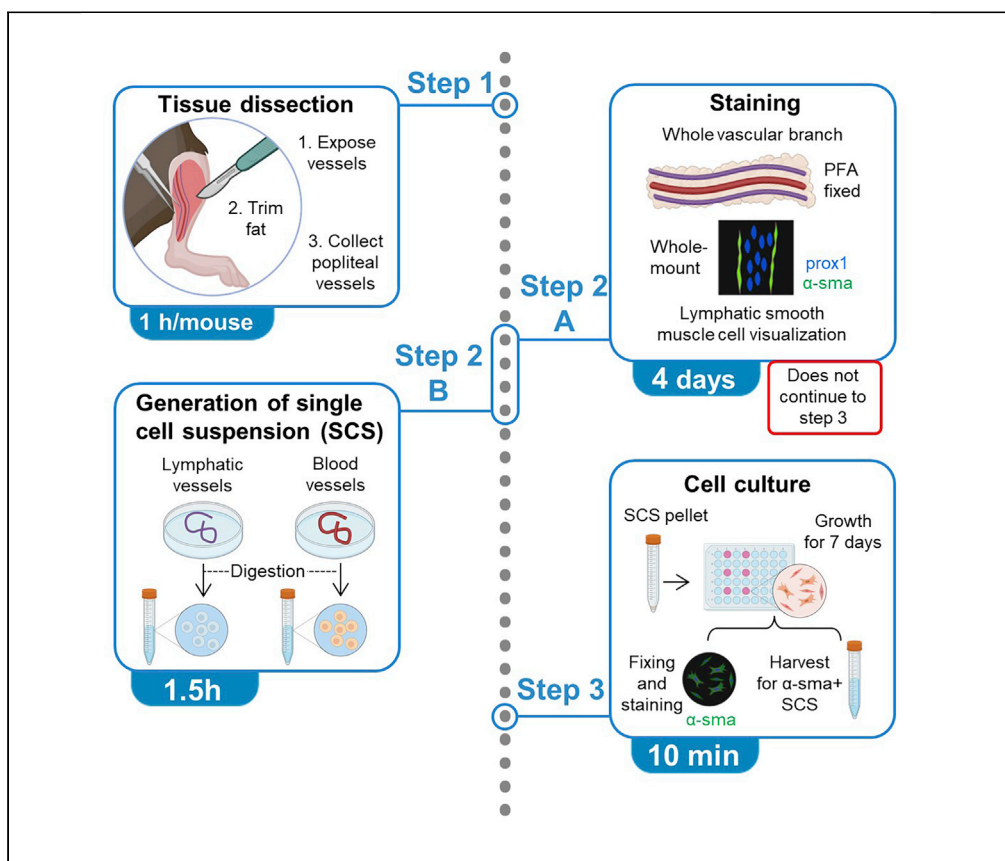


## Protocol

# Dissection of murine collecting lymphatic vessels for imaging, single-cell analysis, and tissue culture of lymphatic muscle cells



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**Highlights**  
Surgical protocol to  
isolate mouse  
collecting lymphatic  
vessels and blood  
vessels

Whole-mount  
immunostaining  
protocol of mouse  
hindlimb vasculature

Digestion protocol to  
obtain single-cell  
suspensions from  
lymphatic and blood  
vessels

Protocol to culture  
 $\alpha$ -smooth muscle  
actin+ cells from  
lymphatic and blood  
vessels

*In vivo* and *in vitro* systems have the potential to provide a framework to study the organization, gene expression, and functionality of lymphatic and blood vessel smooth muscle cells in physiology and disease settings. A series of procedures are described here, including the surgical isolation of mouse collecting lymphatic vessels and blood vessels, whole-mount immunofluorescence staining of muscle cells on the vessels, and the enzymatic digestion and culture of  $\alpha$ -smooth muscle actin+ cells from the vessels.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

## Dissection of murine collecting lymphatic vessels for imaging, single-cell analysis, and tissue culture of lymphatic muscle cells

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<https://doi.org/10.1016/j.xpro.2022.101800>

## SUMMARY

*In vivo* and *in vitro* systems have the potential to provide a framework to study the organization, gene expression, and functionality of lymphatic and blood vessel smooth muscle cells in physiology and disease settings. A series of procedures are described here, including the surgical isolation of mouse collecting lymphatic vessels and blood vessels, whole-mount immunofluorescence staining of muscle cells on the vessels, and the enzymatic digestion and culture of  $\alpha$ -smooth muscle actin+ cells from the vessels.

