Chapter 34

Methods for Forming Human Lymphatic Microvessels In Vitro and Assessing their Drainage Function

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Abstract

This chapter describes methods to engineer human lymphatic microvessels in vitro and to assess their fluid and solute drainage capacities. The lymphatics are formed within micropatterned type I collagen gels that contain a blind-ended channel for the growth of lymphatic endothelial cells. Because the vessels have one blind end and one open end each, they mimic the terminal structure of the native lymphatic microvascular tree. The solute drainage rates that are measured from the engineered lymphatics in vitro can be directly compared with published results from intact vessels in vivo. Practical considerations to increase the accuracy of the drainage assays are discussed.

Key words Microvascular tissue engineering, Microphysiological system, Lymphoscintigraphy, Dextran, Albumin, Interstitial flow

1 Introduction

This chapter describes methods to engineer human lymphatic microvessels in vitro and to characterize the drainage function of these structures. The lymphatics are formed within extracellular matrices (chiefly, type I collagen gels) that are patterned to contain blind-ended microscale channels. These scaffolds provide a template for the growth of lymphatics that are open at one end and thus capable of drainage. The vessels and matrices are housed within microfluidic chambers through which external pressures can be applied to induce physiological flows through the system.

Many of the methods described in this chapter are based on our previously described procedures for patterning gels using a thin needle as a removable element [1, 2] and for forming endothelial tubes within microchannels in patterned gels [3]. The reader is expected to be familiar with these protocols, and the current chapter describes modifications of these methods to make them suitable for engineering lymphatics.

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In vivo, lymphatic microvessels serve as the initial site of solute and fluid drainage from the surrounding interstitium [4]. The rate of solute drainage is estimated by a technique called lymphoscintigraphy, in which time-lapse imaging of a tissue after a tracer is injected into it yields a characteristic time constant for solute removal [5]. The method described in this chapter to assess solute drainage function in vitro is based on an adaptation of lymphoscintigraphy for use with commonly available fluorophores and epifluorescence microscopes, and provides solute removal constants that can be directly compared with in vivo values.

Lymphatic fluid drainage in vivo is mainly assessed indirectly by the accumulation of fluid over time in an affected tissue (e.g., by measuring the circumference of an upper extremity in breast cancer-related lymphedema [6]). In contrast, the method described here to assess fluid drainage function measures the flow rate of interstitial fluid across the lymphatic endothelium and is thus a more direct method.

The microscale human lymphatics that are described in this chapter can serve as a microphysiological system for studying lymphatic biology in a three-dimensional, organotypic setting. We have used them to understand the basic design principles that govern effective drainage in engineered lymphatic microvessels [7]. In principle, these lymphatics can be used as living biosensors to detect external environmental factors that perturb drainage function. We envision that the lymphatics can eventually be coupled with blood vessels and/or parenchymal tissues to create a more complex "organ-on-a-chip" that is perfused and drained in vitro.

2 Materials

2.1 Fabrication of Microfluidic Chambers

1. Polished silicon wafer, 4″ diameter (Montco Silicon).
2. Wafer-handling tweezers (Ted Pella).
3. UV/ozone cleaner (Jelight).
4. SU-8 2150 negative photoresist (Microchem).
5. Digital hot plate (Barnstead Thermolyne).
6. PL-360LP glass plate, 6.5″ × 6.5″ (Omega Optical).
7. Plastic transparency, printed with the design shown in Fig. 1a (Output City).
8. Spin-coater and UV mask aligner suitable for processing 4″ silicon wafers.
9. Propylene glycol methyl ether acetate (PGMEA; Sigma).
10. 1*H*,1*H*,2*H*,2*H*-perfluorooctyltrichlorosilane (Sigma).
11. Sylgard 184 two-component polydimethylsiloxane (PDMS; Dow Corning).
12. Razor blades.

