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Microfabrication of AngioChip, a biodegradable polymer scaffold with microfluidic vasculature

Boyang Zhang[1,6], Benjamin Fook Lun Lai[1,6], Ruoxiao Xie[2], Locke Davenport Huyer[1,3], Miles Montgomery[1,3] and Milica Radisic[1,3,4,5*]

Microengineered biomimetic systems for organ-on-a-chip or tissue engineering purposes often fail as a result of an inability to recapitulate the in vivo environment, specifically the presence of a well-defined vascular system. To address this limitation, we developed an alternative method to cultivate three-dimensional (3D) tissues by incorporating a microfabricated scaffold, termed AngioChip, with a built-in perfusable vascular network. Here, we provide a detailed protocol for fabricating the AngioChip scaffold, populating it with endothelial cells and parenchymal tissues, and applying it in organ-on-a-chip drug testing in vitro and surgical vascular anastomosis in vivo. The fabrication of the AngioChip scaffold is achieved by a 3D stamping technique, in which an intricate microchannel network can be embedded within a 3D scaffold. To develop a vascularized tissue, endothelial cells are cultured in the lumen of the AngioChip network, and parenchymal cells are encapsulated in hydrogels that are amenable to remodeling around the vascular network to form functional tissues. Together, these steps yield a functional, vascularized network in vitro over a 14-d period. Finally, we demonstrate the functionality of AngioChip-vascularized hepatic and cardiac tissues, and describe direct surgical anastomosis of the AngioChip vascular network on the hind limb of a Lewis rat model.

Introduction

Vascular networks are involved in most human tissues, providing essential support for cell survival by delivering oxygen and nutrients, and removing waste products. During human embryonic development, biological growth is governed by the diffusion limit of oxygen and nutrients as the embryo grows in size and its cells increase the metabolic demand for oxygen. The development of a vascular system that delivers oxygen and nutrients deep into the developing tissues overcomes this critical diffusion limit. In fact, the embryonic heart begins beating 21 d post fertilization and is complemented by the circulation pattern established in just 4 weeks[1]. Tissue engineering technologies, which seek to synthesize functional human tissues with combinations of biomaterials, cells and growth factors, have requirements similar to those of the developing embryo[2]. Without a coherent vascular system, the engineered tissue cannot reach a therapeutically relevant functional level[3]. Therefore, clinical success to date has been limited to thinner and in some cases avascular tissues, such as cartilage, skin and bladder[4]. Furthermore, vascular assembly is only part of the challenge; the establishment of a vascular connection upon tissue implantation, the initiation and maintenance of perfusion, and tissue scale-up are all hurdles that must be overcome before tissue engineering technologies can accomplish meaningful therapeutic tissue repair[5,6]. This is especially true for large solid tissues (e.g., myocardium and liver) that are metabolically demanding and necessitate rapid and continuous vascular perfusion from in vitro culture to in vivo implantation.

To engineer functional tissues, it is also important to recapitulate vascular structures on the microscale. Recent advances in 'organ-on-a-chip' engineering highlight the importance of capturing high-level tissue functions for pharmaceutical drug testing through modeling of various tissue interfaces in controlled microenvironments[7–11]. Capturing the barrier function of the endothelium in the control of drug transport under various biomechanical stimuli is essential to demonstrating the relevance of these in vitro models[12–14]. Furthermore, the important role of endothelial cells in enhancing the function and survival of the parenchymal tissue through paracrine and biochemical signaling is also well recognized[15]. Finally, the vascular system functions as a conduit that gives rise to

[1]Institute for Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada. [2]Department of Chemistry, Tsinghua University, Beijing, China. [3]Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada. [4]Toronto General Research Institute, University Health Network, Toronto, Ontario, Canada. [5]The Heart and Stroke/Richard Lewar Centre of Excellence, Toronto, Ontario, Canada. [6]These authors contributed equally: Boyang Zhang, Benjamin Fook Lun Lai. *e-mail: Milica Radisic m.radisic@utoronto.ca

multi-organ interactions by linking multiple tissue models together[16]. The potential of engineered tissue to capture important human physiology that is not easily captured with traditional screening using immortalized cell lines and animal testing is integral to the future success of drug discovery[9].

Various growth factors (e.g., vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF))[17,18] have been found to enhance vascular growth and maturation. However, intricately orchestrated vascular growth in native tissue is inherently challenging to replicate in engineered tissues and is difficult to achieve solely with the application of these molecules. Decellularization of tissue extracellular matrix (ECM) is another promising vascularization approach that makes use of nature's own biological template[19–21]. However, as the decellularized matrix is both complex and dense, repopulating it efficiently and precisely with cells remains challenging. The emergence of microfabrication and 3D bioprinting have expanded the engineers' toolbox, allowing for bottom-up development of intricate biological structures. Building from this knowledge, vascular networks have been molded by subtractive fabrication with sacrificial materials such as carbohydrate–glass lattice[22,23], Pluronic F127[22], dry alginate fibers[24] , Matrigel[25] or gelatin-[26] in hydrogels. Although these methods provide control over the initial spatial positioning of cells with unprecedented precision, formation of high-density tissues still requires extensive cellular remodeling over time[27,28]. Soft hydrogels often lack sufficient mechanical stability to support built-in biological structures while still permitting extensive biological remodeling that could dramatically alter the functional tissue mechanics[29–31]. Synthetic biodegradable polymers, on the other hand, are structurally stable but offer low permeability and are non-permissive to cell migration and intercellular interaction[32–35]. Overcoming these opposing engineering challenges requires a multi-material-based approach.

We developed a scaffold, termed AngioChip, with a built-in endothelialized branched network constructed from a synthetic biodegradable polymer and a parenchymal space that can be filled with hydrogel matrices embedded with a number of parenchymal cell types[36]. This multi-material approach allowed us to provide both stability of the vascular interface (with a synthetic polymer) and amenability of the parenchymal space (with a permissive hydrogel matrix). To overcome limitations in permeability, microholes and nanoporosity were introduced into the channel wall, allowing the passage of small and large molecules, as well as trans-endothelium cell migration and vascular sprouting. Furthermore, the use of structurally defined polymer scaffolds allows us to incorporate anisotropic structures into the parenchymal space to mimic organ-specific tissue mechanics (e.g., cardiac muscles), which would be difficult to achieve with hydrogels alone. Recapitulating vascularized tissues with AngioChip and potential applications of AngioChip are illustrated in Fig. 1. This technology is rooted in a novel 3D stamping fabrication technique that yields a scaffold construction with built-in features of 10-μm resolution, which guides cellular growth at a nearly single-cell level within a 3D tissue[36–38]. This addresses resolution or print-size limitations in current 3D printing methods for synthetic biocompatible polymers.

In this protocol, we describe the step-by-step procedures for creating a microfabricated scaffold, AngioChip, that can be used to generate vascularized tissues for drug screening purposes. Furthermore, AngioChip opens an avenue to overcome limitations faced in producing millimeter-thick vascularized tissues that can directly anastomose with a host vascular system upon implantation for tissue repair.

Overview of the procedure

We provide the basic procedures for the fabrication of an AngioChip scaffold. The approach described in this protocol consists of eight key stages. We include the critical steps from scaffold microfabrication to engineering of the 3D vascularized tissues; these involve polydimethylsioxane (PDMS) mold fabrication (Steps 1–5), poly(octamethylene maleate (anhydride) citrate) (POMaC) prepolymer synthesis (Steps 6–16) and preparation (Steps 17–21), injection molding of the POMaC polymer (Steps 22–26), 3D stamping of AngioChip scaffolds (Steps 27–37), bioreactor assembly (Steps 38–44), scaffold endothelialization (Steps 45–51) and tissue cultivation (Steps 52–59). If the seeding experiments are well planned, a single researcher can readily produce up to ten vascularized tissues on multiple AngioChip bioreactors. With this sampling capability, AngioChip can provide sufficient throughput for drug-target validation. Furthermore, AngioChip tissues tested in vitro can be directly transplanted into animals, creating the prospect of directly translating in vitro testing to in vivo validation.

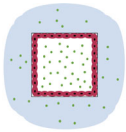
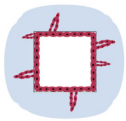
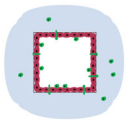
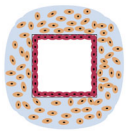
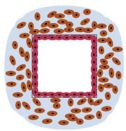
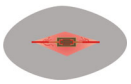
Application	Cell types	Graphical illustration
Permeability	Endothelial cells	
Sprouting	Endothelial cells	
Transendothelial migration	Endothelial cells, monocytes, leukocytes	
3D heart tissue modeling	Endothelial cells, cardiomyocytes	
3D liver tissue modeling	Endothelial cells, hepatocytes	
Anastomosis in vivo	Endothelial cells	

Fig. 1 | Summary of potential applications for AngioChip scaffolds.

Applications of the protocol

This protocol provides a basis for scaffold design and bioreactor setup that can be easily modified to explore a variety of biological applications. The AngioChip scaffold described in this protocol has potential applicability in both in vitro and in vivo scenarios that demand various levels of biological complexity. With this in mind, we developed functional and vascularized cardiac and hepatic tissues that can metabolize clinically relevant drugs delivered through the internal vasculature[16,36]. These tissues are cultured and perfused with gravity-driven flow in a bioreactor that resembles a multi-well plate. This setup is simple and scalable, yielding ease of culturing multiple tissues simultaneously for in vitro application. This platform provides a method of probing tissue-level response to drug input through the vascular system. For instance, functional cardiac muscle grown on the AngioChip scaffold contracts macroscopically without collapsing the built-in vascular network and responds to epinephrine with accelerated contraction[16,36]. Functional liver tissue was shown to metabolize terfenadine, as well as to secrete urea and albumin into the internal vasculature[16,36]. In the absence of parenchymal tissue, the endothelialized network alone can respond to external stimuli to capture biological events such as vascular inflammation with tumor necrosis factor (TNF)-α treatment[39], monocyte trans-endothelium migration, vascular sprouting in response to angiogenic growth factors, and vascular thrombosis under whole-blood perfusion[36].

