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Chapter 24

3D Anastomosed Microvascular Network Model with Living Capillary Networks and Endothelial Cell-Lined Microfluidic Channels

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Abstract

This protocol describes detailed practical procedures for generating 3D intact and perfusable microvascular network that connects to microfluidic channels without appreciable leakage. This advanced 3D microvascular network model incorporates different stages of vascular development including vasculogenesis, endothelial cell (EC) lining, sprouting angiogenesis, and anastomosis in sequential order. The capillary network is first induced via vasculogenesis in a middle tissue chamber and then EC linings along the microfluidic channel on either side serve as artery and vein. The anastomosis is then induced by sprouting angiogenesis to facilitate tight interconnection between the artery/vein and the capillary network. This versatile device design and its robust construction methodology establish a physiological microcirculation transport model of interconnected perfused vessels from artery to vascularized tissue to vein.

Key words 3D microvascular network, Microfluidic chip, Vasculogenesis, EC lining, Sprouting angiogenesis, Anastomosis, Non-physiological leakage, Organ-on-a-chip

1 Introduction

The cardiovascular system plays a vital role in maintaining homeostasis of the human body, which allows blood to circulate throughout body for gas exchange and mass transportation to maintain organ viability. In order to construct a more physiological organ-on-a-chip, it is critical to be able to construct a functional 3D microvasculature system with a closed network of arteries, veins, and capillaries [1, 2].

On that basis, different research groups around the world have applied microfluidics to develop 3D in vitro microvascular models

* These authors contributed equally to this work.

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that physiologically mimic the vascular biology [3]. For cell and tissue engineering applications, microfluidic technologies were first used to confine cells in patterned, restricted regions to create microenvironments with well-controlled cellular interactions. More recently, microfluidic researchers have demonstrated control of the complex microenvironment at physiological levels by regulating chemical factors (molecular gradients etc.) or mechanical factors (interstitial flow etc.). Currently, there are two strategies to develop a 3D microvascular model. One strategy is to create microfabricated vessel scaffolds with specific geometries and dimensions, and line their inner surface with endothelial cells (ECs). The advantage of this method is that the vessel diameter can be precisely controlled, and the tightness of EC junctions can be flexibly adjusted by imposing different shear stress parameters on these lined ECs [4–6]. The other strategy is to seed cells in 3D extracellular matrices (ECMs) to allow spontaneous formation and remodeling of vascular networks through vasculogenesis and angiogenesis, which can closely mimic vascular development in vivo [7–12]. However, the microvascular network pattern embedded in 3D ECMs cannot be controlled and easily perfused [13].

In this protocol, we introduce an advanced 3D microvascular network model with intact perfused physiological blood vessels. By combining the two strategies mentioned above, we have engineered a system with tight interconnections between artery/vein and the capillary microvasculature network without non-physiological leakage. The major procedures involved in this advanced 3D microvascular network model are: (1) The capillary network formation is induced via vasculogenesis in a middle tissue chamber, and ECs are lined along the microfluidic channel on both sides to serve as the artery and vein. (2) Anastomosis is induced bidirectionally through sprouting angiogenesis to guarantee a good connection between the lined ECs along the microfluidic channels and the capillaries inside the tissue chamber [14]. Flow of fluorescent microparticles confirms perfusability of the lumenized microvascular network. In addition, minimal leakage of 70 kDa FITC-dextran confirms physiologic integrity of the interconnections between the artery/vein and the capillary network, which is critically important for drug screening applications [15]. This model can provide a promising avenue for future integration of multiple organs-on-a-chip through the vascular interface between arteries and veins [16, 17].

Building on our previous publications [18–22], this protocol seeks to provide detailed practical procedures to develop a complete and contiguous 3D perfused microvascular network without non-physiological leakage. The enabling technologies detailed here include microfluidic device design and microfabrication, cell-seeded hydrogel preparation and loading, maintenance of cell culture in the microfluidic chip, flow control for different stages of vascular development, and immunostaining of the vascular network.

2 Materials

2.1 Reagents

1. 5% hydrofluoric acid (HF).
2. Photoresist SU-8 2050 (Microchem).
3. Photoresist developer (Microchem).
4. Isopropyl alcohol (IPA).
5. Poly(dimethylsiloxane) (PDMS, Sylgard 184 Silicone Elastomer Kit, Dow Corning).
6. Trichloro(1H,1H,2H,2H-perfluorooctyl)silane.
7. 10 mg/mL fibrinogen: Weight out 28.8 mg of fibrinogen (>75% Clottable Bovine Fibrinogen, Sigma-Aldrich) (*see* **Note 1**) and dissolve in 2 mL of warmed DPBS by flicking the tube (*see* **Note 2**). Put the tube back to water bath for 10–15 min. After fully dissolved, filter fibrinogen solution using 0.22 μm syringe filter. This solution should be made fresh for each experiment.
8. Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+}.
9. 50 U/mL thrombin (from bovine plasma, Sigma-Aldrich) in phosphate-buffered saline (PBS).
10. Full supplement endothelial cell growth medium (EGM-2, Lonza).
11. EGM-2 without VEGF and bFGF. To prepare EGM-2 without VEGF/bFGF, remove the VEGF and bFGF growth factor bullets from the kit, and then add the remaining growth factor bullets. Save the VEGF and bFGF growth factor bullets at −20 °C for future use.
12. 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle's Medium (DMEM).
13. Human endothelial colony forming cell-derived ECs (ECFC-ECs), passages 4–7.
14. Normal human lung fibroblasts (NHLFs, Lonza), passages 5–8.
15. Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}.
16. 0.05% trypsin–EDTA solution.
17. 1 mg/mL laminin solution: Thaw laminin stock solution (mouse natural laminin, Thermo Fisher) slowly at 4 °C and aliquot into 100 μL tubes (*see* **Note 3**). Keep 1–2 aliquots at 4 °C for experiment and short-term storage.
18. 4% paraformaldehyde (PFA).
19. Sterile PBS.
20. 0.05% Tween 20 in PBS.