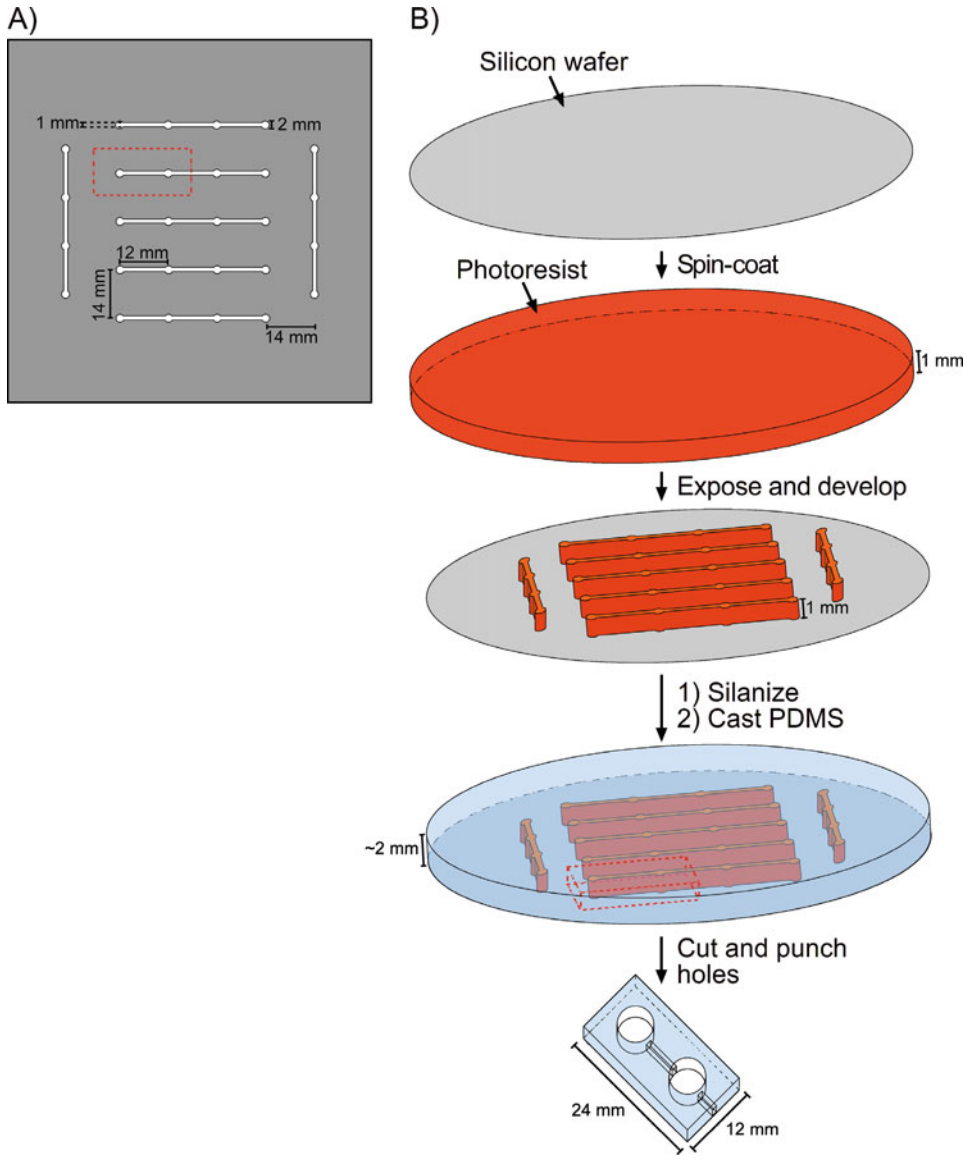

Fig. 1 Schematic diagram of the formation of microfluidic chambers to house lymphatic microvessels. (**a**) Design of the transparency mask used in lithography. Dark areas indicate regions of the transparency that are printed with ink. (**b**) Lithographic procedure to transfer the mask pattern to a 1-mm-thick layer of SU-8 photoresist on a silicon wafer, followed by replication of the photoresist features in PDMS elastomer. Red dotted lines outline the region that corresponds to a single PDMS chamber; with the mask design shown, one wafer can generate fourteen chambers per replication. The small circular features serve as guides to center the hole punch

13. Petri dish, 150 mm in diameter.
14. 6-mm-diameter hole punch.
15. Ultrasonic cleaner.
16. Ethanol (70% and 100%).

2.2 Endothelial Cell Culture

1. Human dermal microvascular lymphatic endothelial cells (LECs; Promocell or Lonza) (*see* **Note 1**).
2. Gelatin, type A from pig skin (Sigma), as a sterile-filtered 0.1% solution in water.
3. Tissue culture polystyrene dishes, 60 mm in diameter (Corning).
4. Sterile water and phosphate-buffered saline (PBS; Invitrogen).
5. *Growth medium*: MCDB 131 medium (Caisson Labs) that contains 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals), 1% penicillin–streptomycin-glutamine (Invitrogen), 80 μM dibutyryl cyclic AMP (db-cAMP; Sigma), 25 μg/mL endothelial cell growth supplement (ECGS; Alfa Aesar), 2 U/mL heparin (Sigma), 1 μg/mL hydrocortisone (Sigma), and 0.2 mM L-ascorbic acid 2-phosphate (Sigma).
6. *Maintenance medium*: growth medium from Subheading 2.2, **item 5**, further supplemented to contain 3% dextran (60–90 kDa; MP Biomedicals) and 400 μM db-cAMP.
7. Trypsin/EDTA (Invitrogen).

2.3 Formation of Lymphatic Microvessels

1. 120-μm-diameter stainless steel needles (Seirin) with PDMS supports (as described in *11.3.3.1* of [1] and in steps 9 through 11 in *3.1* of [2]) (*see* **Note 2**).
2. Glass-bottomed 100-mm-diameter polystyrene dish (as described in *11.3.1* of [1] and in steps 4 through 8 in *3.1* of [2]).
3. Cleaned PDMS chamber (*see* Subheading 3.1).
4. Bovine serum albumin (BSA; Calbiochem), as a sterile-filtered 1% solution in PBS.
5. Poly-D-lysine (PDL; Sigma), as a sterile-filtered 1 mg/mL solution in PBS.
6. Ultrasonic cleaner.
7. UV/ozone cleaner (Jelight).
8. Type I collagen from rat tail (8–10 mg/mL; Corning).
9. Sterile water, PBS, and 10× PBS (Invitrogen).
10. Sodium hydroxide, as a sterile-filtered 0.2 M solution.
11. Genipin (Wako), as a sterile-filtered 20 mM solution in PBS (*see* **Note 3**).
12. Confluent culture of LECs in a 60-mm-diameter dish (*see* Subheading 3.2).
13. Maintenance medium (*see* Subheading 2.2, **item 6**).
14. Ethanol (70% and 100%).
15. 1.7 mL Eppendorf tubes.

2.4 Measurement of Drainage Rates

1. Lymphatic vessel in a micropatterned collagen gel (*see* Subheading 3.3).
2. Cleaned PDMS chamber (*see* Subheading 3.1).
3. Maintenance medium (*see* Subheading 2.2, **item 6**).
4. *Fluorescent solute-containing maintenance medium*: Alexa Fluor 488-conjugated solute (Invitrogen), as a 100 μg/mL solution in maintenance medium, centrifuged at 13,000 × *g* in an Eppendorf tube for 1 min (*see* **Note 4**).
5. Epifluorescence microscope that is equipped with a 37 °C environmental chamber and appropriate time-lapse software.
6. Coverslips, 24 mm × 50 mm, #1½ thickness.
7. 4-mm-thick PDMS spacers, 1.5 cm × 1.5 cm, with 6-mm-diameter holes (*see* **Note 5**).
8. Ultrasonic cleaner.
9. Ethanol (70% and 100%).