For complete details on the use and execution of this protocol, please refer to Jones et al. (2018).

## BEFORE YOU BEGIN

## Institutional permissions

All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of Boston University.

## Preparation before the day (or on the day) of protocol

⌚ Timing: 1 h 10 min

1. Prepare solutions as described in materials and equipment.
  - a. 2% Evans blue.
  - b. Anesthesia solution.
  - c. Dilute stock collagen I solution to 10  $\mu$ g/mL.
2. Coat individual wells of a 48 well cell culture plate with 0.5 mL of diluted collagen I solution (5  $\mu$ g collagen) under a laminar flow hood.
  - a. Add diluted collagen I for 1 h at 18°C–25°C. Aspirate collagen I and wash well with PBS. Aspirate PBS and let air-dry.

**Note:** For each animal, typically 1 well is sufficient for culture of cells recovered from 8 popliteal lymphatic vessels (PLVs). Typically, 4 PLVs per mouse are recovered. An additional well can be used to collect blood vessels as comparators.

3. Sterilize surgical equipment (preferably by autoclaving).



### Preparation right before surgery

⌚ Timing: 10 min

4. Prepare 4 mL of digestion solution (2 mL for each type of vessel harvested) as described in materials and equipment in a 15 mL conical centrifuge tube and place at 37°C when 30 min to an hour of surgery are left.
5. Sterilize surgical equipment (if not autoclaved) and surfaces with EtOH.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Alpha-Smooth Muscle Actin Monoclonal Antibody (1A4), Alexa Fluor™ 488 (1:200)	Invitrogen	Cat # 53-9760-82
Anti-human Prox 1 (1:40)	R&D Systems	Cat# AF2727
Alexa Fluor® 647 AffiniPure Fab Fragment Donkey Anti-Goat IgG (H+L) (1:100)	Jackson ImmunoResearch	Cat# C839L10
<b>Chemicals, peptides, and recombinant proteins</b>		
Collagenase I	Worthington	Cat# LS004196
Dispase II	Gibco	Cat# 17105041
Rat Tail Collagen I	Corning	Cat# CB354249
16% Paraformaldehyde (PFA) Aqueous Solution, EM Grade, Ampoule 10 mL	Electron Microscopy Sciences	Cat# 15710
Glycine	MilliporeSigma	Cat# G8898
Hank's Balanced Salt Solution (HBSS, without calcium, magnesium, phenol red)	Corning	Cat# 21-022-CV
Evans blue Powder	Sigma	Cat# E2129-10G
DMEM 4.5 g/L glucose, L-glutamine and sodium pyruvate	Corning	Supplier# 10-013-CV
Penicillin/Streptomycin Solution 100×	Corning	Cat# 30-002-CI
<b>Experimental models: Organisms/strains</b>		
C57BL/6J wild-type mice, sex: male or female, age: 8–12 weeks	The Jackson Laboratory	Strain code: 000664
<b>Other</b>		
Xylazine injectable solution, 100 mg/mL	Covetrus	SKU# 061035
Ketamine HCl injectable solution, 100 mg/mL C3N	Covetrus	SKU# 071069
Extra Fine Graefe Forceps, 10 cm, 0.5 mm wide tip. Slightly curved, serrated tip. Stainless Steel	Fine Science Tools	Item# 11151-10
Fine Forceps, 120 mm, 0.34 mm wide tip. Straight tip.	Dumont	Cat# 0508-2-PO
Vannas Scissors, 8 cm (3"), 5 mm Blades, 0.1 mm Tips, Curved. Stainless Steel	World Precision Instruments	Order Code 14122-G
McPherson-Vannas Scissors, 8 cm (3"), 5 mm Blades, 0.2 mm Tips, Straight. Stainless Steel	Roboz	Item# RS-5602
Disposable Micro scalpel #15. Stainless Steel	AD Surgical	Item# A301-P15
3M™ Transpore™ Surgical Tape	3M	Ref# 1527-0
BD Insulin syringe (28G, 0.5 mL)	BD	Cat# 14-826-79
Filter 0.22 µm (3.3 cm diameter)	Fisherbrand	Cat# 09-720-004
Costar® 48-well Clear TC-treated Multiple Well Plates	Corning	Product # 3548
Superfrost plus microscope slides (25 × 75 × 1.0 mm)	Fisherbrand	Cat# 12-550-15