When released from the bioreactor culture environment, the mechanically stable vascular network can be surgically connected to the host circulation system with immediate blood perfusion upon implantation. We have used this to demonstrate integration of engineered implants with host tissues[36]. This achievement of surgical connection of a completely synthetic vascular network was the first of its kind. Previously, multiple surgeries were necessary to yield vascular explants to both vascularize and implant an engineered tissue, a clinically impractical approach[40]. Rapid vascular connection is particularly useful for metabolically demanding tissues and opens opportunities to improve viability and accelerate integration of tissue implants. In the absence of tissue, implantation of microfabricated bioscaffolds alone can provide structural guidance for native tissue growth and repair with unprecedented spatial precision. The generic nature of this vascular system suggests

potential for wide technology uptake beyond the fields of cardiac and liver research. In fact, we have expanded the utility of this technology to both kidney and cancer cell culture, along with the modeling of dynamic interorgan biological events[16].

Limitations of the protocol

Given the high optical density of the tissues grown on the AngioChip, imaging deep into the engineered tissue (e.g., the internal vasculature) would require tedious tissue sectioning. However, recent advances in imaging techniques such as CLARITY[41–44] can render a thick tissue transparent and permit optical sectioning of thick tissues without destruction. The combination of this technique with supported cell organization using the AngioChip platform can allow visualization of biological growth in the context of crowded high-density tissues, a more physiologically relevant environment. Furthermore, our engineered vasculature has yet to be tried in the co-culture of smooth muscle cells, which achieve dynamic vascular dilation and contraction in vivo. With future work, smooth muscle cells could be incorporated around the vascular network with localized cell seeding or cell recruitment over time. Differences from the physiological microenvironment are further observed in the inclusion of a channel wall (25–50 μm thick) at the vascular interface that provides necessary structural support. Specifically, the thickness of the vessel wall is still higher than is physiologically desirable. However, this configuration is similar to that of conventional Transwell setups that have been widely used to study cellular barrier function, intercellular communication and migration[45,46]. If close intercellular contact is required, microvasculature could be established through both endothelial cell self-assembly and sprouting, in which direct association of endothelial cells, stromal cells, and parenchymal cells can be assessed[47].

Experimental design

Microfabrication

The AngioChip scaffold is fabricated with a synthetic biodegradable polymer POMaC[48–50], which can be cross-linked with exposure to either UV light or heat energy (Fig. 2). Material cross-linking through UV light is fast and the degree of cross-linking can be well-controlled, which allows repeated patterning and bonding of the polymer material to generate a multi-layered 3D scaffold in minutes (Fig. 3). Additional heat cross-linking through post-processing is an option to fine-tune material properties and achieve material stiffness as desired. The smallest microchannels fabricated using this protocol have a luminal cross-section of 100 × 50 μm. Fabrication techniques could allow for smaller channel dimensions, but endothelial cell seeding limits the channel size to no smaller than the cell diameter. Along the channel wall, we pattern 10–20-μm microholes, for which the hole size was application dependent. Although a 10-μm microhole diameter permits cell migration, a 20-μm diameter is used to encourage endothelial cell sprouting.

Modifiability of the approach

Herein, a generic bifurcating network was designed with ease of modification for a specific application. A lattice matrix was constructed to (i) structurally support the built-in network and

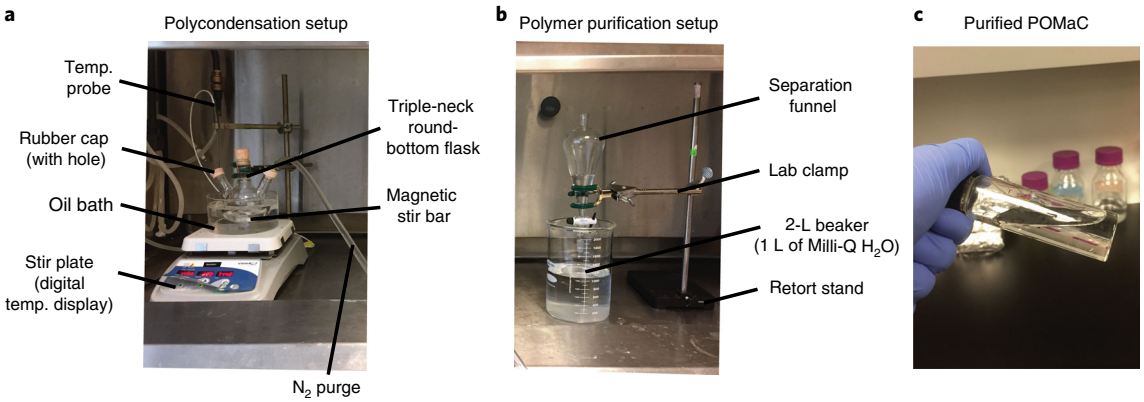


Fig. 2 | POMaC polymer synthesis and purification. a,**b**, Experimental apparatuses for POMaC polymer polycondensation reaction (**a**) and POMaC polymer purification (**b**). **c**, A vial of purified POMaC polymer appears as a transparent viscous solution. temp., temperature.

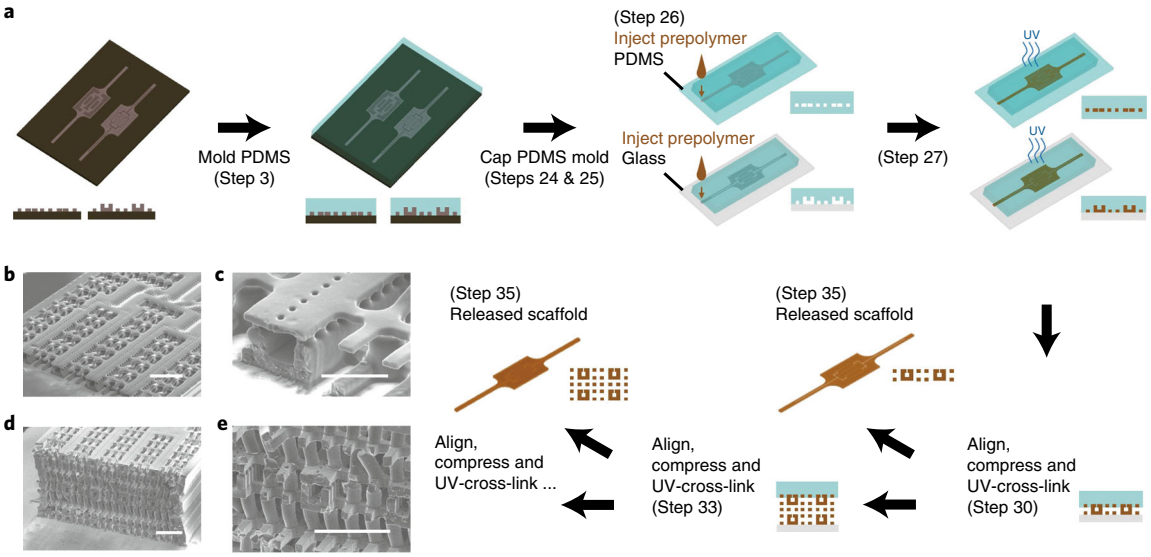


Fig. 3 | Microfabrication of AngioChip scaffold with 3D stamping technique. a, Top, far left, prepare SU-8 masters for the AngioChip; see the Supplementary Data for a CAD file of the design. Top, center-left, develop PDMS negative molds of different AngioChip layers from the SU-8 masters onto PDMS. Top, center-right, cap the PDMS molds with either PDMS (top portion design of AngioChip) or glass (bottom portion design of AngioChip) base and inject POMaC prepolymer (mixed with 40% (wt/wt) PEGDM porogen). Prepolymer injection can be achieved by adding a drop of the prepolymer at the channel inlet and allowing positive pressure to perfuse the prepolymer into the mold, or by pumping prepolymer with a syringe pump directly into the channels. Top, far right, expose the prepolymer to UV to initiate material cross-linking. Bottom right, assemble a single-layer AngioChip scaffold by aligning the top portion to the bottom portion and pressing them together. Expose the assembled scaffold to UV light to initiate the bonding of the two portions. Release the single-layer AngioChip scaffold from the PDMS and glass bases with a razor blade. Bottom center, assemble a multi-layered AngioChip scaffold by aligning multiple scaffold layer portions and building them together layer by layer. Expose the multi-layered AngioChip scaffold to UV light to initiate the bonding of the layers. Release the multi-layered AngioChip scaffold from the PDMS and glass bases with a razor blade. **b**–**e**, Scanning electron micrographs of different areas of the AngioChip scaffold: an AngioChip scaffold with a single layer of networks (**b**), a micro-channel in an AngioChip scaffold (**c**), an AngioChip scaffold with three layers of networks (**d**), and a micro-channel embedded within the AngioChip scaffold with multiple layers of networks (**e**). Scale bars, 500 μm (**b**,**d**,**e**); 200 μm (**c**).