21. DAPI solution: 0.5–1 μg/mL DAPI in sterile PBS.
22. Blocking solution: 5–10% bovine serum albumin (BSA) or goat serum in PBS, sterile filtered.
23. 25 μg/mL 70 kDa-FITC dextran (Sigma-Aldrich) in sterile PBS.
24. Deionized water.
25. Primary and secondary antibodies, as desired.
26. 15 μm fluorescent microspheres (Thermo Fisher), supplied in 10^6 beads/mL concentration.

2.2 Equipment and Tools

1. Silicon wafers, 3 in. in diameter.
2. Spin coater.
3. Vacuum desiccator.
4. Petri dish.
5. Tweezers.
6. Oven.
7. Hotplate.
8. UV lamp with long pass filter (eliminate UV radiation below 350 nm).
9. Oxygen/air plasma cleaner.
10. Glass slides (75 × 50 mm) and glass coverslips.
11. Hole punchers, tip inner diameter of 1 mm.
12. Disposable scalpels.
13. 1.5 mL cryo vials.
14. Autoclave.
15. Incubator with 37 °C, 5% CO_2, 20% O_2.
16. Sterile syringe, 1 mL.
17. 21 gauge blunt end needle.
18. Sterile syringe filter, 0.22 μm.
19. Water bath.
20. T75 flask.
21. Hemacytometer.
22. Benchtop centrifuge.
23. 0.5 mL and 1.5 mL snap cap tube.
24. Double-sided tape.
25. Transparent tape.
26. BSL-2 laminar flow hood.
27. Micropipettors.
28. Inverted epifluorescence microscope.
29. Inverted microscope.
30. High speed cooled CCD camera.

3 Methods

3.1 Fabrication of SU-8 Silicon Mold and Its Silanization

1. After simulation of designed chip (Fig. 1), print out the photomask with high resolution.
2. Put a 3-in. silicon wafer into 5% HF for cleaning its surface, followed by a deionized water rinse, and dehydrate the wafer by placing it into the oven maintained at 120 °C for 10 min.
3. Place the silicon wafer on the spinner chuck of spin coater and apply vacuum (*see* **Note 4**).
4. Pour proper amount of SU-8 2050 on the center of the wafer (*see* **Note 5**).
5. Start the spin coater, and spin the wafer at 500 rpm for 20 s and then increase the speed to 130 × *g* for 30 s to achieve the desired thickness of 100 μm.
6. Place the photoresist-coated wafer into the oven or on the hotplate at 65 °C for 5 min.
7. Transfer the wafer to a hotplate/oven at 95 °C for 20 min (*see* **Note 6**).
8. Remove the baked wafer from the hotplate/oven and cool down to room temperature (RT) gradually.
9. Cover the photoresist-coated wafer with the photomask and bring them in tight contact (*see* **Note 7**).
10. Expose the wafer under UV light at 230 mJ/cm^2.
11. Move the exposed wafer to oven at 65 °C for 5 min, and then bake the wafer at 95 °C for 10 min.
12. Place the wafer into photoresist developer for proper time (*see* **Note 8**).
13. Spray the wafer with developer to remove the undeveloped photoresist, especially at the small features, and then wash the wafer with IPA.
14. Dry the wafer with pressurized filter nitrogen or air.
15. Place the SU-8 silicon mold into the desiccator and put a small drop of silane on coverslip close to the wafer, and evacuate the chamber to induce the evaporation of silane (*see* **Note 9**).
16. Remove the silicon mold from desiccator after overnight silanization.

3.2 Fabrication and Assembly of PDMS Microfluidic Device

1. Pour silicone elastomer base and curing agent at a weight ratio of 10:1 (base: curing agent) into an empty disposable plastic cup.
2. Mix the base and curing agent vigorously using a disposable plastic knife until the PDMS mixture appears opaque.
3. Attach the silicon mold to the bottom surface of petri dish with double-sided tape.