3 Methods

The methods described below consist of the fabrication of microfluidic PDMS chambers to house the lymphatics (*see* Subheading 3.1), the creation of lymphatics that are open at one end within these chambers (*see* Subheadings 3.2 and 3.3), and the measurement of the lymphatic drainage function (*see* Subheading 3.4). Lymphatics are formed by micromolding type I collagen gels within the chambers so that the gels contain blind-ended channels, and by seeding these channels with dense suspensions of LECs. Seeded samples are maintained for the first 3 days with a slight excess of pressure in the lumen of the lymphatic. On the day of the drainage assay, the lymphatics are switched to physiological pressure gradients (i.e., a slight excess of extravascular pressure) before measurement of fluid and/or solute drainage rates.

3.1 Fabrication of Microfluidic Chambers

1. Clean a silicon wafer by oxidizing it for at least 10 min in a UV/ozone cleaner in a class 1000 (or better) cleanroom. Wash the wafer with deionized water, and then dry it under a stream of filtered nitrogen.
2. Spin-coat the wafer with SU-8 photoresist at 500 rpm for 30 s (*see* **Note 6**) (Fig. 1b, *top*).
3. Bake the wafer on a hot plate at 65 °C for 15 min. Increase the temperature of the hot plate to 95 °C and hold it at this temperature to bake the wafer for an additional 8 h (*see* **Note 7**). Allow the wafer to cool slowly overnight to room temperature.

4. Transfer the wafer to the stage of the mask aligner. Place the transparency ink-side down on the photoresist layer, and place the glass plate on the transparency to ensure good contact between the transparency and top of the photoresist.
5. Expose the photoresist through the pattern on the transparency for a total dose of 2.9 mJ/cm^2 at a wavelength of 365 nm (*see* **Note 8**) (Fig. 1b, *middle*).
6. Place the exposed wafer on an unheated hot plate, and raise the temperature of the hot plate to 65 °C at a rate of ~3 °C/min. Bake the wafer at 65 °C for 2 h, and then allow the wafer to cool slowly to room temperature.
7. Develop the photoresist by gently agitating the wafer in PGMEA with tweezers. Refresh the PGMEA several times to remove any dissolved photoresist (*see* **Note 9**). Dry the wafer under a stream of filtered nitrogen.
8. Bake the wafer at 120 °C for 5–10 min to harden the photoresist features.
9. Silanize the surface of the wafer by placing the wafer under vacuum with 0.1 mL of perfluorooctyltrichlorosilane overnight.
10. Mix 40 g of PDMS prepolymer in a 1:10 ratio of cross-linker and base polymer by weight. Place the silanized wafer in a 150-mm-diameter plastic dish, and pour the PDMS prepolymer onto the wafer. Degas the mixture under vacuum to remove any bubbles.
11. Cure the PDMS for at least 10 h at 60 °C (*see* **Note 10**). Carefully peel the cured PDMS from the master, and trim it with a razor blade and hole punch to form PDMS "chambers" that each contain a recessed feature of 1 mm × 1 mm cross-sectional area flanked by two 6 mm-diameter wells (*see* **Note 11**) (Fig. 1b, *bottom* and Fig. 2a, *upper middle*).
12. Clean PDMS chambers by sonicating them in 70% ethanol for at least 1 min. Rinse them thoroughly with 100% ethanol, and allow them to dry in a tissue culture hood.

3.2 Endothelial Cell Culture

1. Coat 60-mm-diameter dishes with 0.1% gelatin for at least 1 h at room temperature. Wash twice with sterile water and dry.
2. Culture LECs in coated dishes in growth medium (*see* Subheading 2.2, **item 5**). Refeed cultures every two days until they are confluent. Passage confluent cultures at a 1:4 split ratio (*see* **Note 12**). Each confluent dish can be used to generate up to ~8 lymphatic vessels.