### MATERIALS AND EQUIPMENT

#### Anesthesia solution

Reagent	Final concentration	Amount
Sterile PBS	N/A	8.9 mL
Ketamine (100 mg/mL)	10 mg/mL	1 mL

(Continued on next page)

### Continued

Reagent	Final concentration	Amount
Xylazine (100 mg/mL)	1 mg/mL	0.1 mL
<b>Total</b>	<b>N/A</b>	<b>10 mL</b>

Keep at 18°C–25°C for up to 1 month.

△ **CRITICAL:** Make solution inside laminar flow hood.

### Enzymatic digestion solution

Reagent	Final concentration	Amount
HBSS	N/A	4 mL
Collagenase I	1 mg / mL	4 mg
Dispase II	1 mg / mL	4 mg
Penicillin/Streptomycin (100×)	1%	40 µL
<b>Total</b>	<b>N/A</b>	<b>4 mL</b>

△ **CRITICAL:** After making the solution, filter through 0.22 µm syringe filter (3.3 cm diameter) fitted onto a 3 mL syringe inside laminar flow hood. The solution should be at 37°C at the time the vessels are added, so 30 min before the digestion begins, transfer to 37°C water bath or cell culture incubator. The digestion solution should be kept on ice until transfer to 37°C. This solution should be used the day of.

### Evans blue

Reagent	Final concentration	Amount
Evans blue Powder	2% (W/V)	0.2 g
Sterile Phosphate-buffered saline (PBS)	N/A	10 mL
<b>Total</b>	<b>N/A</b>	<b>10 mL</b>

Keep at 18°C–25°C indefinitely.

**Note:** After making the solution, filter through 0.22 µm syringe filter (3.3 cm diameter) fitted onto a 3 mL syringe inside laminar flow hood.

### Collagen I Solution

Reagent	Final concentration	Amount
Rat Tail Collagen I (10 mg/mL)	10 µg/mL	10 µL
Sterile PBS	N/A	9.99 mL
<b>Total</b>	<b>N/A</b>	<b>10 mL</b>

Keep at 4°C for up to 1 year.

△ **CRITICAL:** Make solution inside laminar flow hood.

### 4% PFA

Reagent	Final concentration	Amount
16% PFA	4%	10 mL
PBS	N/A	30 mL
<b>Total</b>	<b>N/A</b>	<b>40 mL</b>

Keep at 4°C for up to 1 month.

**Note:** Make this solution in chemical fume hood.

#### 0.5% Triton-X

Reagent	Final concentration	Amount
Triton-X	0.5%	50 $\mu$ L
PBS	N/A	10 mL
<b>Total</b>	<b>N/A</b>	<b>10 mL</b>

Keep at 4°C for up to 1 year.

#### PBS-Glycine Wash Buffer

Reagent	Final concentration	Amount
Glycine	0.2 M	0.15 g
PBS	N/A	10 mL
<b>Total</b>	<b>N/A</b>	<b>10 mL</b>

Keep at 4°C for up to 1 year.

#### Blocking buffer

Reagent	Final concentration	Amount
Donkey Serum	10%	100 $\mu$ L
0.1% Tween in PBS	N/A	900 $\mu$ L
<b>Total</b>	<b>N/A</b>	<b>1 mL</b>

Keep at 4°C for up to 1 year.