(ii) provide control of scaffold apparent elasticity. To allow for scaffold network perfusion with simple gravity-driven flow, the scaffold was installed inside a bioreactor with three compartments (inlet chamber, tissue chamber and outlet chamber), each of which is individually accessible with conventional pipetting (Fig. 4). Generally, a single inlet and outlet are used to perfuse the network, but additional branching can be incorporated to the scaffold structure to simulate additional epithelial networks (e.g., bile ducts in the liver and proximal tubules in the kidney). Scaffold seeding (both endothelial cells and parenchymal cells) and medium exchange are accomplished with simple pipetting techniques, yielding technological amenability to a number of user skillsets. By the nature of the generic scaffold design, our platform is also compatible with various natural ECM types in the parenchymal space, which is important to end users who cannot compromise application-specific material choices. Tissue assessment can be achieved with a variety of techniques such as immunostaining, histology, biological assay of culture medium, optical imaging and image analysis of macroscopic tissue contraction. The large tissue size yields enough cells for conventional biochemical analysis such as quantitative PCR and ELISA.

Materials

Reagents

- heparin (Sigma-Aldrich, cat. no. H3149-100KU)
- 1,8-octanediol (Sigma-Aldrich, cat. no. O3303)
- Citric acid (Caledon Laboratory Chemicals, cat. no. 2980-1) **! CAUTION** Use this chemical in a fume hood. Wear protective goggles, gloves and suitable protective clothing.
- Maleic anhydride (Sigma-Aldrich, cat. no. 63200)
- 1,4-dioxane (Sigma-Aldrich, cat. no. 360481)
- ddH_2O (from a Direct-Q 5 water purification system; Millipore)
- 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959; Sigma-Aldrich, cat. no. 410896)
- Poly(ethylene glycol) dimethyl ether (PEGDM, M_n ~500) (Sigma-Aldrich, cat. no. 445886)

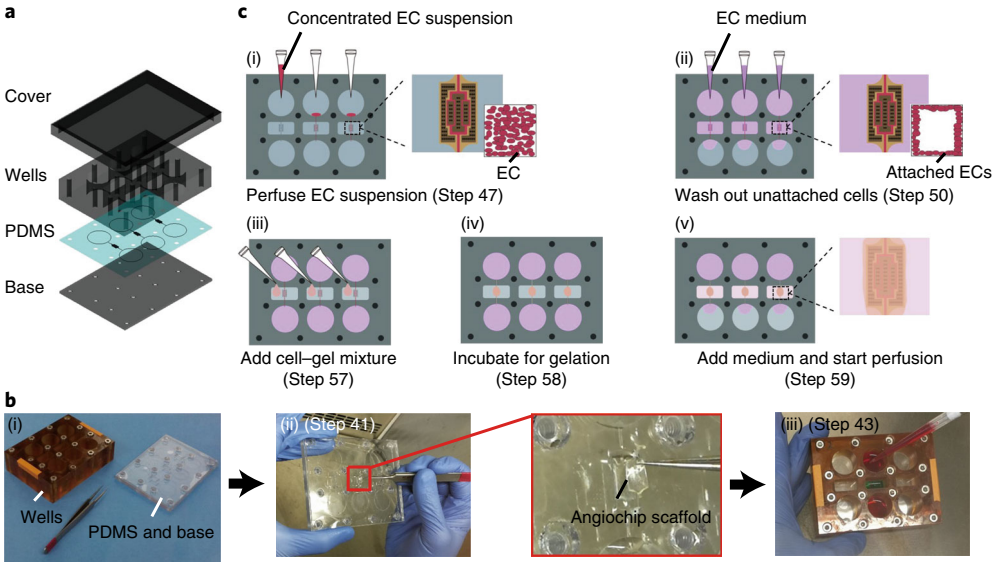


Fig. 4 | Assembly of an AngioChip bioreactor with seeding of endothelial cells and parenchymal tissues. a, Schematic of the AngioChip bioreactor assembly. **b**, Photographs of the complete AngioChip bioreactor assembly. i,ii, Using tweezers, the AngioChip scaffold is placed on top of the patterned PDMS sheet, which is then sandwiched between the polycarbonate body and base. The bioreactor is then screwed shut with an Allen key. iii, One percent agarose can be used to seal any gaps around the connections of inlet and outlet wells. **c**, i,ii, Seeding of endothelial cells (ECs) in the AngioChip scaffold network. i, Concentrated EC (25×10^6 cells per mL) suspension (10–20 μL) is added to the AngioChip inlet. Pressure differences drive the cell suspension to the middle segment of the AngioChip scaffold. After ~1.5 h, which allows the ECs to attach to the lumen, apply EC medium (purple) with a pressure head gradient to wash out unattached ECs. ii, With low-shear fluid flow culture overnight, attached ECs will elongate and provide surface coverage to the lumen of the AngioChip scaffold. **c**, iii–v, Seeding of cardiomyocytes or hepatocytes into the parenchymal space of the AngioChip scaffold. iii, Parenchymal cells are premixed in the desired hydrogel (150×10^6 cells per mL) and added directly on top of the AngioChip scaffold as a droplet. iv, Allow 15–30 min for gelation (at 37 °C) of the cell-hydrogel mixture around the AngioChip scaffold. For seeding of thick AngioChip tissue, multiple seeding and centrifugation steps with the cell-hydrogel mixture will be needed. v, Apply medium with a pressure gradient to initiate perfusion. Inlet and outlet wells contain EC medium (purple) and the AngioChip well contains an appropriate medium (pink) for culturing the seeded tissues.

- Sylgard 184 Silicone Elastomer Kit (polydimethylsiloxane (PDMS); Dow Corning, cat. no. 3097358-1004)
- Photoresist SU-8 2050 (MicroChem, cat. no. NC0060520) **! CAUTION** Wear protective goggles, gloves and suitable protective clothing.
- Silicon wafer (6 inches in diameter; University Wafers, cat. no. 2908)
- Agarose (Sigma-Aldrich, cat. no. A9539)
- Dulbecco's phosphate buffered saline (PBS; Gibco, cat. no. 14190-144)
- Dulbecco's Modified Eagle Medium (DMEM; Gibco, cat. no. 11965-092)
- Heat-inactivated FBS (hi-FBS; Gibco, cat. no. 16000044)
- Penicillin–streptomycin (Gibco, cat. no. 15140122)
- HEPES (Thermo Fisher, cat. no. 15630080)
- Gelatin porcine skin (type A; Sigma-Aldrich, cat. no. G6144)
- Pluronic F-127 (Sigma-Aldrich, cat. no. P2443)
- Corning Matrigel (VWR, cat. no. 354234)
- Rat-tail collagen type 1 (Corning, cat. no. 354236)
- Sodium hydroxide (NaOH, 1 N; VWR, cat. no. BDH7994-4) **! CAUTION** Sodium hydroxide is highly corrosive. Wear protective goggles, gloves and suitable protective clothing.
- M199 medium (10×; Thermo Fisher, cat. no. 11825015)
- Glucose (Sigma-Aldrich, cat. no. A00040)
- Sodium bicarbonate ($NaHCO_3$; EMD Chemicals, cat. no. SX0320-1)
- Fibrinogen (Sigma-Aldrich, cat. no. F3879)
- Thrombin (Sigma-Aldrich, cat. no. T6884)
- Aprotinin (Sigma-Aldrich, cat. no. A3428)
- Tissue glue (cyanoacrylate, Vetbond; 3M, cat. no. 1469SB)

- Tetramethylrhodamine isothiocyanate dextran (TRITC-Dextran, ~70 kDa; Sigma-Aldrich, cat. no. T1162)
- Hematoxylin solution (Harris modified; Sigma-Aldrich, cat. no. HHS16)
- Eosin Y solution, alcoholic (Sigma-Aldrich, cat. no. HT110116)
- Formalin, neutral buffered (10% (vol/vol); Sigma-Aldrich, cat. no. HT501128)
- Paraformaldehyde (Sigma-Aldrich, cat. no. P6148)
- Triton X-100 (0.25% (wt/vol); Alfa Aesar, cat. no. 9002-93-1)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650)
- 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA; Life Technologies, cat. no. C369)
- Hank's Balanced Salt Solution with Ca^{2+}/Mg^{2+} (HBSS; Gibco, cat. no. 14025-092)
- Carboxyfluorescein diacetate, acetoxymethyl ester (CFDA, AM, 557 Da; Invitrogen, cat. no. C1354)
- Propidium iodide (PI; Life Technologies, cat. no. P3566)
- Ammonium bicarbonate (Sigma-Aldrich, cat. no. A6141)
- Terfenadine (Sigma-Aldrich, cat. no. T9652)
- Epinephrine (Sigma-Aldrich, cat. no. E4250)
- 4-(2-(6-(dibuytlamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl) pyridinium hydroxide inner salt (di-4-ANEPPS; Sigma-Aldrich, cat. no. D8064)
- Lewis rats (optional for surgical anastomosis procedures): hind limbs of adult male Lewis rats (150–250 g) from Charles River were used for surgical anastomosis
- Ketoprofen (10 mg/mL; Anafen Injection, Merial Canada, Inc., cat. no. 02150999)
- Isoflurane (Fresenius Kabi Canada, Ltd., cat. no. 02237518)
- Ethanol (100%; Commercial Alcohol, University of Toronto MedStore, cat. no. 39752-P016-EAAN)

! CAUTION All procedures involving live rats should be performed in agreement with institutional and national regulations. All surgical procedures described in this protocol were performed at the Department of Comparative Medicine Animal Facility, University of Toronto, under a protocol approved by the University of Toronto Committee on Animal Care.