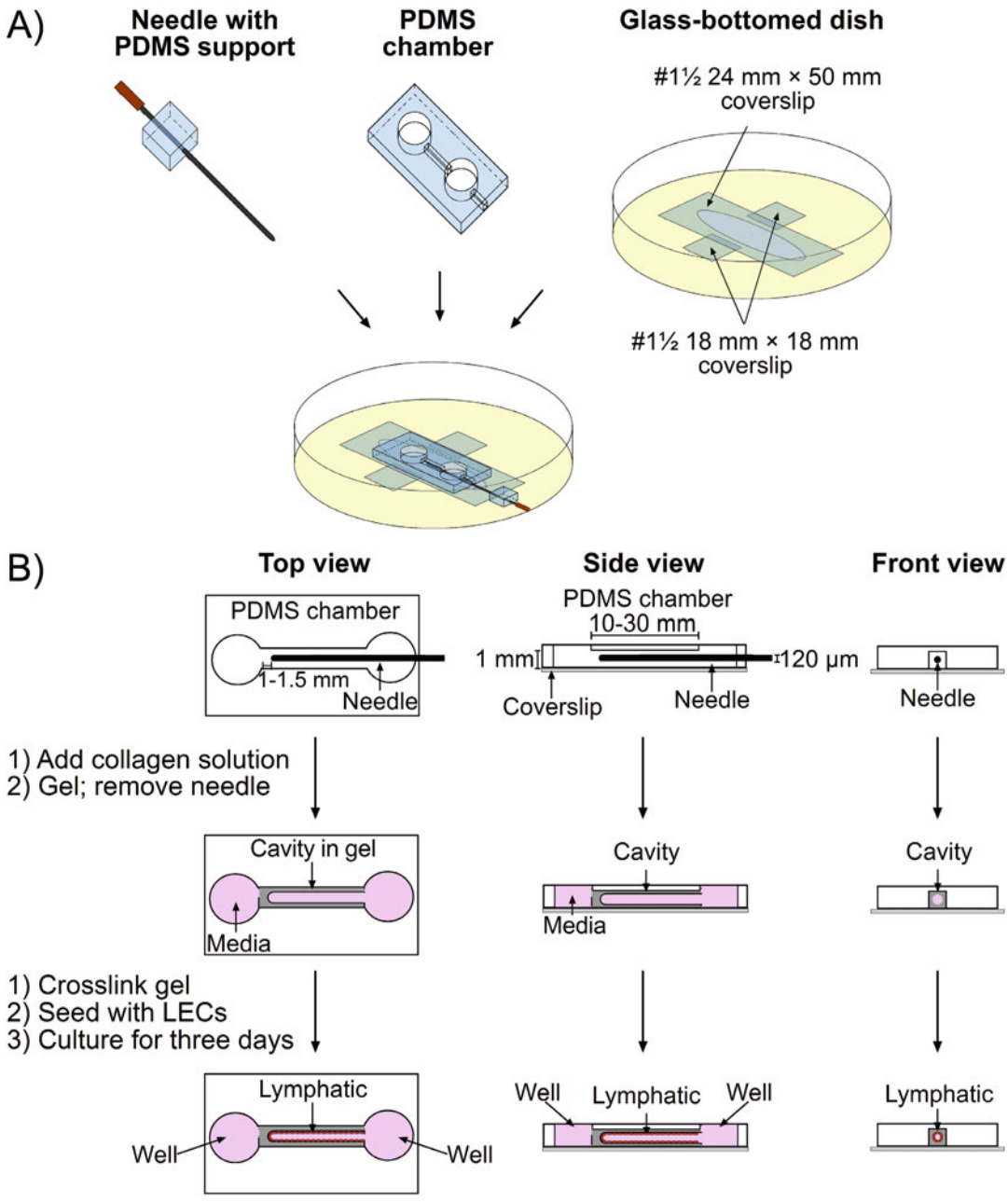

Fig. 2 Schematic diagram of the formation of a blind-ended lymphatic microvessel. (**a**) Assembly of a PDMS chamber, a needle with PDMS support, and a glass-bottomed dish, before addition of collagen gel. (**b**) Generation of a blind-ended channel within a collagen gel and seeding of the channel with LECs to create a lymphatic

3.3 Formation of Lymphatic Microvessels

3.3.1 Formation of Collagen Gels that Contain a Blind-Ended Channel

1. Drag the tips of needles in a circular motion across a hard surface (e.g., a lab bench). This motion will slowly grind down the tips into a rounded shape (*see* **Note 13**).
2. Clean rounded needles (Fig. 2a, *upper left*) by sonicating them in 70% ethanol for at least 1 min. Rinse them thoroughly with 100% ethanol, and allow them to dry in a tissue culture hood.
3. Cover the needles with 1% BSA for at least 1 h at room temperature. Wash the needles twice with water, and allow them to dry. An adsorbed layer of BSA decreases the adhesion between the surface of the needle and collagen gel [8].
4. Oxidize the PDMS chambers, recessed side up, in a UV/ozone cleaner for 10–15 min. Place the oxidize chambers, recessed side down, onto a glass-bottomed dish (Fig. 2a, *upper right*).
5. Immediately introduce PDL solution into the space defined by a PDMS chamber and the underlying glass. After 1 h at room temperature, wash out the solution with water, and aspirate any remaining liquid. An adsorbed layer of PDL increases the adhesion of collagen gel to oxidized PDMS and glass (*see* **Note 14**) [9].
6. Carefully thread one BSA-coated needle into the space between a PDMS chamber and the underlying glass, using the needle's PDMS support as a guide (Fig. 2a, *bottom*). The needle should be situated so that it is surrounded on three sides by PDMS and on one side by glass (Fig. 2b, *top row*). The needle should not be in direct contact with the PDMS chamber or glass. The rounded tip of the needle should be situated so that it is 1–1.5 mm away from the distal well at one end of the PDMS chamber (*see* **Note 15**). Place the dish with the assembled needle and PDMS chamber in a 4 °C refrigerator for at least 30 min.
7. Neutralize the collagen solution to pH 7–7.5 on ice with an appropriate volume of 0.2 M sodium hydroxide, and supplement the solution with PBS and 10× PBS to obtain the desired collagen concentration and ionic composition (*see* **Note 16**).
8. Add collagen solution to fill the space around a needle between a PDMS chamber and glass, and carefully aspirate any excess. Add ~1 mL of PBS in droplets around the dish to maintain a hydrated atmosphere. Allow the collagen to gel at room temperature for at least 20 min (*see* **Note 17**).
9. Add ~20 μL of PBS to each end of a collagen gel. Be careful not to disturb the needle when adding the PBS, as the gel around the needle is very fragile and easily distorted.
10. While gently pressing the PDMS support, carefully remove the needle to yield a collagen gel that contains a blind-ended, 120-μm-diameter channel (Fig. 2b, *middle row*). The channel

will be open at one end, while its tip will face a solid section of gel (thickness of 1–1.5 mm) (*see* **Note 18**).