## STEP-BY-STEP METHOD DETAILS

### Mouse preparation for surgery

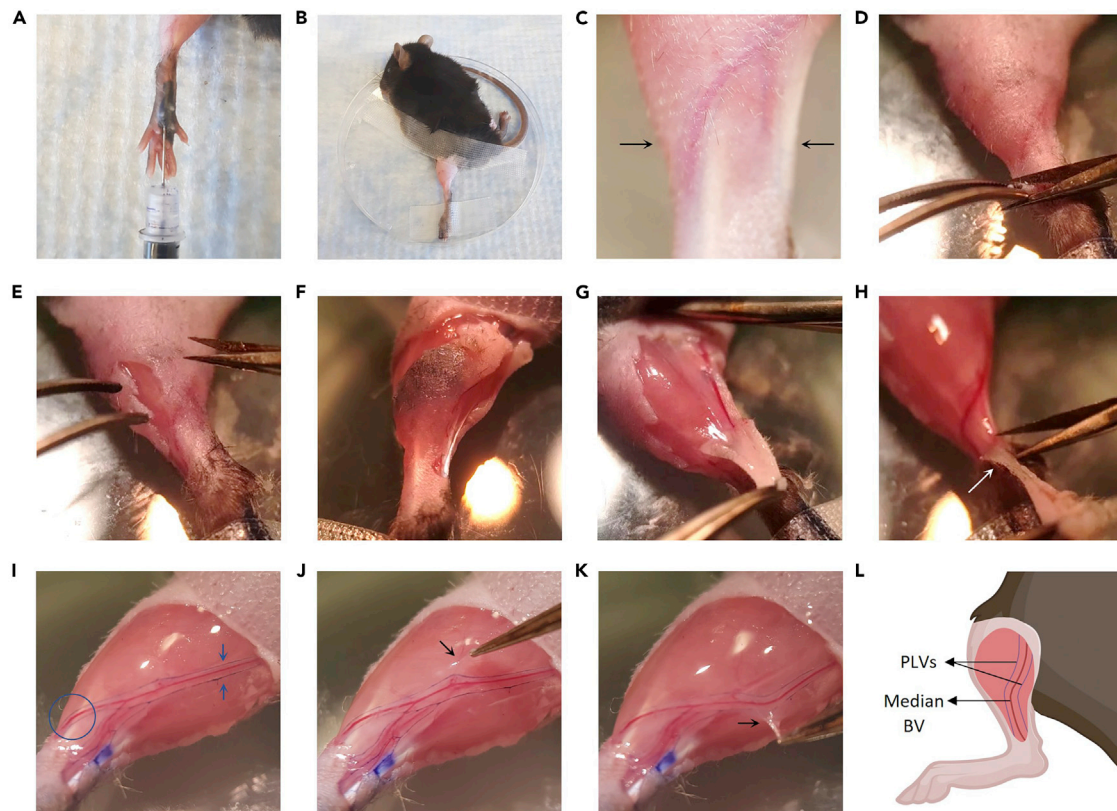
⌚ Timing: 10 min

In this section, the mouse is anesthetized, partially shaved, and has the contrast agent for lymphatics injected.

1. Anesthetize mouse using an intraperitoneal injection (IP) of ketamine/xylazine cocktail (K/X).
  - a. Recommended dose of K/X is 0.2 mL/20 g mouse weight for IP injection with syringe containing a 26G  $\times$  5/8-inch needle.
  - b. Wait till the mouse does not react to a toe pinch (around 5 min).

**Note:** After 60 min, an additional dose might be required.

2. Remove fur from hindlimbs to minimize possibility of microorganism contamination and improve visualization of the area.
  - a. Use mechanical clippers first.
  - b. Apply hair removal cream for 30 s using a cotton tip applicator. Use a paper towel wetted with water to wipe away cream. Then dry the area.
3. Inject 2% Evans blue in foot pad of both limbs subcutaneously (Figure 1A).
  - a. Inject in the middle point of back of the paw between digital and metatarsal pads with a depth of 3–5 mm.
  - b. We recommend using a volume of 10–20  $\mu$ L (enough to make a visible bolus beneath the skin) injected with insulin syringe containing a 28G  $\times$  1/2 inch needle.
4. Place the mouse in lid of sterile Petri dish in prone position. Tape the ankle and upper part of the thigh to immobilize the surgical area (Figure 1B).



**Figure 1. Exposing vessels of the hind limb**

- (A) Evans blue subcutaneous injection (left paw).  
 (B) Shaved mouse immobilized for surgery (left limb).  
 (C) View of vessels of ankle under stereomicroscope. Arrows point to where the cuts should be made.  
 (D) Initial cut in the left side of the left ankle.  
 (E) Cut extensions from ankle to upper thigh in left side of left hind limb.  
 (F) Transverse cut joining incisions from both sides of the left hind limb.  
 (G) Pulling of the skin flap to expose vessels.  
 (H) Cut skin flap to reveal vessels. Arrow pointing to part of the skin flap to be cut.  
 (I) Vessels of the left hind limb exposed. Circled area is where the vascular branch bends. Blue arrow points to PLV.  
 (J) Pulling upwards of the fat layer on top of vascular branch. Black arrow points to fat.  
 (K) Pulling downwards of the fat layer on top of vascular branch. Black arrow points to fat.  
 (L) Diagram showing the distribution of the main lymphatic vessels in the mouse hind limb.