Cell sources

- Human umbilical vein endothelial cells (HUVECs; Lonza, cat. no. C2519A) **! CAUTION** The cells used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- Lewis rat vein endothelial cells (Cell Biologics, cat. no. R2133-LS) **! CAUTION** The cells used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- Bone marrow-derived mesenchymal stem cells; normal, human (ATCC, cat. no. PCS-500-012) **! CAUTION** The cells used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- Primary adult rat hepatocytes: primary adult rat hepatocytes were isolated from an 8-week-old male Sprague Dawley rat according to a two-step isolation procedure described previously[51,52] **! CAUTION** All procedures involving live rats should be performed in agreement with institutional and national regulations. All surgical procedures described in this protocol were performed at the Department of Comparative Medicine Animal Facility, University of Toronto, under a protocol approved by the University of Toronto Committee on Animal Care.
- Neonatal rat cardiomyocytes: neonatal rat cardiomyocytes were extracted and digested according to a two-step isolation procedure described previously[53,54] **! CAUTION** All procedures involving live rats should be performed in agreement with institutional and national regulations. All surgical procedures described in this protocol were performed at the Department of Comparative Medicine Animal Facility, University of Toronto, under a protocol approved by the University of Toronto Committee on Animal Care.
- Human hepatocytes: human hepatocytes were differentiated from human embryonic stem cells (H9, the Wicell Research Institute) and cultured according to the differentiation protocol described previously[55,56].
- Human cardiomyocytes: human cardiomyocytes were differentiated from NKX2-5-GFP human embryonic stem cells (HES3-NKX2-5GFP) and cultured according to a described differentiation protocol[57]
- HES3-NKX2-5GFP was a generous gift from E. Stanley and A. Elefanty (Monash University, Australia). **! CAUTION** Caution All procedures involving human embryonic stem cells should be performed in agreement with institutional and national regulations.

Cell culture medium
- Endothelial Growth Medium-2 Bullet Kit (EGM-2 medium; Lonza, cat. no. cc-3162)
- Rat endothelial cell medium (Cell Biologics, cat. no. M1226)
- Clonetics HCMTM hepatocyte culture medium (Lonza, cat. no. CC-3198)

Assay kits
- QuantiChrom Urea Assay Kit (Cedarlane Labs, cat. no. DIUR-500)
- Lactate Dehydrogenase Toxicity Assay Kit (Cayman Chemical Company, cat. no. 601170-96)
- Quant-iTTM PicoGreen dsDNA Assay Kit (Thermo Fisher, cat. no. P11496)

Antibodies
- Anti-rat CD68 (Cedarlane Labs, cat. no. MCA341 GA)
- Anti-rat CD163 (Cedarlane Labs, cat. no. MCA342 GA)
- Anti-human sarcomeric-α-actinin (Abcam, cat. no. ab9465)
- Donkey Alexa Fluor 488 anti-mouse IgG (Life Technologies, cat. no. A21202)
- Goat tetramethylrhodamine isothiocyanate (TRITC) anti-mouse IgG (Abcam, cat. no. ab7065)
- F-actin phalloidin Alexa Fluor 660, conjugated (Life Technologies, cat. no. A22285)
- Anti-human vascular endothelial (VE)-cadherin (Abcam, cat. no. ab33168)
- Goat anti-human albumin (Abcam, cat. no. 2406)
- Donkey Alexa Fluor 647 anti-mouse IgG (Sigma-Aldrich, cat. no. AP192SA6)
- TRITC anti-goat IgG (Sigma-Aldrich, cat. no. T4530)
- Mouse anti-human CD31 (BD Biosciences, cat. no. 557555)
- DyLight anti-mouse IgG (Rockland, cat. no. 610-143-121)
- Anti-alpha smooth muscle actin (Abcam, cat. no. ab5694)

Equipment
- Square glass cover slip, 20 × 20 mm (VWR, cat. no. 10118-641)
- 25-gauge polyimide surgical tube (SmallParts, cat. no. TWPT-0201-30)
- Scotch tape (Staples, cat. no. STP.17995)
- 7-0 and 4-0 silk sutures (Ethicon, cat. no. 7733-G)
- Ultraviolet mask aligner (OAI, model no. 200)
- Spin coater (Specialty Coating Systems, model no. G3P-8)
- Myograph force transducer (Kent Scientific)
- Bright-field microscope (Olympus, CKX41)
- Confocal microscope (×10 and ×40 objectives; Olympus, model no. FV5-PSU)
- Scanning electron microscope (Hitachi, model no. S-3400)
- Fluorescence plate reader (SpectraMax i3; Molecular Devices)
- Electrical stimulator (Astro Med, model no. Grass s88x)
- Oil bath equipped with a heating unit and a temperature controller (VWR, cat. no. 97042-714)
- Electric warming pad (Kaz)

Bioreactor
- Polycarbonate body and base (McMaster-Carr, cat. nos. 8574K54 and 8574K6)
- Polycarbonate cap (McMaster-Carr, cat. no. 8574K54)
- Stainless-steel screws (McMaster-Carr, cat. no. 91292A131)

Reagent setup
Cardiomyocyte culture medium
To make cardiomyocyte culture medium, add 50 mL of hi-FBS, 5 mL of HEPES and 5 mL of penicillin–streptomycin to 440 mL of DMEM. Mix the components gently to avoid bubble formation. Filter-sterilize the medium using a 500-mL 0.22-μm PES filter system by attaching the filter system to the vacuum pump. The medium may be stored for up to 1 month at 4 °C.

Agarose solution
Dilute agarose to 1.0% (wt/vol) with deionized water. Filter-sterilize the solution with a 0.22-μm sterile syringe filter. It can be stored for up to 6 months at 4 °C.

Gelatin solution
Dilute gelatin to 0.2% (wt/vol) with deionized water. Autoclave the solution to sterilize. It can be stored for up to 6 months at 4 °C.

Pluronic solution
Dilute pluronic F-127 to 5.0% (wt/vol) with deionized water. Filter-sterilize the solution with a 0.22-μm sterile syringe filter. The solution can be stored for up to 1 year at 4 °C.

Paraformaldehyde solution
Dilute paraformaldehyde to 4.0% (wt/vol) in PBS. The solution can be stored for up to 1 year at 4 °C.

Triton X-100 solution
Dilute Triton X-100 to 0.25% (vol/vol) in PBS. The solution can be stored for up to 1 year at 4 °C.

Procedure

Fabrication of PDMS molds for AngioChip ● Timing 3 d

1 Fabricate the AngioChip master using the photomask provided in the Supplementary Data, using standard SU-8 photolithographic techniques as described previously (see Steps 1–15 in ref. [58]) or via outsourcing (Fig. 3a, top, far left). Silanize the wafer overnight in a vacuum chamber according to a standard protocol, as described previously (see Steps 21 and 22 in ref. [59]).

2 Mix 30 g of PDMS silicone elastomer base with curing agent in a disposable plastic cup at a 15:1 (polymer base/curing agent) weight ratio. This can be used to prepare 16 AngioChips.
▲ **CRITICAL STEP** In parallel, prepare 30 g of PDMS silicone elastomer base with curing agent at a 10:1 (polymer base/curing agent) weight ratio. This will be used to prepare a flat PDMS sheet layer for capping in Step 25.

3 Pour 30 g of the 15:1 PDMS mixture onto the silanized SU-8 mold from Step 1 and distribute evenly to cover the surface of the features (Fig. 3a, top, center-left).
▲ **CRITICAL STEP** Pour the 10:1 PDMS mixture on a blank SU-mold and treat this in parallel with the 15:1 PDMS-coated SU-8 mold in Steps 4 and 5.

4 Place the SU-8 molds with PDMS mixture in a vacuum desiccator for 1 h at <60 mTorr.

5 Remove the PDMS-cast SU-8 molds from the vacuum desiccator after degassing (such that all bubbles are removed from the PDMS) and cure the PDMS at room temperature (25 °C) on a level surface.
▲ **CRITICAL STEP** The PDMS curing process is complete when PDMS is not sticky. This usually takes 2–3 d.
■ **PAUSE POINT** The PDMS-cast SU-8 molds can be stored at room temperature for up to 1 d to prevent the PDMS cast from becoming too hard.

Chemical synthesis of POMaC prepolymer ● Timing 2 d

6 Dry a 250-mL three-necked round-bottom flask overnight at T >90 °C.
▲ **CRITICAL STEP** This is to remove water condensation from the flask before the reaction.

7 Turn on the silicon oil bath, set the temperature to 160 °C and set shaking at 200 r.p.m. on a hot plate stirrer fitted with an external temperature probe submerged in the heating oil (Fig. 2a).
▲ **CRITICAL STEP** It will take 30 min to warm the oil bath and ensure even oil heating.

8 Weigh out the reactants—1,8 octanediol, citric acid and maleic anhydride—at a molar ratio of 5:1:4. We recommend preparing a total mixture of 30 g; this is sufficient for making at least 20 g of POMaC prepolymer, which can be used to build at least 100 AngioChips.

9 Add a magnetic stir bar to the three-necked flask.

10 Add and mix the reactants in the three-necked flask.

11 Place the flask in the oil bath and begin a nitrogen purge. Visually observe complete melting of the reagents, then reduce the temperature to 140 °C for the remainder of the reaction.
▲ **CRITICAL STEP** Ensure that the reactant mixture, which is in liquid phase, is submerged in the oil bath.

12 React at 140 °C while maintaining stirring under a nitrogen purge for 3 h.

13 Turn off the heating bath and lift the flask out of the oil to allow cooling to room temperature. Then add 30 mL of 1,4-dioxane to the flask to completely dissolve the generated POMaC prepolymer.