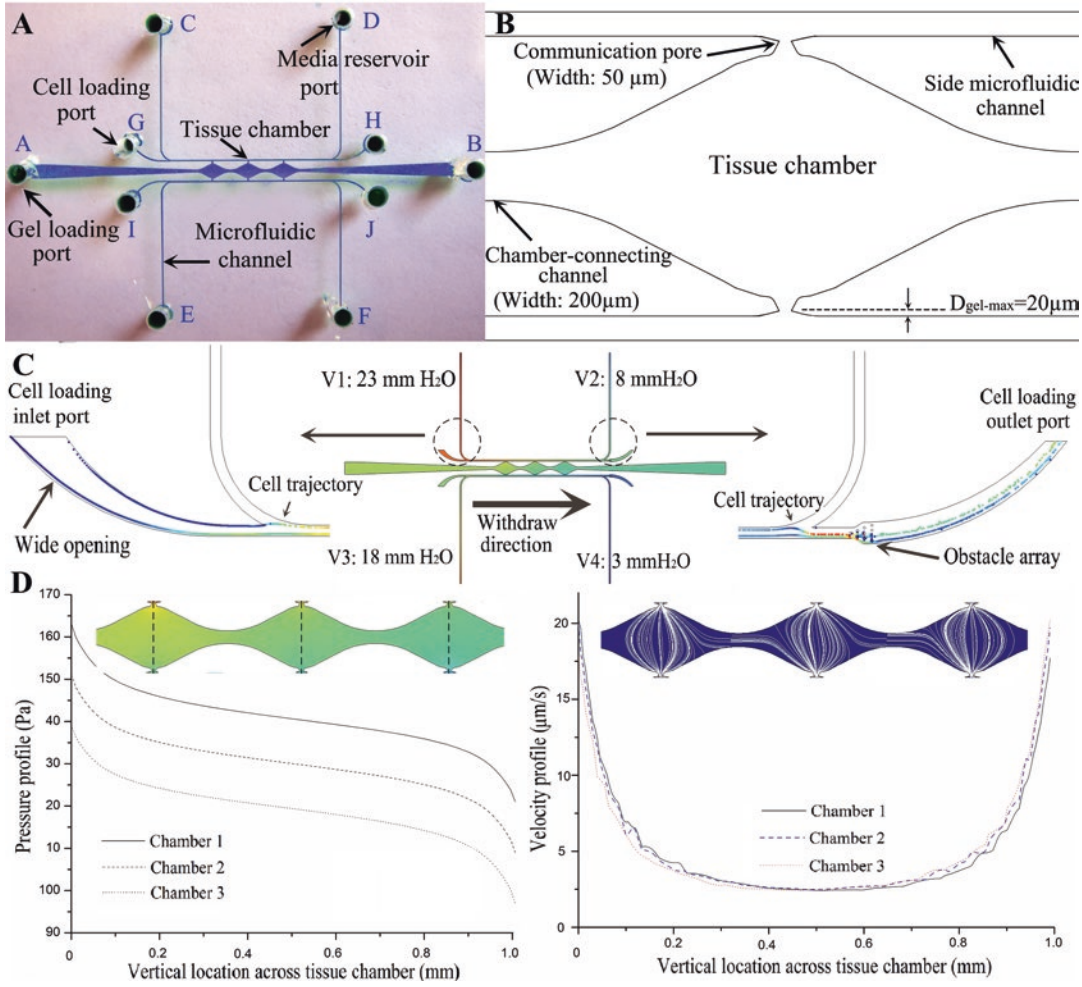

Fig. 1 Chip design and simulation. (**a**) Top-view of channel structure and ports for gel loading, cell loading and media reservoir. The entire device structure consists of three central millimeter-sized diamond tissue chambers (1 × 2 mm) and two side square cross-sectional microfluidic channels (100 × 100 μm) that connect to the tissue chambers through a series of communication pores. (**b**) Schematic and dimensions of one tissue chamber with optimized communication pore design to prevent gel bursting (or leaking) into side microfluidic channels from pressure built-up during the gel loading process. (**c**) Simulation result on pressure distribution inside the entire device, and the particle tracing from cell loading inlet port with wide opening to cell loading outlet port with obstacle array through withdrawal mode. (**d**) Simulation results on pressure drop and velocity profile of interstitial flow across three tissue chambers. Due to the fully symmetrical configuration of communication pores along both side microfluidic channels, the pressure drop across each tissue chamber from the top communication pore to the bottom one is almost the same ($\Delta P_{chamber} \sim 5$ mmH_2O), which induces a uniform interstitial flow profile inside each tissue chamber to stimulate vasculogenesis. Reproduced from Wang et al. [21] with permission from Royal Society of Chemistry (RSC)

4. Use compressed nitrogen to gently blow off dust particles from the silicon mold.
5. Pour approximately 20–25 g of the PDMS mixture onto the silicon mold inside the petri dish, and approximately 5 g of the PDMS mixture into an empty petri dish to fabricate a thin PDMS sheet.
6. Place both petri dishes into a vacuum desiccator to degas until the PDMS mixture becomes transparent.
7. Transfer the petri dishes covered with lid to the oven at 60 °C for 4–5 h to fully cure PDMS.
8. Remove the petri dishes from the oven and cool down to RT.
9. Carefully remove the PDMS microfluidic chip from the silicon mold and the PDMS sheet from the petri dish by cutting around the perimeter of the device using a scalpel and trim to proper size.
10. Punch holes at inlets and outlets of the PDMS microfluidic chip using a hole puncher.
11. Remove debris inside the punched holes using a compressed nitrogen gun.
12. Clean both sides of the PDMS microfluidic chip and PDMS sheet with transparent tape.
13. Treat the cleaned PDMS microfluidic chip and PDMS sheet inside a plasma cleaner for 3 min at 250–300 mTorr.
14. Quickly remove these two plasma-treated pieces from the plasma cleaner and bond them together.
15. Place the bonded two-layer device into the oven at 120 °C for 10 min.
16. Treat the PDMS sheet of bonded device and a glass slide (or a glass coverslip) inside the plasma cleaner for 1 min at 250–300 mTorr.
17. Quickly bond the PDMS sheet and the glass slide to make a three-layer microfluidic device (Fig. 2a).
18. Place the bonded three-layer device into the oven at 120 °C for 10 min.
19. Place pipette tips into all inlets and outlets of bonded microfluidic device to prevent PDMS seeping into these punched holes.
20. Cut off the bottom of plastic cryo vials using a scalpel.
21. Immerse the edge of bottomless plastic vials into PDMS mixture and attach them to all medium inlets and outlets of the microfluidic device.

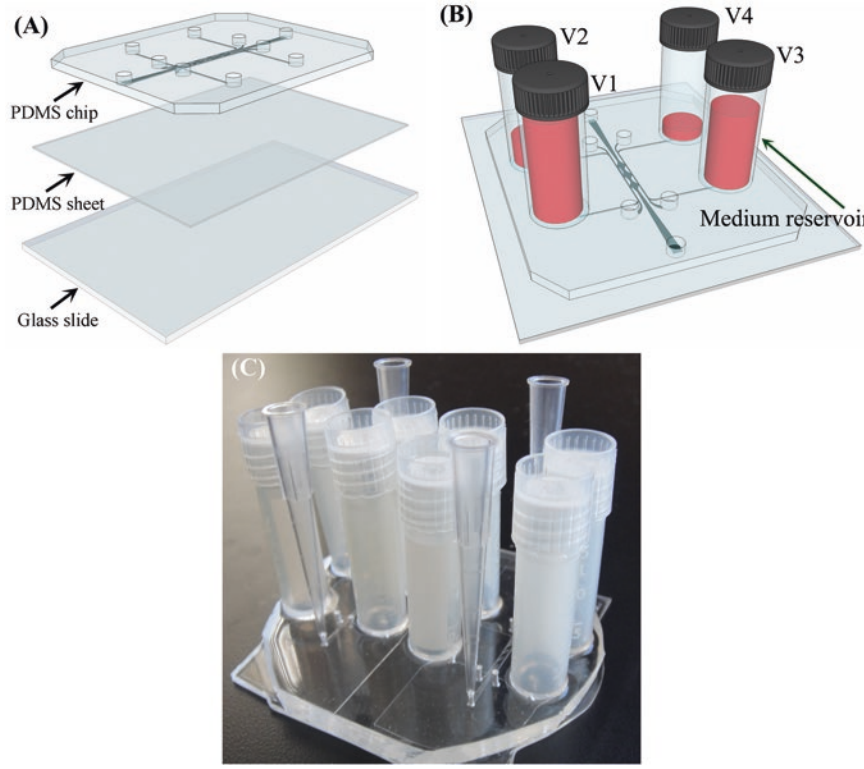

Fig. 2 Fabrication and assembly of PDMS microfluidic device. (**a**) Three layer microfluidic device design. *Top layer* is the PDMS microfluidic chip, the *middle layer* is the thin PDMS sheet, and the *bottom layer* is the glass slide. (**b**) Schematic of decoupling design with four media reservoirs containing different medium volume that could separately control flow to the tissue chamber for vasculogenesis and along the microfluidic channel for EC lining. (**c**) Prototype of assembled microfluidic device with plastic vials. Reproduced from Wang et al. [21] with permission from RSC

22. Place the assembled microfluidic device into the oven at 60 °C overnight to fully cure PDMS (Fig. 2b).
23. Sterilize the microfluidic device by autoclaving at 121 °C for 30 min before experiment (Fig. 2c).