5. Sanitize surgical area with iodine and alcohol wipes.
  - a. Add a drop of iodine to the alcohol pad.

### Exposure of vessels

⌚ Timing: 5 min

In this section, the skin of the hind limb is removed to expose the vessels in the area. The popliteal lymphatic vessel (PLV) exposure described in this section has been optimized from [Liao et al. \(2014\)](#) for procedures in this protocol but can also be used for measuring PLV contraction. The mouse should be placed in the orientation depicted in [Figure 1B](#) and a stereomicroscope is preferred for removing the skin and the underlying connective tissue.

6. Locate the PLVs in the ankle area ([Figure 1C](#)).

**Note:** The PLVs should be visible through the skin after Evans blue injection into the footpad.

7. On one side of the ankle, pinch a portion of the skin without PLVs using blunt forceps and make a cut with micro scissors. Repeat on other side (Figure 1D).
  - a. Be careful not to cut any of the PLVs (this would interrupt the flow of Evans blue within the collecting lymphatic vessel to be isolated).
  - b. If the PLVs are not visible, make the incisions as laterally as possible on both sides of the ankle, using the blood vessel as a reference.
  - c. Extend both cuts towards the end of the thigh (Figure 1E).
8. Once the end of the thigh is reached with both cuts, make a transverse cut to join the incisions and pull downwards to expose the PLVs and BVs (Figures 1F and 1G).

**Note:** It is preferred that the skin flap made during these steps remains small (around 0.3 × 1 cm) since this is conducive to pulling of the skin to expose the vessels. More resistance is encountered with large skin flaps and with diseased mice.

**△ CRITICAL:** Avoid pulling too forcefully, since this could lead to the rupture of vessels. See troubleshooting 1 and troubleshooting 2.

9. Cut the skin flap to reveal the vessels from the lymph node to the ankle (Figures 1H and 1I).
10. After removing the skin, use a sterile cotton applicator to remove any blood coming from broken capillaries and immediately hydrate the area with sterile PBS.

**Note:** During the surgery, add sterile PBS to keep the exposed area hydrated. PBS also improves visibility of surrounding fat to be extracted.

11. Place the mouse at an oblique angle and using a different set of forceps (fine forceps instead of blunt) and micro scissors, remove the membrane of fat covering the vessels.
  - a. This can be achieved by pulling the membrane with fine forceps and making small incisions in the fat with micro scissors or microscalpel (Figures 1J and 1K).

**Note:** Take caution to not pull on any of the vessels while removing the fat. The area of the limb where the vascular branch bends is usually covered in more fat than the rest. This fat layer can be pulled with forceps and cut with micro scissors to expose the vessels.