14 Pour the dissolved prepolymer into a separatory funnel and place above a beaker filled with ddH_2O. Purify the POMaC prepolymer by dropwise precipitation (Fig. 2b).
▲ **CRITICAL STEP** This is done at room temperature and at a rate of one drop/s. Slowly drip the POMaC solution into the beaker to ensure separation of low-molecular-weight or monomer reactants from higher-molecular-weight POMaC prepolymer. High-molecular-weight POMaC prepolymer will precipitate to the bottom of the beaker and appear white in color.
15 Decant the water from the beaker and collect the precipitated POMaC prepolymer from the bottom of the beaker.
16 Dry the POMaC prepolymer solution in a new glass container under compressed air flow overnight to evaporate residual water and 1,4-dioxane. The final POMaC prepolymer solution appears as a transparent viscous solution (Fig. 2c). The successful synthesis and purity of the polymer can be determined with 1H NMR following standard procedure, as shown previously[36].
■ **PAUSE POINT** The purified POMaC prepolymer can be stored at 4 °C for up to 3 months.

Preparation of POMaC polymer mixture for photo-cross-linking ● Timing 1 h

17 Mix POMaC prepolymer with PEGDM in a glass vial at a 2:3 (POMaC/PEGDM porogen) weight ratio. We recommend preparing a total mixture of 50 g; this is sufficient to coat PDMS molds for 3D stamping of at least 100 AngioChips.
18 Add UV initiator (Irgacure 2959) at a final concentration of 5% (wt/vol) to the vial of POMaC–PEGDM mixture.
19 Mix the reagents with a spatula for 3 min on a hot plate set at 120 °C.
▲ **CRITICAL STEP** Warming the polymer solution temporarily will ensure appropriate mixing of POMaC with other reagents, as this will reduce solution viscosity and completely dissolve the UV initiator in the polymer solution.
▲ **CRITICAL STEP** Minimize the heating time to reduce the risk of undesired polymerization at high temperature.
20 Pour the well-mixed reagents into a 10–30-mL syringe.
▲ **CRITICAL STEP** Cover the syringe with tape to protect the reagents from light.
21 Store the syringe at 4 °C until use.
■ **PAUSE POINT** The POMaC–PEGDM–UV initiator mixture will be stable for at least 1 month when stored at 4 °C in the dark. Warm the solution to room temperature and remix the reagents before use if phase separation of the polymer solution occurs.

Injection molding of POMaC polymer ● Timing 1 d

22 Cut out the fully cured AngioChip molds from the PDMS layer generated from the SU-8 masters from Step 5 (Supplementary Video 1).
▲ **CRITICAL STEP** Trim the PDMS molds so that the inlet and outlet channels are exposed.
23 Clean the PDMS surface with Scotch tape to remove deposited dust.
24 Cap the feature side of the PDMS mold for layer 1 of the AngioChip scaffold with a square glass coverslip without any plasma treatments (Fig. 3a, top, center-right).
25 Cap the feature side of the PDMS mold for all other layers of the AngioChip scaffold with a piece of flat, cleaned 10:1 PDMS (slightly larger than molded piece) without any plasma treatments. (Fig. 3a, top, center-right).
▲ **CRITICAL STEP** This step should be done slowly to prevent air bubbles from becoming trapped between the PDMS layers.
26 Perfuse POMaC polymer mixture into the assembled PDMS molds with capillary action by applying a drop of POMaC mixture at the inlet of the PDMS mold (Fig. 3a, top, center-right).
▲ **CRITICAL STEP** POMaC mixture solution will slowly fill the channels of the patterned PDMS molds overnight.
? TROUBLESHOOTING
■ **PAUSE POINT** The POMaC solution–coated AngioChip molds can be stored at room temperature in the dark for up to 1 d.

AngioChip scaffold construction by 3D stamping ● Timing 1 d

▲ CRITICAL Steps 27–37 describe the procedure for 3D stamping of the AngioChip scaffold and should be performed in a clean room to avoid dust deposition onto the AngioChip channels.

27 Expose the injected POMaC solution to UV light under a UV mask aligner (OAI) at an intensity of 10 mJ/s for 4 min (Fig. 3a, top, far-right) to cross-link the polymer (Supplementary Video 1).
▲ CRITICAL STEP The exact timing of the UV exposure must be fine-tuned to account for batch-to-batch variation of the POMaC polycondensation reaction, which leads to variation in the rate of photo-cross-linking.

28 Uncap the PDMS mold from the glass slide to expose the cross-linked POMaC features, which should remain on the glass slide during PDMS mold removal.
▲ CRITICAL STEP To ensure that the patterned POMaC polymer is transferred to the glass slide, the UV-exposed mold can be baked for 5 min at 90 °C to improve adhesion of POMaC to the glass slide before uncapping the PDMS mold from the glass slide. The mold should be removed slowly in a direction parallel to the scaffold features.
? TROUBLESHOOTING

29 Uncap the PDMS mold from the flat PDMS sheet to expose the cross-linked POMaC features, which should stay on the PDMS mold.

30 Align and stamp the PDMS mold with patterned POMaC polymer onto the glass slide affixed with patterned POMaC polymer under a UV mask aligner, using an alignment stage and an upright microscope (Fig. 3a, bottom right). After the two components are aligned, gently press them together by moving the *z* axis of the alignment stage (Supplementary Video 1).

31 Apply additional UV light at an intensity of 10 mJ/s for 4 min to bond the two layers.

32 Detach the PDMS mold slowly with fine-tip tweezers. The two layers of patterned POMaC polymers should be bonded together and firmly attached on the glass slide.
? TROUBLESHOOTING

33 (Optional) If desired, repeat Steps 29–32 with additional UV-cross-linked POMaC sheets on PDMS molds to build additional layers on the AngioChip scaffold for engineering thick tissue (Fig. 3a, bottom center). At the conclusion of layer-by-layer stamping, continue to Step 34.

34 Place the assembled POMaC AngioChip scaffolds in an 80 °C oven and incubate overnight to enhance bonding between layers.

35 Release the assembled AngioChip scaffold from the glass slide by immersing it in phosphate buffered saline for 1 h.
▲ CRITICAL STEP A razor blade can be used to slowly and lightly scrape the scaffolds from the surface of the glass slide.

36 Incubate the AngioChip scaffold in 70% (vol/vol) ethanol overnight to sterilize the scaffold and leach out any residual PEGDM and UV initiator. The scaffold will swell in ethanol but will not disintegrate.

37 Wash the AngioChip scaffolds with PBS three times.
■ PAUSE POINT Store the AngioChip scaffolds in PBS at 4 °C in a sealed container until use.

Assembly of the AngioChip scaffold into a bioreactor ● Timing 1 d

38 Sterilize the components of the bioreactor by autoclaving (Fig. 4a,b,i). The bioreactor can be machined according to the dimensions shown in Supplementary Fig. 1.

39 Coat the bioreactor PDMS base with 5% (wt/vol) pluronic acid (F-127) in PBS for 2 h by adding it dropwise to the tissue chambers on the PDMS base.

40 Wash off excessive pluronic acid solution with PBS.

41 Place the AngioChip scaffold on the tissue chamber (Fig. 4b,ii).

42 Assemble the bioreactor with built-in screws.
▲ CRITICAL STEP To improve the seal of the AngioChip network to the inlet and outlet wells, apply 10–20 µL of 1% (wt/vol) agarose in PBS (heated to >50 °C) around the AngioChip inlet and outlet connections, and then allow the agarose gel to cool to room temperature for 5 min.
? TROUBLESHOOTING

43 Fill the bioreactor chambers with 0.2% (wt/vol) gelatin in PBS for 2 h to coat the polymer surfaces for facilitating cell attachment (Fig. 4b,iii).

44 Replace the 0.2% (wt/vol) gelatin solution with EGM-2 medium and place the bioreactor in an incubator overnight to prime the scaffold for cell seeding and eliminate bubbles in the AngioChip networks.
? TROUBLESHOOTING

Endothelialization of the AngioChip network ● Timing 2 d

45 Prepare a concentrated endothelial cell suspension (25 × 106 cells per mL) in EGM-2 medium. A total of 10–20 µL of concentrated cell suspension is needed per AngioChip.
46 Aspirate out all medium from the bioreactor.
▲ CRITICAL STEP Be careful to not completely dry out the AngioChip scaffold.
47 Perfuse 10–20 µL of concentrated endothelial cell suspension from the inlet well of the AngioChip scaffold for 1 min by tilting the bioreactor at 45° (Fig. 4c,i).
48 Level the bioreactor to stop the flow after the endothelial cells are perfused into the AngioChip network.
49 Place the AngioChip bioreactor in an incubator at 37 °C and 5% CO_2 for 2 h under static conditions to allow cell attachment.
50 Add 1 mL of endothelial cell culture medium to the inlet well to initiate slow perfusion and place the bioreactor back in the incubator for another 2 h (Fig. 4c,ii).
51 Aspirate all medium from the bioreactor and replace it with EGM-2 medium (4 mL in inlet wells, 1 mL in middle wells and 0.2 mL in outlet wells).
▲ CRITICAL STEP To increase the perfusion rate, the bioreactor can also be tilted at 45°.
? TROUBLESHOOTING