11. Crosslink the gel by adding ~80 μL and ~20 μL of 20 mM genipin solution to the open and solid ends of the channel, respectively, and allowing the solution to flow into the channel for 2 h at room temperature. Exhaustively rinse the gel with PBS overnight to remove excess genipin (*see* **Note 19**).
12. Condition the crosslinked gels with dextran-containing maintenance medium (*see* Subheading 2.2, **item 6**) by adding ~80 μL and ~20 μL of medium to the open and solid ends of the channel, respectively, and allowing the solution to flow into the channel for 1 day at room temperature.
13. Seal one side of the PDMS chamber so that the patterned gel and its two adjacent wells form a fluidically isolated unit (*see* **Note 20**).
14. Add ~60 μL of maintenance medium to both wells around a gel, fill the dish with ~5 mL of PBS, and place the dish in a 37 °C CO_2 incubator.

3.3.2 Formation of Blind-Ended Lymphatics in Patterned Gels

1. Trypsinize a confluent 60-mm-diameter dish of LECs, and resuspend the cells in 1 mL of maintenance medium in an Eppendorf tube.
2. Centrifuge the cell suspension at ~100 × *g* for 1 min. If the cells do not form a pellet, then reorient the tube and centrifuge again.
3. Carefully remove all but ~20 μL of supernatant. Resuspend the cell pellet in the remaining volume (*see* **Note 21**).
4. Add ~2 μL of cell suspension to the open end of a channel. Cells should begin to flow slowly into the channel. Observe the process at 10× magnification on a standard tissue culture microscope, and tilt the dish slightly to stop flow once the cell suspension has reached the tip of the channel. Allow the cells to settle for 1–2 min, during which they will adhere to the lower half of the channel surface (*see* **Note 22**).
5. Flush out any nonadherent cells by adding ~60 μL of maintenance medium to the solid end of the channel (*see* **Note 23**). Rinse the well that is adjacent to the open end of the channel three times with maintenance medium to remove excess cells. Return the dish to the 37 °C incubator.
6. Feed the samples twice daily for 3 days by removing media from the open and solid ends of the channel and adding ~80 μL and ~40 μL to them, respectively (*see* **Note 24**). By day 3 postseeding, the LECs should have proliferated and migrated to form a confluent, blind-ended "lymphatic" (Fig. 2b, *bottom row*, and Fig. 3).

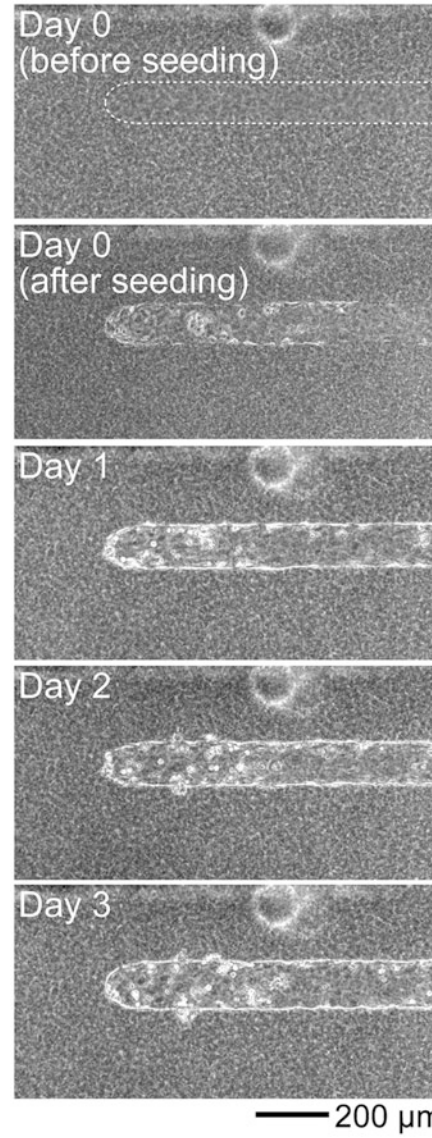

Fig. 3 Time-lapse phase-contrast images of the formation of a lymphatic microvessel in a blind-ended channel, from the day of seeding (day 0) to 3 days after seeding (day 3). Dotted lines outline the border of the channel

3.4 Measurement of Drainage Rates

3.4.1 Measurement of Fluid Drainage Rates

1. Clean PDMS spacers by sonicating them in 70% ethanol for at least 1 min. Rinse them thoroughly with 100% ethanol, and allow them to dry in a tissue culture hood.
2. Four hours before the intended measurement, place a PDMS spacer on top of the well adjacent to the blind end of the lymphatic, with the hole in the spacer aligned with the well (*see* **Note 25**).

3. Fill both wells (including the PDMS spacer) flush with maintenance medium, and allow the medium to flow in a retrograde direction (i.e., from the blind end to the open end of the lymphatic) at 37 °C. The hydrostatic pressures at the blind and open ends of the lymphatic will be ~6 mm H_2O and ~2 mm H_2O, respectively.
4. After 4 h of retrograde flow, refill both wells so that they are again flush with the height of the PDMS spacer or chamber.
5. Measure the volume of fluid that accumulates in the well next to the open end of the lymphatic after 2–5 h at 37 °C (*see* **Note 26**).
6. Calculate the fluid drainage rate as the ratio of the fluid volume to the time interval.
7. *Data interpretation:* Typical fluid drainage rates are on the order of 1–3 μL/h for lymphatics in a 7 mg/mL collagen gel [7]. Assuming that the fluid drainage is restricted largely to the upstream ~0.5 mm^2 of lymphatic endothelium, these drainage rates are equivalent to trans-endothelial fluid resorption speeds on the order of 0.6–1.7 μm/s.