### Dissection of hindlimb tissue for immunofluorescence staining

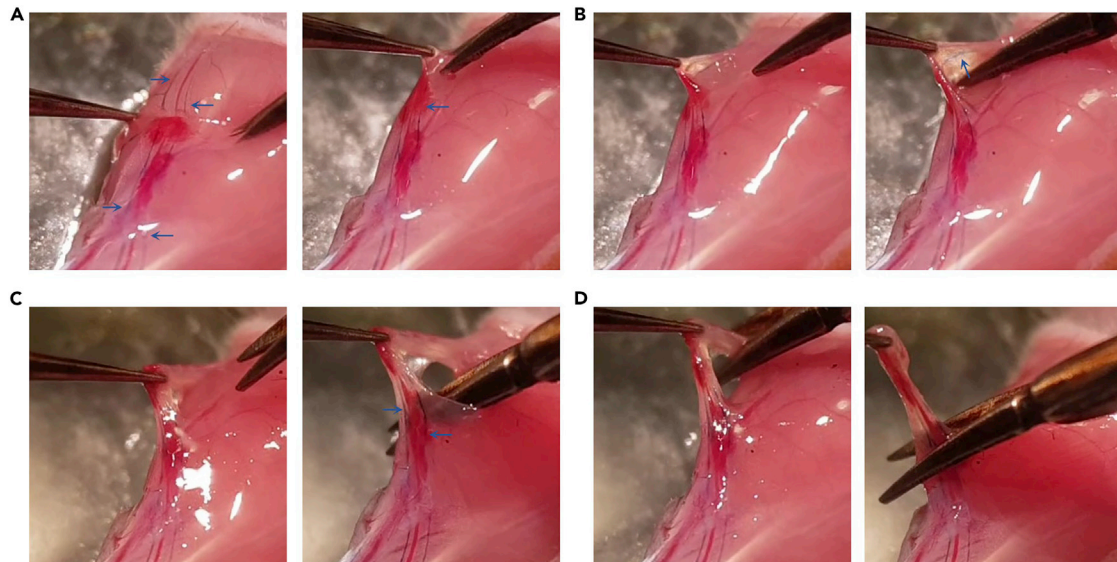
⌚ **Timing:** 4 days

Perform this section if the structure of the vessels needs to be preserved for histological studies. We recommend performing steps 12–15 with the help of a stereomicroscope.

12. Locate the fat within the area of vascular branch. Use micro scissors to make a cut that runs parallel to either PLV (Figure 2A).
  - a. Pull the fat on the exterior side of the PLV upwards to aid the process.

**Note:** Although the upper layer of fat has been removed in step 11, a layer of fat surrounding the vessels will still be present and can be cut.

13. Once a cut is made on the side of the PLV, trim the fat underneath the PLV, BV, and the PLV of the opposite side (Figure 2B).
  - a. Pull the fat in between the BV and PLVs to aid the process.



**Figure 2. Isolation of vascular branch of the hind limb**

(A) Vessels of the right hind limb exposed and initial cut parallel to the right PLV. Blue arrow points to PLVs.  
 (B) Progressive cut of the fat below the vascular branch from the right side of the right PLV to the left side of the left PLV.  
 (C) Extension of separation of vascular branch from underlying tissue with micro scissors. Blue arrow points to PLVs.  
 (D) Extraction of vascular branch by making two cuts with micro scissors.

- b. Keep cutting until the three vessels are separated from the underlying tissue. If necessary, cut from the other side of the vascular branch.
14. Once a portion of the vascular branch is separated from the fat and the BV, the separation can be widened with the aid of a microscalpel or micro scissors (Figure 2C).
15. Once the area of interest of the vascular branch has been separated from the underlying tissue, cut with micro scissors to retrieve it (Figure 2D).
16. Place in PBS to wash off the blood.
17. Place in 4% PFA for 1–4 h at 18°C–25°C.
18. Wash with PBS for at least 1 h at 18°C–25°C (or 12–14 h at 4°C).
  - a. For sectioning, an appropriate protocol for OCT (cryosections) or paraffin embedding can be followed. The latter is recommended to preserve tissue architecture.
  - b. For whole-mount, continue performing steps 19–26.

**Note:** For OCT embedding, samples usually undergo a glucose/OCT gradient, while for paraffin, samples usually undergo ethanol and xylene/paraffin gradients before the final embedding. For sectioning, the vessels should be embedded in vertical orientation to obtain cross-sections of the lumen. For paraffin sectioning, we recommend making 5–8  $\mu\text{m}$  sections.

19. Permeate with 0.5% triton (in PBS) at 4°C for 12–14 h in a microcentrifuge tube.

**Note:** If the tissues are derived from a mouse expressing fluorescent reporters, use an opaque microcentrifuge tube.

20. Incubate with PBS-glycine wash buffer for 20 min at 18°C–25°C. Repeat 3 times.
21. Incubate samples with blocking buffer (10% normal donkey serum in immunofluorescence wash buffer (PBS, 0.1% Tween)) for 1 h at 18°C–25°C.
22. Incubate samples with primary antibody for 24–48 h at 4°C.



- a. We typically combine a fluorophore-conjugated anti- $\alpha$ -sma antibody to visualize LMCs with and anti-Prox1 antibody to detect lymphatic endothelial cells.