Culture of 3D tissue on AngioChip scaffolds ● Timing 7 d

52 Supplement the stem cell–derived hepatocytes or cardiomyocytes (depending on the downstream applications; see Introduction for details) with a 20% human mesenchymal stem cell population. A total of 1.5–2.25 million parenchymal cells are needed for culturing a 3D tissue on AngioChip scaffolds (1.2–1.8 million of which are hepatocytes or cardiomyocytes and 0.3–0.45 million of which are human mesenchymal stem cells).
53 Prepare a collagen–Matrigel hydrogel mixture as follows: neutralize (pH 7.4) 2.5 mg/mL of rat tail collagen type 1 with 1 N NaOH and 10× M199 medium supplemented with 4.5 µg/mL of glucose, 1% (vol/vol) HEPES, 10% (vol/vol) Matrigel and 2 µg/mL $NaHCO_3$. Prepare a total of 15 µL for each AngioChip.
▲ CRITICAL STEP Alternatively, fibrinogen (33 mg/mL) or Matrigel can be used.
54 Suspend the cells in 15 µL of collagen–Matrigel mixture at a concentration of 100–150 million cells per mL in a conical tube on ice.
55 Before seeding the cell-gel mixture into the AngioChip scaffold, aspirate the endothelial medium from the AngioChip scaffold compartment of the bioreactor.
56 Gently dry the AngioChip scaffold with autoclaved Kimwipes.
▲ CRITICAL STEP To avoid bubble generation and hydrogel deposition into the lumen of the AngioChip network after drying, maintain an equal pressure head (0.5 mL of culture medium) in the inlet and outlet wells of the bioreactor.
57 Withdraw 15 µL of cell-gel mixture (100–150 × 10^6 cells per mL) and pipette it onto the AngioChip scaffold (Fig. 4c,iii).
▲ CRITICAL STEP For thick AngioChip scaffolds, pipette 40 µL of cell–gel solution and seed directly on top of the AngioChip scaffold, then centrifuge the bioreactor at 87 *g* for 3 min at 4 °C.
58 Place the bioreactor in an incubator with a built-in humidified chamber for 30 min to achieve gelation of collagen/Matrigel (Fig. 4c,iv). If such a feature is not available, it is possible to locally humidify the environment around the AngioChip scaffold by placing the AngioChip bioreactor in a 100-mm Petri dish that is partially immersed in another container partially filled with dH_2O.
59 After gelation, add 4 mL of endothelial medium to the inlet well and 0.2 mL of endothelial medium to the outlet well. Add 1 mL of hepatocyte medium or cardiac medium (as appropriate) to the tissue in the middle well of the AngioChip bioreactor (Fig. 4c,v).
Change the medium in the bioreactor daily for 7 d to grow a vasculature network.
? TROUBLESHOOTING
■ PAUSE POINT The 3D tissues on the AngioChip scaffolds can be cultured in the respective media for at least 2 weeks.

AngioChip perfusion and permeation analysis ● Timing 1 d

▲ CRITICAL After seeding endothelial cells in the built-in network and parenchymal cardiac cells on the AngioChip scaffold, permeability analysis is performed by perfusing with large (70-KDa TRITC-Dextran, Steps 60 and 61) or small molecules (557-Da CFDA, Steps 62 and 63).

60 Prepare 10 μM TRITC–Dextran solubilized in endothelial medium and perfuse 4 mL of the solution from the inlet well of bioreactor for 20 h. The middle well and outlet well of the AngioChip bioreactor contain 1 and 0.2 mL, respectively, of endothelium medium only.

61 At the conclusion, collect all medium from the middle well and measure the accumulated fluorescent molecules with a fluorescence plate reader at excitation (ex) = 557/emission (em) = 576 nm. A standard curve of known TRITC–Dextran concentration is generated to assess the measured amounts. We usually observe lower permeability on endothelialized AngioChips than non-endothelialized AngioChips.

62 Perfuse the AngioChip bioreactor with 100 μM CFDA dissolved in PBS by adding 4 mL of the solution to the inlet well. The middle well and outlet well of the AngioChip bioreactor contain 1 and 0.2 mL, respectively, of PBS without CFDA.

63 Monitor permeation of the small molecule through the AngioChip scaffold under a fluorescence microscope at ex 492/em 517 nm. Time-lapse images of the AngioChip scaffold are taken every 5 min for a duration of 1 h. We usually observe that the parenchymal cells closely surrounding the AngioChip network light up first.

(Optional) Immunostaining of AngioChip tissues ● Timing 1 d

▲ CRITICAL Steps 64–69 describe the procedures for immunostaining of AngioChip tissues.

64 Before staining, remove the tissues on day 7 by dissembling the bioreactor.

65 Conduct live and dead staining by staining of the AngioChip tissue models with CFDA and propidium iodide. Wash the AngioChip tissue models with PBS one time. Submerge the AngioChip tissue models in 1 mL of (10 μM) CFDA and propidium iodide (75 μg/mL) staining mixture. The staining molecules are prepared in PBS according to the manufacturer's protocol. Perform staining for 30 min at 37 °C. After this, wash the tissues three times with PBS before imaging with a confocal microscope.

66 When imaging surface and intracellular protein markers, first fix the tissues with 4% (wt/vol) paraformaldehyde for 15 min at room temperature.

67 To stain for intracellular cardiac markers, permeate the cardiac tissues with 0.25% (vol/vol) Triton X-100 in PBS for 10 min. Skip the permeation step when staining for surface protein markers.

68 To stain for intracellular hepatic markers, permeate the hepatic tissue models with cold methanol for 2 min. Skip the permeation step when staining for surface protein markers.

69 Immunostain the tissues using specific antibodies according to published protocols[16,37,39,56].

(Optional) Functional tissue analysis ● Timing 1 d

70 Depending on the tissue model, several functional assays can be performed, starting on day 7 after seeding cells into the AngioChip (Step 59). For assessing urea secretion by liver tissues, follow option A. For studying terfenadine drug metabolism by liver tissues, follow option B. For studying the drug-stimulated response of cardiac tissue, follow option C.

(A) Assessment of urea secretion by liver tissues ● Timing 1 d

(i) Perfuse 4 mL of ammonium bicarbonate (10 mM) in EGM-2 medium from the inlet well on day 7 after seeding parenchymal cells into the AngioChip (Step 59). Add 1 mL of ammonium bicarbonate (10 mM) in hepatocyte medium to the middle well of the hepatic tissue model. Add 0.2 mL of normal EGM-2 medium to the outlet well.

(ii) After 24 h of incubation, collect all media from the middle and outlet wells.

(iii) Centrifuge the medium at 300*g* for 5 min at room temperature to remove cells and cell debris.

(iv) Determine the amount of urea produced by using a QuantiChrom Urea Assay Kit according to the manufacturer's procedure.

(B) Study of terfenadine drug metabolism by liver tissues ● Timing 1 d

(i) Perfuse 4 mL of terfenadine (10 μM) in EGM-2 medium from the inlet well on day 7 after seeding parenchymal cells into the AngioChip (Step 59). Add 1 mL of normal hepatocyte

medium to the middle well of the hepatic tissue model. Add 0.2 mL of normal EGM-2 medium to the outlet well.

(ii) After 24 h of incubation, collect all media from the inlet, middle, and outlet wells.

(iii) Perform standard mass spectrometry to determine the amount of fexofenadine secreted by each hepatic tissue model[60,61].

(C) Study of drug-stimulated response of cardiac tissues ● Timing 1 d

(i) On day 7 after seeding parenchymal cells into the AngioChip (Step 59), record spontaneous tissue contraction under a bright-field microscope to set a baseline value.

(ii) Expose the AngioChip cardiac tissue by perfusing 4 mL of 10 μM epinephrine in EGM-2 medium from the inlet well. Add 1 mL of cardiomyocyte culture medium to the middle well of the cardiac tissue model. Add 0.2 mL of EGM-2 medium to the outlet well.

(iii) Continuously Record the contraction of the cardiac tissue under a bright-field microscope for 30 min following the application of drugs. We usually observe an increasing frequency of tissue contraction during this period.

(Optional) Direct surgical anastomosis of AngioChip tissues and immunohistochemical analysis of implanted AngioChip tissues ● Timing 10 d

! CAUTION All procedures involving live rats should be performed in agreement with institutional and national regulations. All surgical procedures described in this protocol were performed at the Department of Comparative Medicine Animal Facility, University of Toronto, under a protocol approved by the University of Toronto Committee on Animal Care.

▲ CRITICAL Steps 71–84 describe the surgical procedure involved in the implantation of AngioChip tissues with surgical vascular anastomosis on the rat hind limb. This type of assay is useful for studying the survival and integration of engineered tissues in the body.

71 Systematically heparinize Lewis rats with a dosage of 1 mg/kg heparin (subcutaneous injection) before surgery (for detailed procedures, see ref. [62]).

72 Anesthetize the rats in an induction chamber containing 5% (vol/vol) isoflurane at a flow rate of 1 L/min. Once the animal becomes unresponsive, place it in the supine position over an electric warming pad and transfer it to a portable anesthetic machine for maintenance (5% (vol/vol) isoflurane at flow rate of 1 L/min).

73 Administer analgesic (5 mg/kg ketoprofen, s.c.) and prepare both hind limbs for surgery by removal of hair, using electric clippers, and skin disinfection, using three alternating scrubs of 70% (vol/vol) ethanol and betadine.

74 Cover the animal with sterile drapes and place it under a dissection microscope to obtain an enlarged view of the hind-limb region. Extend and fix the hind limbs with surgical tape.

75 Make an ~1-cm-long incision into the skin with a scalpel, starting from the knee and extending to the medial thigh. Use a retractor to open the wound and gain a better view of the underlying vasculature. Dissect the subcutaneous fat tissue to reveal the underlying neurovascular bundle.