3.3 Cell Harvesting and Cell-Seeded Hydrogel Preparation

1. Warm EGM-2, DPBS, and trypsin in water bath at 37 °C for 20–30 min before experiment.
2. Wash ECFC-ECs and NHLFs with 5–10 mL of HBSS 1–2 times.
3. Aspirate wash solution and add 2 mL of trypsin for each T75 flask to harvest cells.
4. Agitate and place the flasks in incubator for 20–30 s.
5. Remove the flasks from incubator and check under microscope for cell detachment.

6. Stop trypsin with 4 mL of EGM-2 per flask, transfer the cell suspensions into separate conical tubes and rinse the flasks with another 4 mL of EGM-2. Combine the rinsed media into conical tubes for a final volume of 10 mL.
7. Count both ECFC-ECs and NHLFs using a hemocytometer to determine the amount of cells harvested.
8. Centrifuge cells at 300 × *g* for 3 min.
9. Aspirate the supernatants and reconstitute cells in EGM-2 at a density of 10^6 cells/mL.
10. Prepare cell matrix solution: The desired cell concentration for ECFC-ECs and NHLFs is both 5×10^6 cells/mL of fibrinogen solution, for a combined concentration of 10^7 cells/mL. For example, after harvesting each cell type, resuspend the cell pellet in medium at 10^6 cells/mL, take out 500 μL of ECFC-ECs and 500 μL of NHLFs and combine in a single tube.
11. Centrifuge cells at 300 × *g* for 3 min and resuspend the combined pellet in 100 μL of sterile filtered fibrinogen solution (*see* **Note 10**).

3.4 Cell-Seeded Hydrogel and Medium Loading into Device

1. Aliquot 2 μL of thrombin into a 0.5 mL snap cap tube for each device planned to load (*see* **Note 11**).
2. Put a filter pipette tip of 200 μL at one end of gel loading pore to prevent spilled out hydrogel.
3. Gently mix and take out 15 μL of cell-seeded fibrinogen suspension without any air bubble (*see* **Note 12**).
4. Quickly mix with the thrombin droplet 2–3 times in the 0.5 mL snap cap tube without generating air bubbles (*see* **Note 13**).
5. Quickly inject the mixed suspension into the loading pore and gently push the gel through to the other side with steady pipette pressure (*see* **Note 14**).
6. Slowly place the microfluidic device down and twist the pipette tip out of the micropipettor to eject (*see* **Note 15**).
7. Leave the device inside the hood for 1–2 min before bringing out to check under the microscope. During this time, load the next device.
8. After checking under microscope, transfer the device into incubator at 37 °C and incubate for 10–15 min to let the gel fully polymerize (Fig. 3). While waiting for gel polymerization, thaw laminin solution from 4 °C to RT 30–45 min before loading medium.
9. After incubation, pipette 3–4 μL of laminin solution into the cell lining inlets to coat the inner surface of both microfluidic channels adjacent to the tissue chamber (*see* **Note 16**).

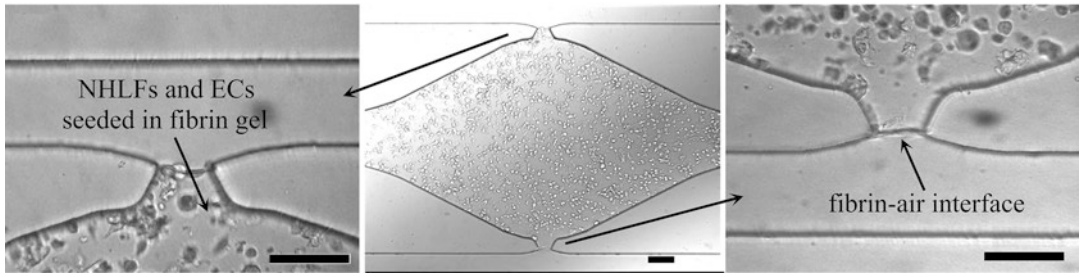

Fig. 3 Cell-seeded fibrinogen gel loading. The optimized communication pore design can precisely control the gel at the vertexes of communication pores with a flat gel-air interface without bursting into the microfluidic channels. This enabled the formation of a smooth EC monolayer on the gel interface at the communication pore, a critical condition for EC lining and sprouting angiogenesis. Scale bars, 100 μm. Reproduced from Wang et al. [21] with permission from RSC

10. Block all cell lining inlets and outlets with PDMS plugs (*see* **Note 17**).
11. Take 200 μL of full supplement EGM-2 and load through one medium inlet inside the plastic vial (*see* **Note 18**).
12. Wait until medium droplet spills out to the medium outlet inside the other plastic vial.
13. Repeat **steps 11** and **12** to load medium into the other side channel.
14. Slowly twist pipette tips out of the medium inlets and let medium form droplets.
15. Add appropriate amount of EGM-2 into each plastic vial to achieve desire pressure drop (V1: 23 mm H_2O, V2: 8 mm H_2O, V3: 18 mm H_2O, V4: 3 mm H_2O) and mark the medium levels with a marker (*see* **Note 19**).
16. Place caps onto all plastic vials and unscrew halfway to allow gas exchange.
17. Place device into a secondary container with sterile water underneath to maintain humidity and transfer to a 37 °C, 20% O_2, 5% CO_2 incubator (*see* **Note 20**).
18. After 1 h, check back to see if there is any air bubble in both side channels. If there is, remove it by pipetting medium through the medium inlet to push air bubble out (*see* **Note 21**).

