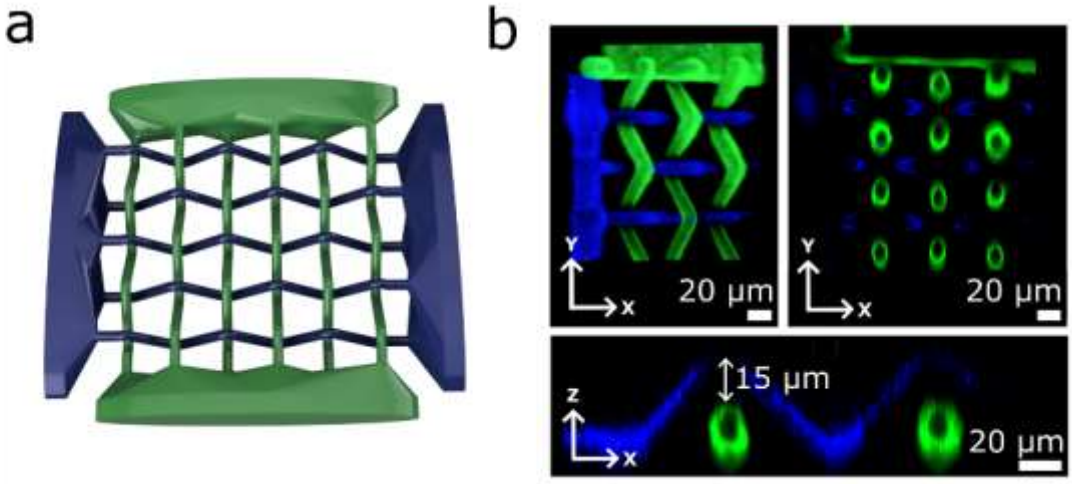


**Figure S3: Design and ablation of an interwoven meshwork. a,** Model CAD structure with blue and green colors each representing a distinct and separately perfusable channel. **b,** Bead perfusion of an ablated meshwork. Channels were separately perfused with fluorescent beads of two different colors, demonstrating that channels remain intact and individually perfusable despite being interwoven and closely apposed. XY optical sections (top, right) show round, open lumens. XZ sections (bottom) highlight achievement of the intended 15 µm separation between the interwoven lumens.

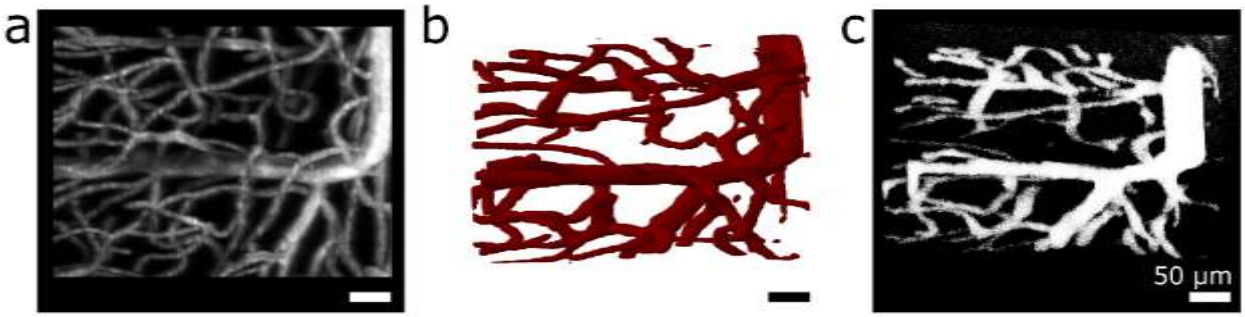


**Figure S4: Ablation of mouse cerebral cortical microvasculature. a,** Projection image obtained via two-photon microscopy of mouse cerebral cortical microvasculature. **b,** 3D surface model created from in vivo microvascular imaging. **c**, Image of mouse cerebral cortical microvascular model ablated into collagen. Obtained image is shown here as a 3D projection with inverted lookup tables (luminal spaces displayed in white) showing replication of the modeled complex microvascular geometries.