▲ CRITICAL STEP The surgical area should be periodically irrigated with heparinized saline to prevent clotting.

76 Dissect the femoral artery and separate it from the femoral vein and nerve at the proximal location near the groin. Fully expose a segment of the femoral artery and vein (~1 cm in length). Clamp the upper and lower ends of the segments with microsurgical approximator clamps to temporarily stop the blood flow during the surgery.

77 Expose the vessel lumen and wash it with 3–5 mL of heparinized saline. Insert one 25-gauge polyimide tube (surgical cuff) into each end of the artery and vein and secure this with 7-0 silk sutures.

78 Insert the inlet and outlet of the engineered cardiac tissue with the built-in networks into the cuffs and seal them with tissue glue (cyanoacrylate).

79 Remove the clamps to re-establish blood perfusion. Becaue the vascular scaffold is transparent, any blood perfusion will be visible.

80 Close the skin incision with 4-0 silk or surgical staples.

81 Once the incision is closed, transfer the animal to a recovery cage and place it under a heat lamp. For postoperative pain management, administer ketoprofen (5 mg/kg, 24 h, s.c.) for 2 d.

! CAUTION In the event of postoperative bleeding or wound infections, kill the afflicted animal(s) by cervical dislocation under anesthesia (isoflurane).

82 7 d after the surgery, kill the animals by cervical dislocation under anesthesia (isoflurane) and surgically extract the AngioChip cardiac tissue implant. Partial hind-limb tissue of the animal is explanted with the AngioChip cardiac tissue implant during extraction.
! CAUTION All procedures involving live rats should be performed in agreement with institutional and national regulations. Animal euthanasia and surgical extraction procedures described in this step were performed at the Department of Comparative Medicine Animal Facility, University of Toronto, under a protocol approved by the University of Toronto Committee on Animal Care.

83 Immediately place the extracted implant in 10% neutral buffered formalin for 3 d at 4 °C. After this, wash the fixed implant excessively with PBS.

84 After fixation, use standard pathology services to embed the implant in paraffin, section the paraffin blocks into 3-μm sections and place them onto glass slides. After deparaffinizing the tissue sections, perform standard hematoxylin and eosin staining, as well as immunostaining for smooth-muscle actin. In our case, we used the Pathology Research Program Laboratory, University Health Network, Toronto, for tissue embedding, sectioning and staining. Histological preparation and immunostaining of the tissue implant can also be performed in-house, according to published protocols[63–65].

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
26	POMaC prepolymer solution does not fill the PDMS mold completely	POMaC prepolymer is too viscous and requires more time to fill the mold. Alternatively, the outlet is blocked	For a PDMS mold with features that are more complex or that have large footprint, a longer perfusion time might be required. Increase the perfusion time to 2–3 d. Alternatively, multiple inlets and outlets can be built into the design to accelerate perfusion
28	Detachment of UV-cross-linked POMaC from glass slides	Overexposure of POMaC prepolymer or the PDMS mold is too stiff	Reduce the time of UV exposure to achieve the minimal threshold of cross-linking in the POMaC prepolymer to allow for mold removal. Additional UV cross-linking can be applied after 3D stamping, if desired. Alternatively, reduce the stiffness of the PDMS by reducing the curing agent/polymer base ratio or curing time. A softer PDMS mold will encourage the release of patterned POMaC features
32	Bond failure between two patterned POMaC sheets	Underexposure in the bonding step (Step 31) or overexposure of molded POMaC in the initial UV-cross-linking step (Step 27).	A thin layer of non-cross-linked POMaC prepolymer should appear on the PDMS mold in Step 29 after mold release. If not, reduce UV exposure in Step 27 and increase UV exposure in Step 31
42	Collapse of AngioChip inlet and outlet after bioreactor assembly	Excessive pressure was applied when tightening the screws of the bioreactor	Reduce the pressure by loosening the screws. Avoid large torque by using a small Allen key to tighten the screws
44	Trapped air bubbles within AngioChip channels	Generation of air bubbles as a result of elevation of temperature when transferring the bioreactor to the incubator	Centrifuge the bioreactor at 1,000 revolution per minute for 1 min to flush out the air bubbles. Tilt the bioreactor at a 45° angle for a longer period of time in the incubator in Step 44
51	Insufficient endothelial cell coverage in the AngioChip network	Insufficient cell attachment at the seeding stage (Step 47) or lack of medium perfusion	Assess whether there is indeed no flow in the AngioChip network after endothelial cell seeding in Step 48. If not, adjust the inlet and outlet pressure by removing or adding more culture medium to maintain a static condition during cell attachment in Step 48. Alternatively, tilt the bioreactor at a 45° anlge in Step 51 to increase the perfusion rate, which accelerates endothelial proliferation
59	Failure to re-establish flow after seeding of parenchymal cells	Blockage of AngioChip networks due to leakage of hydrogels from the parenchymal space into the microchannels	When seeding, apply a gentle pressure head to both the inlet and outlet to prevent the hydrogel from flowing into the AngioChip network. This can also be improved by ensuring a confluent endothelium coverage before parenchymal cell seeding

Timing

Steps 1–5, fabrication of PDMS molds for AngioChip: 3 d
Steps 6–16, chemical synthesis of POMaC prepolymer: 2 d
Steps 17–21, preparation of POMaC polymer mixture for photo-cross-linking: 1 h
Step 22–26, injection molding of POMaC polymer: 1 d
Steps 27–37, AngioChip scaffold construction by 3D stamping: 1 d
Steps 38–44, assembly of the AngioChip scaffold on a bioreactor: 1 d
Steps 45–51, endothelialization of the AngioChip network: 2 d
Steps 52–59, culture of 3D tissue on AngioChip scaffolds: 7 d
Steps 60–63, AngioChip perfusion and permeation analysis: 1 d
Steps 64–69, (optional) immunostaining of AngioChip tissues: 1 d
Step 70A, (optional) assessment of urea secretion by liver tissues: 1 d
Step 70B, (optional) study of terfenadine drug metabolism by liver tissues: 1 d
Step 70C, (optional) study of drug-stimulated response of cardiac tissues: 1 d
Steps 71–84, (optional) direct surgical anastomosis of AngioChip tissues and immunohistochemical analysis of implanted AngioChip tissues: 10 d

Anticipated results

In this protocol, we demonstrate the ability of the AngioChip platform to achieve its anticipated functionality and desired responsiveness of viable tissues to different drug stimuli. Specifically, when characterizing the engineered tissues, seeded endothelial cells can cover the entire inner luminal surface of the AngioChip network under low-shear-stress (<1 dyne/cm^2) medium perfusion. This is visualized with endothelial cell surface marker (CD31) staining of the endothelialized AngioChip network on day 7 (Fig. 5a–d). Endothelial cells also express intercellular tight junctions (highlighted by staining withVE-cadherin) over the entire inner luminal surface of the AngioChip network (Supplementary Fig. 2). Coated endothelium forms a biological barrier that effectively shields the passage of large proteins, as shown by the perfusion of high-molecular-weight fluorescent dextran (Fig. 5e). We also illustrate that the endothelial layer can sprout out into the parenchymal space through the patterned microholes of the channel walls, an important first step in angiogenesis (Fig. 5f, Supplementary Fig. 3). However, long-term cultivation, in addition to the inclusion of stromal cells, might be required to mature the newly sprouted microvasculature. When the endothelium is stimulated with TNF-α, human monocytes perfuse through the network and can readily attach to the endothelium, mimicking the vascular inflammation response (Fig. 5g).

Encapsulated parenchymal cells remodel their ECM and form into 3D cardiac or hepatic tissues around the AngioChip scaffolds (Fig. 6a,d). Cardiac tissues begin to spontaneously beat 2 d post cell seeding. Continuous cultivation for 7 d yields AngioChip cardiac tissues with contractile and structural protein characteristics similar to those of native striated cardiac tissues, as visualized by immunostaining (Fig. 6b). AngioChip cardiac tissues also display active contractile properties responsive to epinephrine drug stimulation and electrical impulse; these properties propagate throughout the entire tissue, as characterized by optical mapping (Fig. 6c). Hepatic tissues display metabolic activity, as characterized by the immunostaining of secreted albumin in the tissues and mature morphology, with tight intercellular junctions, as shown by immunostaining with E-cadherin (Fig. 6d–f).

Finally, the demonstration of direct surgical anastomosis (Fig. 7) shows the versatility of AngioChip as an implantable tissue scaffold for regenerative medicine. The implanted AngioChip is perfused immediately after the surgical procedure, and the internal endothelium can be maintained for at least 1 week in vivo. At the termination of the study, implanted AngioChip cardiac tissues demonstrate the presence of myofibroblast around the tissue construct, a sign of tissue remodeling during integration (Supplementary Fig. 4). In this protocol, we offer a generic design of the AngioChip scaffold. However, with the increasing 3D complexity achieved by 3D stamping, AngioChip presents a wide range of opportunities to tackle important engineering challenges in both regenerative medicine and drug discovery.