3.5 Medium Changing and Vasculogenesis

1. Keep the device inside incubator with full supplement EGM-2 for 2 days before changing medium.
2. On day 2 post-embedding, warm EGM-2 without VEGF/bFGF for 20–30 min in the water bath.
3. Aspirate old medium, leaving a small amount of liquid inside each plastic vial to avoid air bubble.

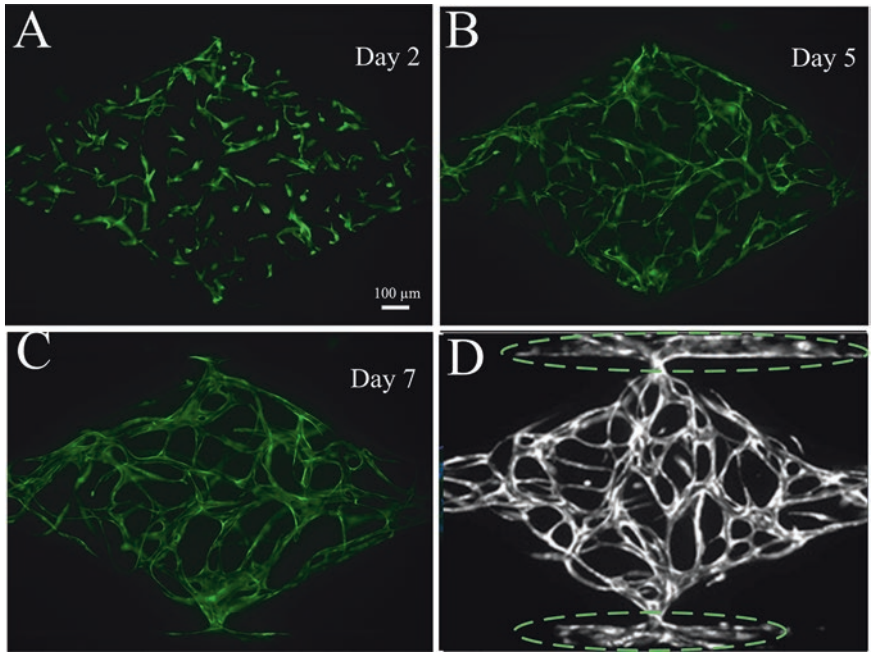

Fig. 4 Progression of vasculogenesis over time. (**a**) After establishing interstitial flow across the tissue chamber, ECFC-ECs formed vascular fragments as early as day 2. (**b**) A capillary network continued to develop through day 5. (**c**) By day 7, the capillary network was lumenized and interconnected. (**d**) The laminin coating inside the microfluidic channels promoted migration of ECs out from the tissue chamber through the communication pore and onto the surface of the channels, thereby facilitating interconnection of the capillary network with the outer channels. Reproduced from Wang et al. [21] with permission from RSC

4. Add fresh medium (EGM-2 without VEGF/bFGF) to each vial with the same height as that of **step 15** in Subheading 3.4.
5. Change medium for the device in similar manner every other day.
6. ECFC-ECs will form vascular fragments as early as day 2, and continue to develop into a capillary network through day 5. By day 7, the capillary network is lumenized and interconnected (Fig. 4a–c).
7. Laminin coating inside the microfluidic channels promotes ECs from the tissue chamber migrating through the communication pore outward and partially lining the inner surface of the channels (Fig. 4d).

3.6 EC Lining, Sprouting Angiogenesis, and Anastomosis

1. Harvest ECFC-ECs and resuspend cells in EGM-2 for a concentration of 2×10^7 cells/mL.
2. Remove medium from the plastic vials, leaving a small amount to avoid air bubble.

3. Block medium inlet and outlet of one side microfluidic channel with plugs.
4. Slowly remove plugs from cell lining inlet and outlet and add 1–2 μL of medium to cover the inlet and outlet and prevent air bubbles.
5. Mix and take out 5 μL of cell suspension using a micropipettor and insert the pipette tip into the cell lining inlet.
6. Insert a bent syringe needle connected with an empty 1 mL syringe into the cell lining outlet (*see* **Note 22**).
7. Place the device under the microscope.
8. Slowly pull syringe to withdraw cells from cell lining inlet toward cell lining outlet. Cells will flow through and accumulate inside the side microfluidic channel by the obstacle array near the cell lining outlet (*see* **Note 23**).
9. Monitor under the microscope and stop withdrawing when cells have filled up a whole microfluidic channel.
10. Alternatively, cells can be withdrawn through the cell lining outlet using a micropipettor instead of the bent syringe needle. Set micropipettor for 5 μL volume, insert an empty pipette tip to the cell lining outlet, and withdraw cells by releasing the micropipettor button.
11. Bring device into the incubator and incubate for 2 h to allow ECs to adhere to all inner side microfluidic channel walls.
12. Reverse flow gently from the cell lining outlet toward cell loading inlet to remove non-adhering ECs by gently pipetting 1–2 μL of medium through the cell lining outlet blocked with a filtered, empty pipette tip.
13. Remove pipette tips at the cell lining inlet and outlet and block with plugs.
14. Reestablish the same medium flow as that of **step 15** in Subheading 3.4.
15. Adhered ECs elongate to form a monolayer in response to shear stress generated by the pressure drop along the side microfluidic channel (Fig. 5).
16. After the EC lining process, a confluent EC monolayer will form on the gel interface at the communication pore (Fig. 6a).
17. Reverse the interstitial flow direction across tissue chamber by changing the hydrostatic pressure distribution (V1: 18 mm H_2O, V2: 3 mm H_2O, V3: 23 mm H_2O, V4: 8 mm H_2O) to induce the basal-to-apical transendothelial flow across tissue chamber.
18. Switch medium supplied to the microfluidic channel with higher pressure to full supplement EGM-2 to induce a positive VEGF gradient that promotes sprouting angiogenesis.