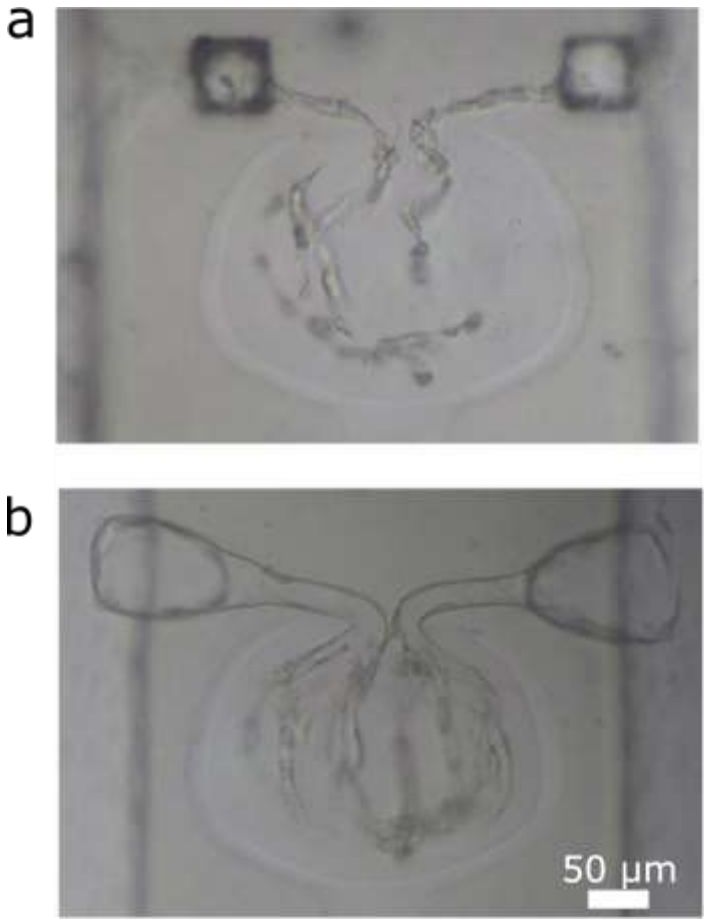


**Figure S5: Endothelialization of a model glomerulus via laser-guided angiogenesis.** Brightfield image of HUVEC ingrowth into an ablated glomerular structure at two days (a) and seven days (b) post-ablation. Cells had begun migrating into the channels by day 2 and had fully grown into the ablated spaces and formed confluent, patent lumens by day 7.

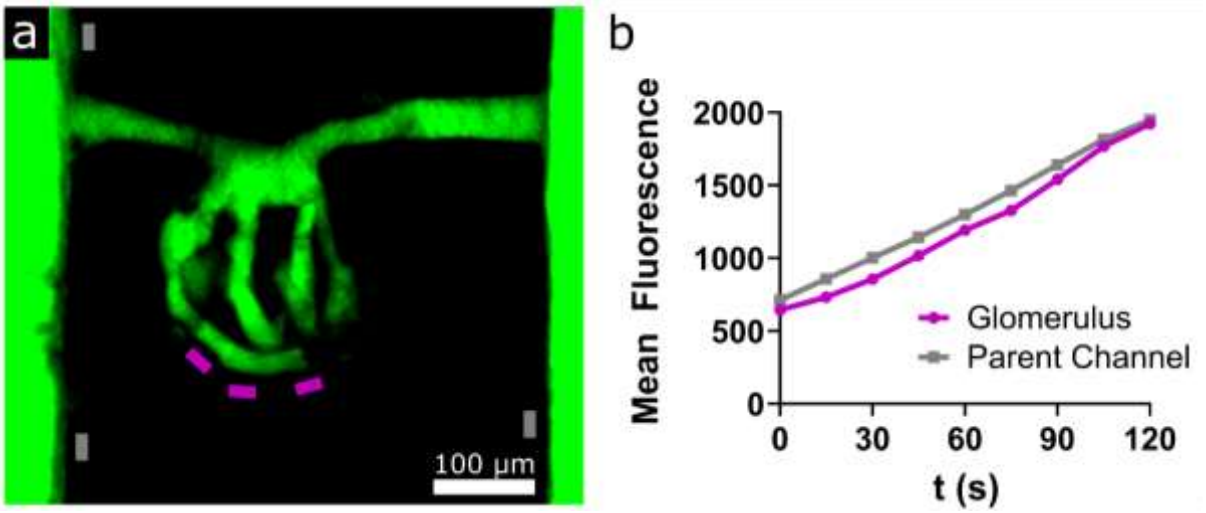


**Figure S6: Dextran perfusion of an endothelialized model glomerulus**. **a**, Z-stack image of a model glomerulus cultured for ten days following ablation and perfused with 500 kDa dextran during live imaging with a spinning disk microscope. Image was taken 30 seconds into perfusion. Mean fluorescence over time for the average of three regions of interest each next to either the parent channel (grey) or a limb of the ablated glomerulus (magenta) were calculated for a single z-slice. **b**, Graph of mean fluorescence over time for the average of chosen regions of interest shows similar change in mean fluorescence outside of the parent channel and ablated glomerulus.