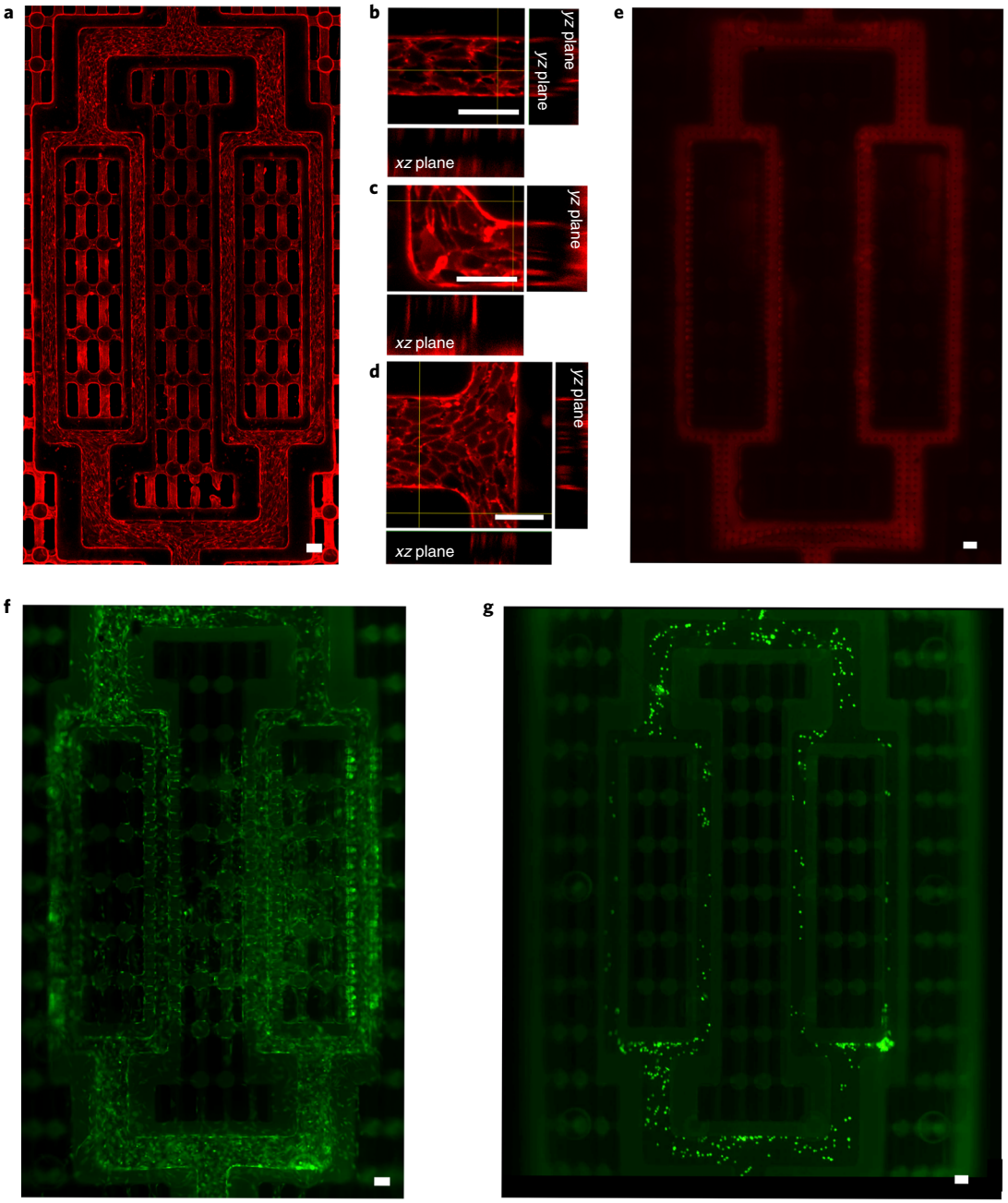


Fig. 5 | Endothelialized lumen of AngioChip scaffold and sprouting of endothelial cells. a–d, Full view of immunostained (CD31, red) internal vascular network of AngioChip scaffolds populated with human umbilical cord endothelial cells 7 d after cell seeding. Endothelial cells elongate and self-assemble into interconnected networks, providing coverage to a straight segment (**b**), a corner (**c**) and a branch (**d**) of the AngioChip scaffold. **e**, Image of 70 kDa fluorescent dextran (red) perfusing an endothelialized AngioChip network on day 2 after cell seeding. **f**, Sprouting of human umbilical cord endothelial cells (labeled green with CFDA according to the manufacturer's protocol) through patterned microholes (20 μm) on the side walls can be clearly identified by fluorescence microscopy. **g**, Image of THP-1 human monocytes (labeled green with CFDA) attaching to the endothelial cell-coated AngioChip network after TNF-α treatment (50 ng/mL). Scale bars, 100 μm.

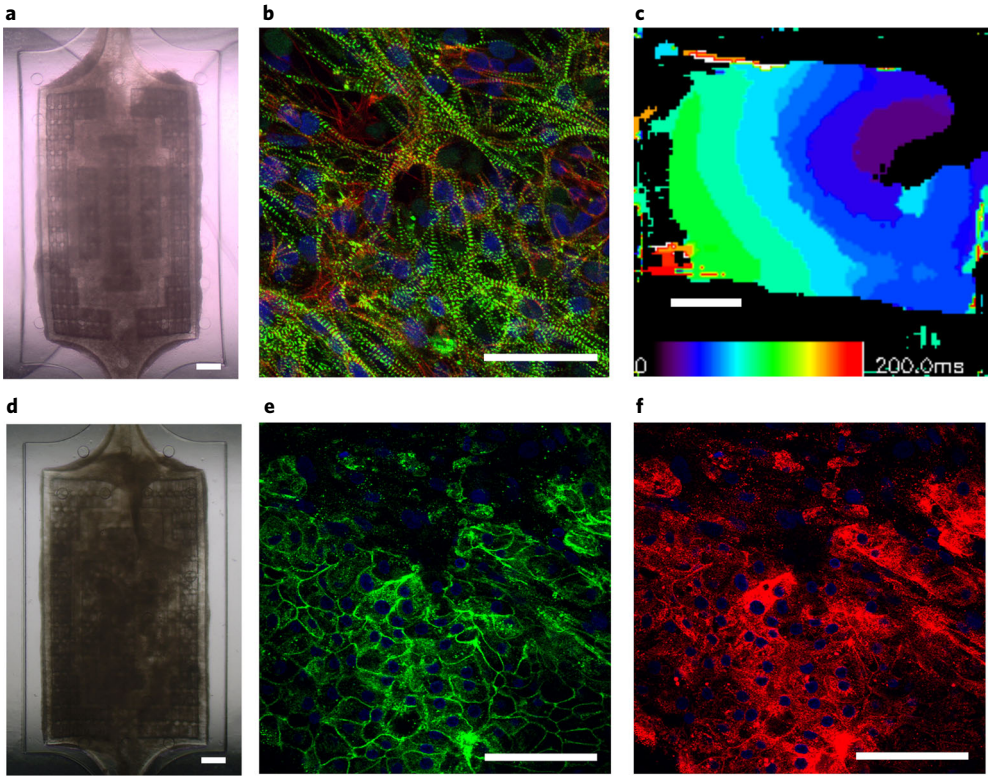


Fig. 6 | Characterization of self-assembled tissues after 7 d of cultivation. a, Bright-field image of cardiomyocytes remodeled into a contractile cardiac tissue. **b**, Immunostaining for sarcomeric-α-actinin (contractile protein, green) and F-actin (structural protein, red) of elongated cardiomyocytes on an AngioChip scaffold. **c**, Optical mapping of the activity of cardiac tissue labeled with DI-4-ANEPPS dyes after 7 d of self-assembly on AngioChip scaffolds. **d**, Bright-field image of hepatocytes remodeled into a hepatic tissue. **e**,**f**, Immunostaining of E-cadherin (**e**, green) and albumin (**f**, red) secreted from a functionally active hepatic tissue after 7 d of cultivation. Scale bars, 200 μm (**a**,**d**); 50 μm (**b**); 1 mm (**c**);100 μm (**e**,**f**).

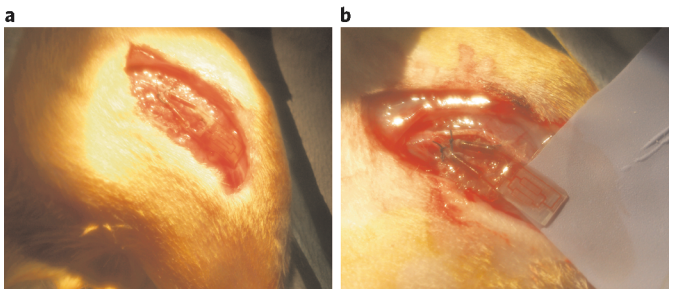


Fig. 7 | Direct surgical anastomosis of vascularized cardiac AngioChip scaffolds to hind limbs of an adult Lewis rat. **a**,**b**, Representative images of surgical implantation of an AngioChip cardiac tissue and vascular anastomosis into the femoral artery and vein of a Lewis rat. The AngioChip is endothelialized with endothelial cells isolated from inferior venae cavae of 6–8-week-old Lewis rats. Neonatal cardiomyocytes from Lewis rat donors were used to assemble the cardiac tissue. The vascularized cardiac AngioChip is cultured in the bioreactor for 7 d before implantation. Surgical procedures were performed at the Department of Comparative Medicine Animal Facility, University of Toronto, under a protocol approved by the University of Toronto Committee on Animal Care.

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Author contributions

B.Z. and M.R. designed the research; B.Z., L.D.H., and M.M. performed the research; B.Z. and M.R. analyzed the data; B.Z. and R.X. prepared the figures for the paper; B.Z., B.F.L.L., and R.X. prepared the supplementary video; B.Z., B.F.L.L., and M.R. wrote the manuscript.

Competing interests

M.R. and B.Z. are among the co-founders of TARA Biosystems and they hold equity in this company. The AngioChip is licensed to TARA Biosystems. The remaining authors declare no competing interests.

Additional information

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