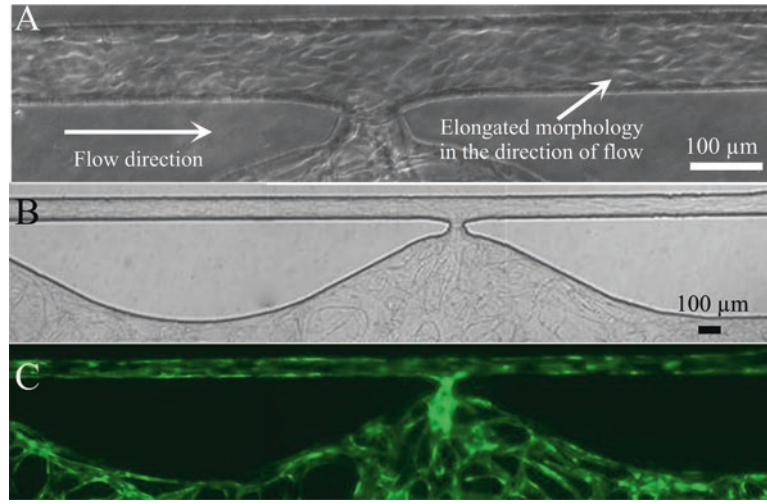

Fig. 5 EC lining along microfluidic channel. (**a**) ECs with elongated morphology in the direction of fluidic flow. (**b**) Bright field image of EC lining along entire microfluidic channel after vasculogenesis, which represent the artery (high pressure) and vein (low pressure) of our 3D microvascular model. (**c**) Corresponding fluorescent image. Reproduced from Wang et al. [21] with permission from RSC

19. Lined ECs at the cell–matrix interface invade into the ECM and form microvascular sprouts as early as 24 h post-lining (Fig. 6b).
20. Highly branched and abundant sprouts extend into ECM (Fig. 6c).
21. Anastomosis with tight interconnection is a bidirectional process. From inside the tissue chamber, ECs migrate outward to the laminin-coated microfluidic channel to connect with the EC-lined microfluidic channel during vasculogenesis. From the outer microfluidic channel, the lined ECs invade into the ECM and form sprouts to connect with the capillaries inside the tissue chamber during sprouting angiogenesis (Fig. 6d).
22. Repeat the EC lining process for the other side microfluidic channel 2 days after lining the first channel. An intact and perfusable microvascular network of artery/vein and capillaries can be developed in this microfluidic device via a multistep process through 12 days (Fig. 7).

3.7 Microparticle Perfusion

1. Remove medium from all plastic vials, leaving a small amount at the bottom to avoid air bubble.
2. Add microparticle solution to the plastic vial with the highest hydrostatic pressure (23 mm H_2O) and sterile PBS to the other plastic vials.
3. Monitor microparticle moving inside vessel lumen under an inverted (epifluorescent) microscope (Fig. 8a).

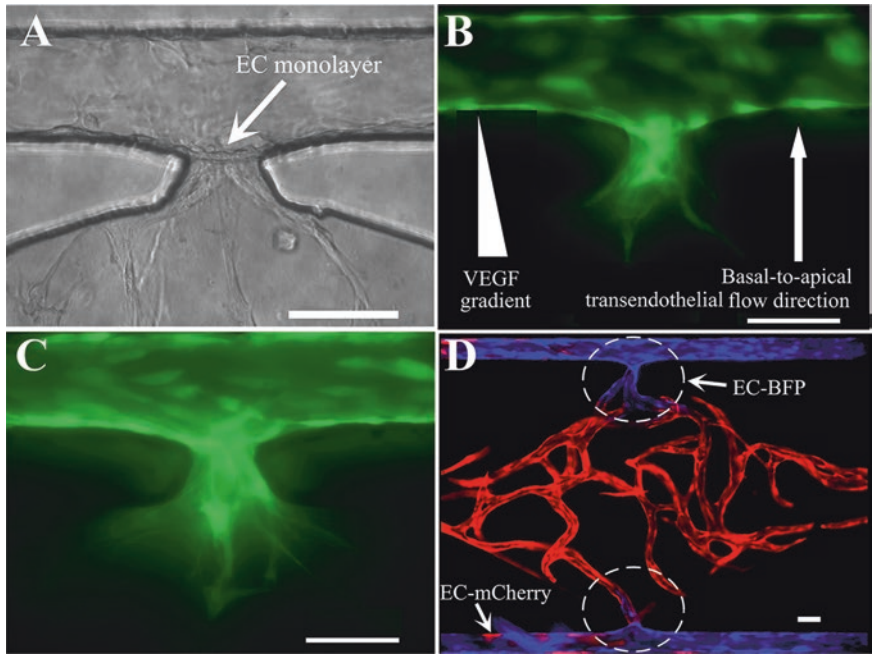

Fig. 6 Sprouting angiogenesis and anastomosis. (**a**) Formation of an EC monolayer on the gel interface at the communication pore after EC lining. (**b**) Invasion of microvascular sprouts from the lined EC monolayer into the gel after 24 h. (**c**) More sprouts and deep invasion after 48 h post-lining, which provided a precondition for anastomosis with the capillary network inside the tissue chamber. (**d**) Tight interconnection between EC lining along the outer channel and the capillary network inside tissue chamber by anastomosis with bidirectional migration. From inside the tissue chamber, mCherry-expressing (red) ECs (ECs-mCherry) migrated outward to the laminin-coated microfluidic channel to connect with the lined BFP-labeled ECs (ECs-BFP) in the channels. From the outer microfluidic channels, the lined ECs-BFP invaded into the 3D gel and formed sprouts to connect with the capillaries in the tissue chamber. Scale bars, 100 μm. Reproduced from Wang et al. [21] with permission from RSC

4. After microparticle perfusion, replace old PBS containing microparticles with sterile PBS and allow flow through for 20 min to flush away microparticles.
5. Replace sterile PBS with fresh medium and continue culture in incubator.

3.8 70 kDa-FITC Dextran Perfusion

1. Remove medium from all plastic vials, leaving a small amount at the bottom to avoid air bubble.
2. Add dextran solution to the plastic vial with the highest hydrostatic pressure (23 mm H_2O) and sterile PBS to the other plastic vials.
3. Monitor dextran flow under an inverted epifluorescence microscope (*see* **Note 24**).
4. After dextran perfusion, replace old PBS containing dextran with sterile PBS and allow flow through for 20 min (Fig. 8b).
5. Replace sterile PBS with fresh medium and continue culture in incubator.