3.4.2 Measurement of Solute Drainage Rates

1. Ensure that the microscope stage is heated to 37 °C for 1–2 h until the temperature is stable.
2. Calibrate the microscope camera for a dark reference by imaging in the absence of light to the camera.
3. Calibrate the microscope camera for a flat-field reference by imaging a uniformly fluorescent material, such as the bottom of a tissue culture dish.
4. Place a PDMS chamber, recessed side down, onto a coverslip. Fill the resulting space with the fluorescent solute-containing maintenance medium (*see* Subheading 2.4, **item 4**). Capture an image of the solution at an exposure time that does not saturate the image. Repeat with various dilutions of the fluorescent solution and with nonfluorescent maintenance medium, all at the same exposure times, to confirm that the fluorescence signal is proportional to solute concentration.
5. Clean PDMS spacers by sonicating them in 70% ethanol for at least 1 min. Rinse them thoroughly with 100% ethanol, and allow them to dry in a tissue culture hood.
6. Four hours before the intended drainage measurement, place a PDMS spacer on the well adjacent to the blind end of the lymphatic (*see* **Note 25**).
7. Fill both wells (including the PDMS spacer) flush with maintenance medium, and allow the medium to flow in a retrograde direction (i.e., from the blind end to the open end of the lymphatic) at 37 °C. The hydrostatic pressures at the blind

and open ends of the lymphatic will be ~6 mm H_2O and ~2 mm H_2O, respectively.

8. After 4 h of retrograde flow, remove media from both wells (including from the PDMS spacer). Fill the well and PDMS spacer adjacent to the blind end of the lymphatic with fluorescent solute-containing maintenance medium. Fill the well adjacent to the open end of the lymphatic with nonfluorescent maintenance medium (Fig. 4a).
9. Immediately place the dish that contains the vessel on the heated microscope stage.
10. Move the microscope stage to focus on a region-of-interest (ROI) in the well and immediately adjacent to the open end of the lymphatic (*see* **Note 27**).
11. Capture fluorescence images of the ROI every 2 min for at least 90 min (*see* Fig. 4b, *top* for representative images). Integrate the signal over the ROI at each time to obtain the average ROI fluorescence intensity I_{ROI} as a function of time after introduction of solute upstream (*see* **Note 28**) (Fig. 4b, *bottom left*).
12. Calculate the solute drainage rate with the following expression:

$$Solute\ \ drainage\ \ rate = \frac{dI_{ROI}}{dt} \times \frac{\delta t_{bulk}}{\delta t_{ROI}} \times \frac{1}{I_{bulk} - I_{background}}$$

Here, dI_{ROI}/dt is the least-squares estimate of slope of the linear region in the intensity versus time plot, δt_{ROI} and δt_{bulk} are the exposure times used to obtain the time-lapse images (**step 11**) and the image of the fluorescent medium (**step 4**), and I_{bulk} and $I_{background}$ are the average intensities of the fluorescent and nonfluorescent maintenance media taken at an exposure time of δt_{bulk} (*see* Fig. 4b, *bottom right* for a sample calculation).

13. Repeat **steps 6–12** in a separate lymphatic, but with nonfluorescent medium. The calculated solute drainage rate (which should, in principle, equal zero, since no fluorescent solute is added) provides an estimate of the detection limit of the solute drainage assay (*see* **Note 29**).
14. *Data interpretation:* Solute drainage rates depend on many factors, including the fluid drainage rate, the length of the lymphatic microvessel, and the molecular weight of the solute [7]. In general, higher solute drainage rates are observed with higher fluid drainage rates, shorter lymphatics, and higher solute molecular weights. For drainage of 10 kDa dextran in a ~2-cm-long lymphatic under a fluid drainage rate of ~2 μL/h, the solute drainage rate is on the order of 10^{-4} to 10^{-3}/min, which is comparable to values reported with lymphoscintigraphy in vivo [5].