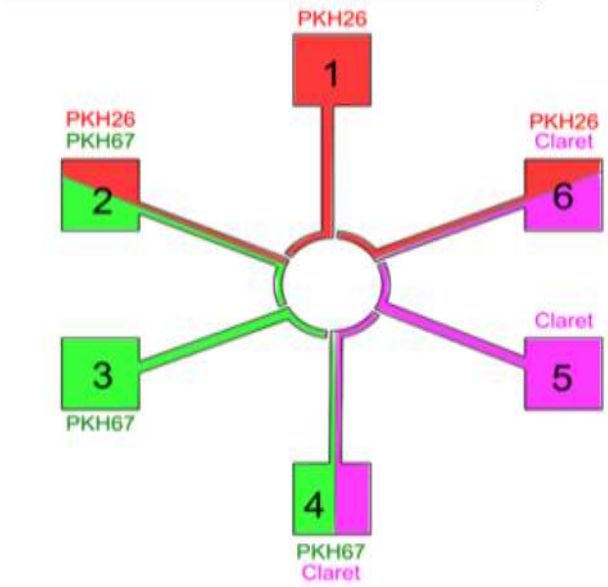


**Figure S7: Cell-labeling protocol for creation of a continuous heterogeneous vessel.** Schematic for the labeling of the “color wheel” microvascular structure. Cells were labeled with one or two membrane dyes to create 6 color combinations as shown. Each of the six compartments was seeded with a cell population pre-labeled with one of these 6 color combinations. Populations initially grew individually, without connection to each other, but were later united when discontinuities were removed via ablation, as described in the main text.

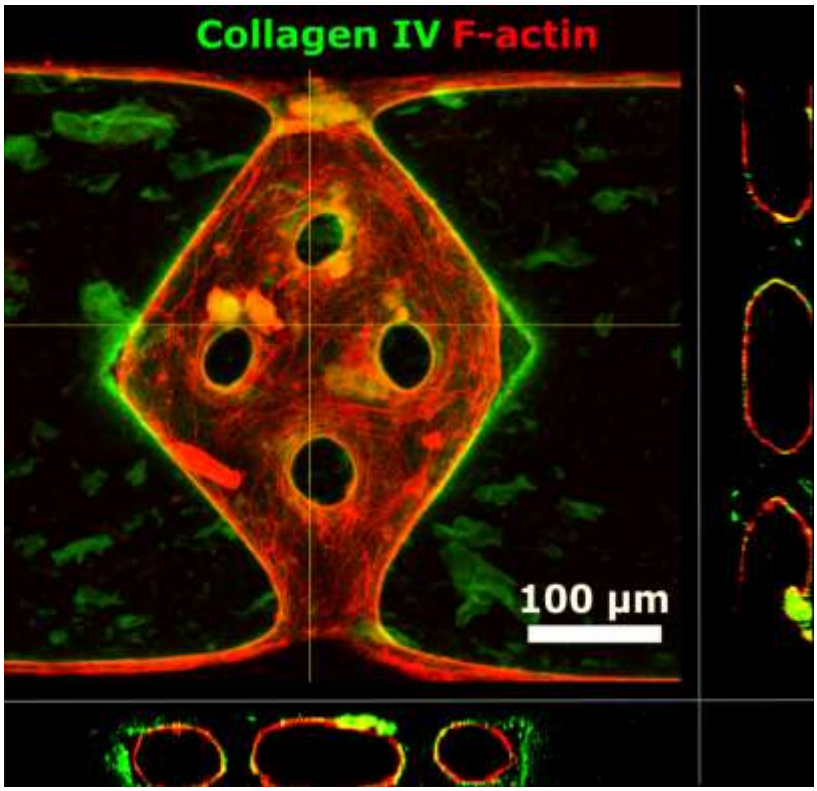


**Figure S8: Ablation and laser-guided angiogenesis within decellularized kidney extracellular matrix (kECM).** Projection image of a capillary "grid" formed through laser-guided angiogenesis performed on a parent channel lined with HUVECs cast within a blend of type 1 collagen and kECM. Image obtained by multiphoton imaging following immunostaining shows both cellular structures (F-actin) and presence of kECM material (Collagen IV). Note that initially square supports within the capillary "grid" have been rounded through cellular remodeling of kECM/collagen mixture over 72 hours of culture. Cross-sectional views through the XY plane (bottom) and YZ plane (right) are shown along the planes in yellow.

**Movie S1**: Three-dimensional rotation of a cellularized model glomerulus. Red, F-actin; Blue, nuclei

**Movie S2**: RBC perfusion of engineered glomerular capillaries