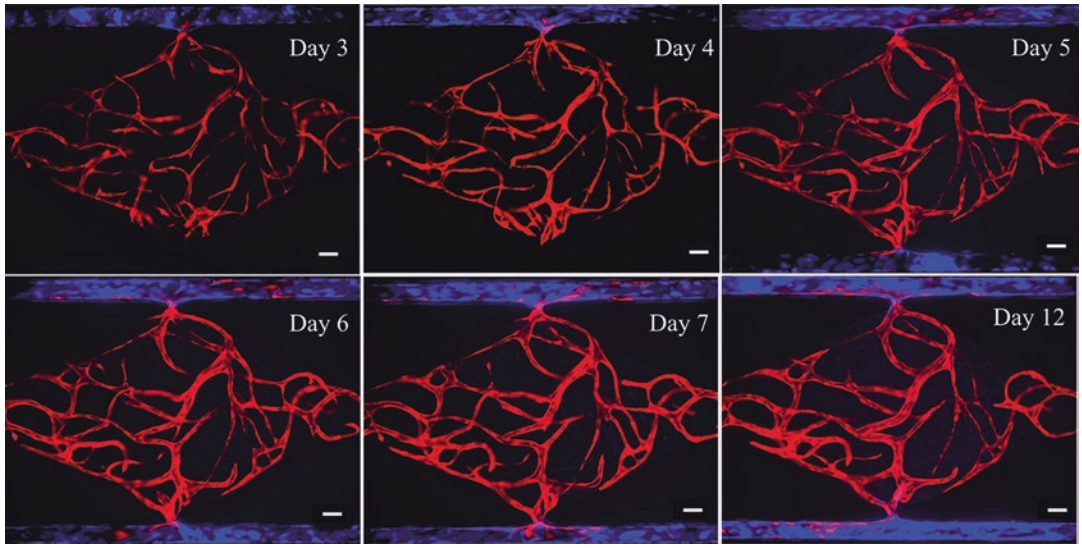

Fig. 7 Formation of intact and perfusable microvascular network with artery/vein and capillary network by multistep processes throughout 12 days. Vasculogenesis of ECs-mCherry started to occur 3 days after introducing cell-seeded fibrin gels into the tissue chamber. ECs-BFP were added to an outer channel at this point. After 24 h post-lining, these lined ECs-BFP began to elongate and form connections with ECs-mCherry through the communication pore. On day 5, the other channel was lined with ECs-BFP and the direction of interstitial flow, which was also the transendothelial flow direction, was reversed to promote remodeling of the capillaries inside the tissue chamber and to stimulate angiogenic sprouting of lined ECs. In addition, ECs-mCherry also migrated out from the tissue chamber and interlaced with ECs-BFP lining the microfluidic channel. ECs-BFP along both microfluidic channels continued to proliferate, elongate, and form connections with the microvascular network inside the tissue chamber thereafter. By day 12, a fully lumenized capillary network inside the tissue chamber had anastomosed with both EC-lined microfluidic channels to form the intact microvascular network. Scale bars, 100 μm. Reproduced from Wang et al. [21] with permission from RSC

3.9 Immunostaining of Vascular Network

1. Replace medium in all plastic vials with freshly thawed 4% PFA (*see* **Note 25**).
2. Fix device with 4% PFA for 15–20 min at RT inside a chemical fume hood.
3. Replace 4% PFA with sterile PBS and wash the device at RT for 1–2 h.
4. Replace washed PBS with sterile PBS (*see* **Note 26**).
5. For intracellular staining that requires cell membrane permeabilization, replace PBS with 0.05% Tween 20 in PBS and permeabilize for 1 h at RT.
6. After permeabilization, wash the device with sterile PBS.
7. Replace PBS with blocking solution and leave at 4 °C overnight.
8. Prepare primary antibody staining solution, 100–150 μL per device. Typically, start with antibody dilution 1:200 in blocking solution and optimize if needed.

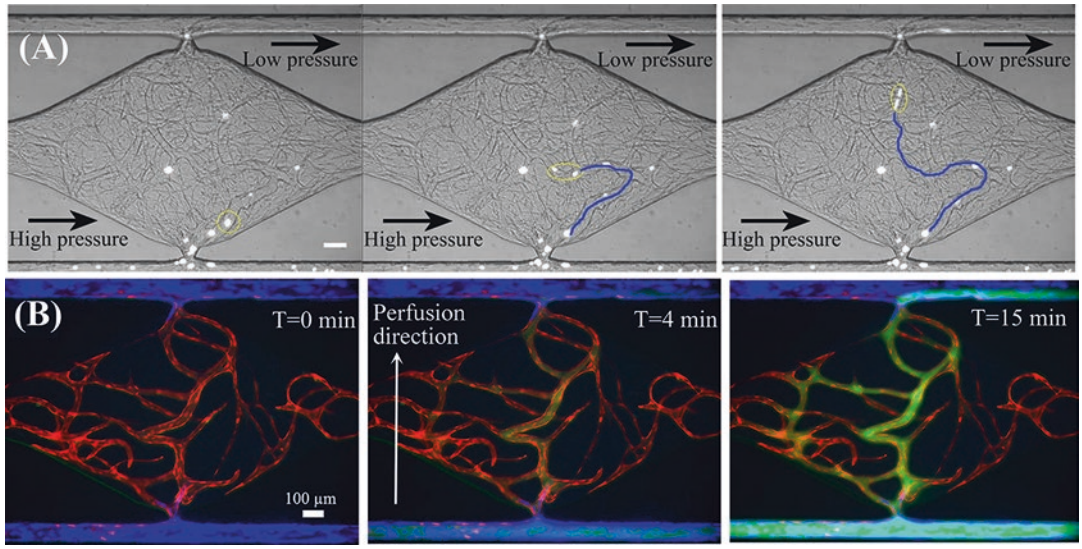

Fig. 8 Particle and dextran perfusion. (**a**) Perfusion of 15 μm fluorescent microparticles into the high pressure outer channel and these then flowed through the microvascular network from one communication pore to the other and out into the low pressure outer channel. (**b**) Fluorescence images showing 70 kDa FITC-dextran perfusion in the absence of non-physiological leakage. After loading dextran from left to right in an EC-lined microfluidic channel for 4 min (the lower channel in the figure), it quickly flowed into the capillary network through the communication pore. Over time, the fluorescence intensity in the lumen of microvessels increased due to the intensive dextran influx. Finally, dextran passed through the capillaries and flowed into the low pressure side microfluidic channel and exited within 15 min, validating the connectivity and perfusability of the microvascular network. More importantly, no dextran was observed outside the vessel during the 15 min perfusion, including the communication pore region where lined ECs connect to the microvascular network. This demonstrated the strong barrier property of the microvascular network, and the tightness of the anastomosis. Reproduced from Wang et al. [21] with permission from RSC