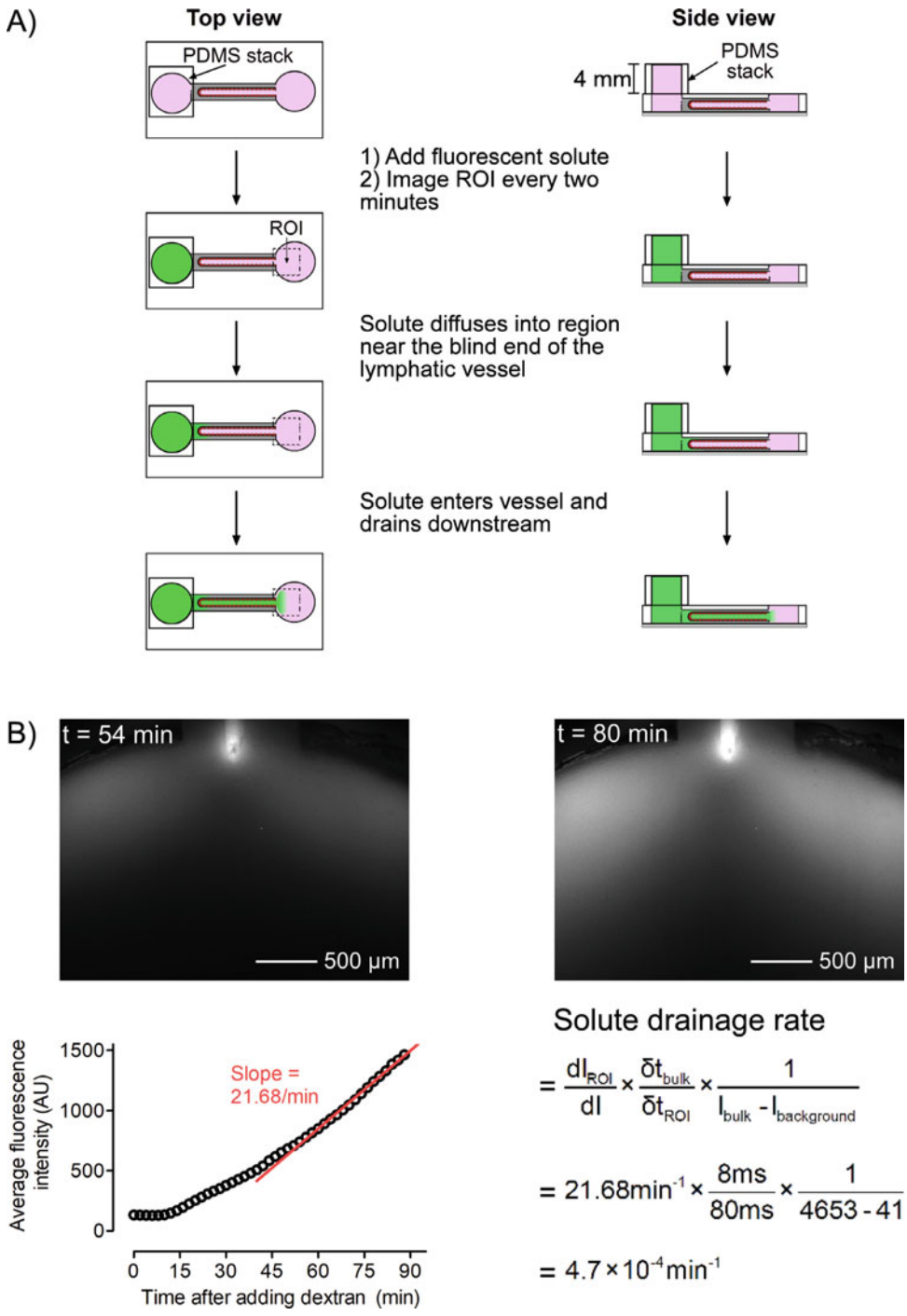

Fig. 4 Determination of the solute drainage rate from time-lapse imaging at the open end of the lymphatic after introduction of fluorescent solute to the collagen gel near the blind end of the lymphatic. (**a**) Schematic diagram of the solute drainage assay. (**b**) Plot of I_{ROI} versus time, and fluorescence images at $t = 54$ min and 80 min (the open end of the lymphatic is at the top of these images). In this example, the background and bulk fluorescence intensities $I_{background}$ and I_{bulk} were 41 and 4653 arbitrary units (AU), respectively, at an exposure time of 8 ms. Fluorescence images of the ROI were obtained at an exposure time of 80 ms

4 Notes

1. Commercially available LECs can vary substantially in their purity. Routine staining for the LEC-specific transcription factor Prox1 is highly recommended. We have had good results with a rabbit antibody to human Prox1 (Millipore) on methanol-fixed cells; we only use cultures that stain positively in at least 90% of cell nuclei.
2. The length of the needle should be at least as large as the desired length of the lymphatic vessel. A needle length of 40 mm should be sufficient for most applications.
3. Genipin dissolves slowly in PBS, and we have found that sonication of genipin powder in PBS can greatly decrease the time needed to obtain a uniform solution for sterile-filtration.
4. Suitable solutes include dextrans of molecular weight 3 kDa and 10 kDa and BSA [7]. Red fluorescent tags, such as Alexa Fluor 594, are less desirable, since the red autofluorescence of genipin-crosslinked collagen can interfere with the fluorescence imaging needed for the solute drainage assay.
5. Curing 60 g of PDMS in a 150-mm-diameter plastic dish yields a flat, ~4-mm-thick slab of PDMS from which the spacers can be cut. The circular hole in a spacer is generated with a hole punch.
6. SU-8 2150 photoresist is extremely viscous. To generate a layer of photoresist that does not contain bubbles or other flaws, the rotational speed of the spin-coater should be slowly increased to the final speed of 500 rpm over a span of 5 s, before the speed is held at 500 rpm for an additional 30 s. This procedure will yield a photoresist thickness of ~1 mm.
7. As the wafer is heated, the photoresist layer will buckle from thermal gradients. Over several hours of baking, the buckles will dissipate to yield a smooth film. It is important that the hot plate be allowed to cool slowly back to room temperature so that the photoresist layer remains smooth. To obtain a layer of uniform thickness, the hot plate should be leveled beforehand (e.g., with a bubble level). Wafer-handling tweezers can help in transferring the wafer from the spin-coater to the hot plate.
8. The total exposure time depends on the intensity of the UV light source in the mask aligner. We typically use a light source that provides an intensity of 1.7 mW/cm^2 at 365 nm, which is equivalent to an exposure time of 1700 s. To avoid overheating the photoresist, the exposure is split into 85 cycles of 20 s, separated by 20 s rest periods.
9. Because SU-8 is a negative photoresist, the exposed regions (which correspond to the clear areas in the transparency) will be

crosslinked and insoluble in PGMEA. Complete removal of photoresist from the unexposed regions (which corresponds to the dark areas in the transparency) will require at least 10 min under agitation in PGMEA.

10. Although PDMS will cure in less than an hour at 60 °C, we use a longer curing time to increase the rigidity of the polymer, which facilitates subsequent trimming with a razor blade and hole punch. A thickness of 2 mm corresponds to ~40 g PDMS in a 150 mm-diameter dish.
11. The length of the PDMS chamber will define the length of the collagen gel and lymphatic vessel. We have used recessed features of up to ~30 mm in length, which is roughly the maximum that is compatible with a 40 mm-long needle. Manual handling of PDMS should always be performed while wearing gloves.
12. Confluent cultures should either be passaged or used for seeding of collagen gels on the day of confluence. If held until the next day, LECs tend to trypsinize poorly and seed as clumps, which often clog the collagen channels. We routinely discard cultures when the cells begin to appear excessively large, typically around passage 8–10.
13. Commercially available needles from Seirin have a pointy tip. Collagen gels that are cast against unrounded needles will contain channels that replicate the sharp geometry at their ends, and LECs tend to adhere poorly at these features.
14. We typically use PDL of 30–70 kDa molecular weight. Larger molecular weights should also be suitable for adsorption and may lead to better results [10].
15. The distance between the needle tip and the well will equal the thickness of the solid collagen gel that is upstream of the lymphatic. When the distance is greater than ~1.5 mm, the lymphatic drainage rates become impracticably small. In principle, distances smaller than ~1 mm would enhance drainage rates, but they may be difficult to achieve with manual alignment of the needle.
16. We have found that the volume of sodium hydroxide required to neutralize collagen solution varies with each batch of sodium hydroxide solution. One should not rely on the nominal 0.2 M concentration to calculate the required volume, which must be determined by trial-and-error. We typically make enough neutralized collagen solution for 6–8 samples, which is equivalent to 0.2–0.3 mL of solution, depending on the desired length of the gel. Collagen concentrations of at least 6 mg/mL work well.