9. Remove blocking solution from all plastic vials, leaving a small amount at the bottom to avoid air bubbles.
10. Carefully remove the plastic vials without disrupting the blocking solution droplets.
11. Insert pipette tips with antibody staining solution to the medium inlets. Place empty pipette tips to the medium outlets (*see* **Note 27**).
12. Gently withdraw antibody dilution from the outlets using a micropipettor. Monitor for a small amount of liquid moving upward through the empty pipette tips.
13. Aspirate excess blocking solution at the inlets and outlets and leave the device at 4 °C overnight.
14. Reverse the flow direction by switching antibody dilution from the inlet to the outlet and leave at 4 °C for 4–6 h.
15. After primary antibody staining, wash the device with sterile PBS for 4–6 h (Fig. 9a).

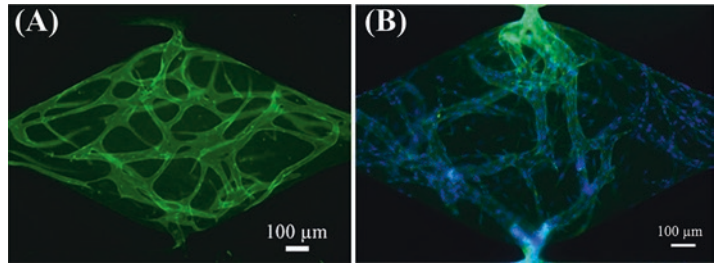

Fig. 9 Immunostaining image of a capillary network inside a tissue chamber. (**a**) CD31 immunostaining. CD31 (PECAM-1) is a cellular adhesion and signaling receptor that is highly expressed at endothelial cell–cell junctions, and has been shown to play an important role in maintenance of vascular barrier integrity. This CD31 immunostaining highlighted the integrity of the vascular network formed in our device. (**b**) DAPI nuclear staining. Reproduced from Wang et al. [21] with permission from RSC

16. Prepare secondary antibody staining solution, 100–150 μL per device. Typically for secondary antibody, start with 1:300 or 1:400 dilution in blocking solution.
17. Repeat the antibody staining process from **steps 11–13**.
18. After secondary antibody staining, wash device multiple times with sterile PBS to remove background.
19. For nuclei staining, replace washing PBS with DAPI solution and leave at 4 °C overnight.
20. Wash device multiple times with sterile PBS to remove excess DAPI (Fig. 9b).

4 Notes

1. Make extra fibrinogen to account for sterile filter after dissolved in DPBS.
2. Do not vortex the tube to dissolve the fibrinogen powder.
3. Do not thaw laminin stock solution directly to RT. For long-term storage, keep laminin at −80 °C.
4. Place silicon wafer at the center of spinner chuck to ensure a uniform thickness of the photoresist layer.
5. Slowly perform this step to prevent air bubble formation in the poured photoresist layer.
6. Keep the hotplate/oven on a flat surface to prevent the unwanted changes of photoresist film.
7. Ensure the ink printed side of photomask is in contact with the photoresist layer on the wafer.

8. Placing the container with the developer and wafer into an ultrasonic instrument will highly accelerate the development speed.
9. Perform the silanization procedure inside a ventilated chemical fume hood.
10. Prepare no more than 200 μL of cell–fibrinogen mixture per aliquot. If more cell-matrix volume is needed, divide combined cell pellet into multiple aliquots to assure desired cell density.
11. Ensure thrombin droplet is at the bottom of the tube.
12. Change pipette tips between mixing the solution and loading the solution into the device.
13. Perform this step quickly to avoid damaging the gel or creating uneven polymerization, as the cell matrix solution will begin polymerizing quickly upon mixing with thrombin.
14. Press the push-button smoothly and evenly.
15. Do not eject pipette tip by pushing the eject button on the micropipettor. Otherwise, gel will burst out.
16. Pre-coating side microfluidic channels with laminin before adding culture medium is crucial to ensure EC adherence to PDMS for EC lining, and stimulate EC migration outward from the tissue chamber to anastomose with the EC-lined microfluidic channels.
17. PDMS plug is fabricated by dipping pipette tip into PDMS mixture and allowing to fully cure at 60 °C overnight.
18. Perform this step slowly to avoid damaging the gel inside tissue chamber.
19. Calculate the volume based on the diameter of vial.
20. To minimize the medium evaporation from the plastic vials and maintain humidity, prepare a large pipette tip box with sterile water and a smaller pipette tip rack (or a small plastic petri dish) to place the device on top and separate from the water. Sterile water should be changed every other day.
21. Removing air bubbles formed inside the microfluidic channel as soon as detected is crucial after loading device. Air bubbles can block medium flow and damage gel inside the tissue chamber.
22. Bend a 21-gauge blunt end needle and attach to the syringe. Do not use slant end needle.
23. Withdraw slowly to avoid introducing air bubble into the microfluidic channel.
24. Do not perfuse dextran for more than 90 min under fluorescent microscope.
25. Thaw fresh 4% PFA for optimal fixing. Do not freeze and thaw 4% PFA multiple times.

26. Device with sterile PBS can be stored at 4 °C for 1–2 weeks after fixing. Seal storage container with Parafilm to prevent evaporation.
27. Prepare 100–150 μL of antibody dilution per device. Set up a temporary hydrostatic pressure to allow antibodies flowing through the device. Typically, 70–80 μL of antibody dilution is for the highest pressure inlet, and 30–40 μL is required for the second highest pressure inlet.

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