17. The temperature of gelation determines the structure of the resulting gel. We have found that gelation at room temperature (20–24 °C) yields gels that have sufficiently large pores to enable brisk drainage. In general, the lower the gelation temperature, the larger the pore size.
18. By phase-contrast microscopy, the sidewalls of the collagen channel should appear sharp, straight, and uniform. Defects in the sidewalls are usually caused by vibration of the needle as it is removed, and we discard gels that display these defects. It is important to confirm, by changing the focal plane, that the upper and bottom surface of the channel are completely surrounded by collagen gel (which is visible as a faint fibrous mat). If any part of the channel is in direct contact with PDMS or glass (which can occur if the needle was initially poorly aligned), then LECs will be able to migrate from the channel onto the PDMS or glass, thereby destroying the integrity of the vessel.
19. Although genipin is generally viewed as a much less cytotoxic crosslinker compared to dialdehydes, it is by no means benign and must be removed after crosslinking, by exhaustively washing the gel with PBS. Tilting the dish can increase the rate of washing. Appropriately crosslinked gels will exhibit a pale blue hue, and LECs will spread readily on them. Crosslinking is needed to enhance the stability of cell adhesion to the collagen gel [11].
20. Semicured PDMS or autoclaved silicone vacuum grease (Dow Corning) can be used to plug the chambers. This step is performed to ensure that the wells do not leak when placed under modest pressures of ~6 mm H_2O.
21. The volumes should be scaled proportionally if multiple or larger dishes are used. For instance, cells from a confluent 100-mm-diameter dish should be resuspended in ~40 μL of maintenance medium. The dextran and db-cAMP in the maintenance medium are included to enhance stability of cell adhesion to the collagen gel [12, 13].
22. If the initial seeding density is too low, then the channel can be reseeded by un-tilting the dish and allowing additional cell suspension to enter the channel. Depending on how much time has elapsed since the cell suspension was first added, it may be necessary to add more cell suspension to the open end of the channel.
23. We typically seed 6–8 channels in one experiment, one dish at a time. By the time the second or third dish is seeded, the first dish is ready to be flushed. The time of 5–10 min between seeding and flushing is sufficiently long to allow firm cell adhesion to the collagen without hindering the removal of

excess cells. Small clumps of cells will gradually resolve as the cells migrate over time, but large clumps (i.e., ones that completely clog the channel) generally will not; samples that are clogged should be discarded.

24. The maintenance pressures are designed to expose the lymphatic endothelium to a slightly positive trans-endothelial pressure. Although such pressures are not physiological, they help to maintain the stability of the endothelial adhesion to the collagen gel [14].
25. The thickness of the spacer controls the pressure difference that can be applied to the gel. For instance, a 4 mm-thick spacer generates an extra pressure of 4 mm H_2O when the hole is filled flush with medium. If desired, spacers can be stacked to enable greater pressures. The "blind end" of the lymphatic refers to the end that is separated from the neighboring well by collagen gel. In Figs. 2b and 3, this end is on the left side of the diagrams and images. In a drainage assay, the PDMS spacer is placed on the blind-ended side so that flow can be directed from the blind end to the open end of the lymphatic.
26. A simple way to measure the flow-through volume is to pipette liquid from the downstream well so that the meniscus is again flat, measure the mass of that liquid, and convert mass to volume using the density of maintenance medium (~1.0 mg/μL). Similarly, one can measure the mass of liquid that restores a flat meniscus when added to the upstream PDMS spacer. In general, these liquid volumes will differ slightly as a result of evaporation and measurement error, and we use the average value when calculating the fluid drainage rate.
27. It is important to capture as much as possible of the fluorescence signal from solute that has drained out the open end of the lymphatic. We typically use a 5× objective to maximize the field-of-view (2.1 mm × 2.8 mm) and light capture efficiency for time-lapse imaging, as well as for the calibration imaging in **steps 2–4** in Subheading 3.4.2.
28. A plot of I_{ROI} versus time will display several characteristic features (Fig. 4b, *bottom left*). Depending on the fluid drainage rate, I_{ROI} will be close to the background value for the first 10–20 min. Then I_{ROI} will rise slowly as solute begins to enter the lymphatic at the blind end and exit at the open end. Finally, I_{ROI} will rise more rapidly and increase linearly with time. In most cases, a total time-lapse duration of 90 min is sufficiently long to capture the linear trend without appreciable loss of signal from diffusion of fluorescent solute out of the ROI. For samples with high drainage rates, a shorter time-lapse duration may be needed.

29. Genipin-crosslinked collagen is weakly autofluorescent in the green channel. Photobleaching of this autofluorescence will yield a slowly decreasing I_{ROI} versus time plot, which will result in an artefactual negative solute drainage rate. We take the magnitude of this negative drainage rate (~10^{-6}/min) as the detection limit of the solute drainage assay.

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