Antigen	Fluorophore	Host organism	Clone	Stock concentration	Final concentration
$\alpha$ -sma	488	Mouse	Monoclonal (1A4)	0.5 mg/mL	2.5 $\mu$ g/mL
Prox1	N/A	Goat	Polyclonal	0.2 mg/mL	5 $\mu$ g/mL

23. Wash three times with PBS 20 min each time.  
 24. Incubate with secondary antibody with desired fluorophore for 12–14 h at 4°C.  
 a. 1:100 of anti-goat 647 for Prox1.  
 25. Rinse samples with PBS. For 20 min. Repeat 3 times.  
 26. To image using confocal microscopy, place the stained sample on a glass slide with enough PBS for hydration. Use a glass coverslip to flatten tissue for imaging.

### Isolation of popliteal lymphatic and blood vessels

⌚ Timing: 20 min

Perform this section if the separation of PLVs and BVs is needed for downstream applications. This section continues from step 11 and requires the use of a different set of forceps (fine forceps) and micro scissors. We recommend performing steps 27–31 with the help of a stereomicroscope.

27. Cut the fat between one of the PLVs and the BV (Figure 3, Methods video S1).  
 a. Start with the PLV most distal from the BV. This distance between vessels is greater in the area between the popliteal lymph node (pLN) and where the vascular branch bends.  
 b. Pull the fat adjacent to either PLV and make a small downward incision in the fat in between the PLV and BV with the tip of a microsurgical scalpel (Figure 3A, Methods video S1).  
 c. Repeat step 27b till the PLV is completely separated from the underlying tissue (Figure 3B).

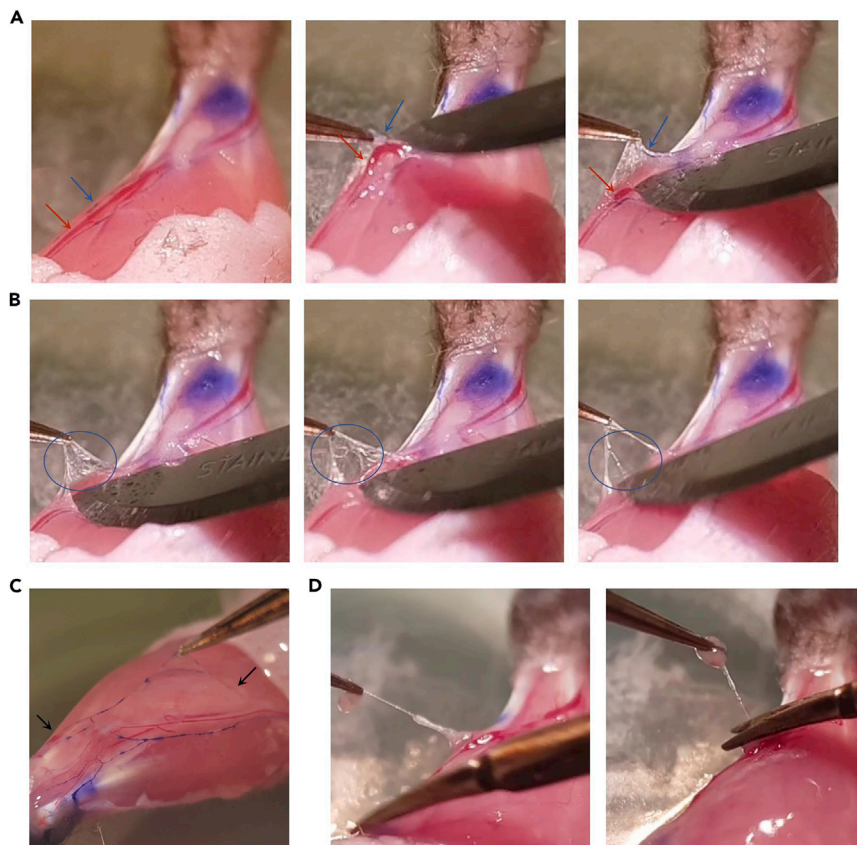
**Note:** The fat on the exterior side of the PLV can be cut with micro scissors to aid step 27c.

**⚠ CRITICAL:** In step 27b, be gentle when pulling the PLV and keep the blade of the scalpel horizontal to not slice through the BV. See troubleshooting 2.

28. Once a portion of the PLV is separated from the fat and the BV, widen the interior incision with the aid of the microsurgical scalpel or micro scissors.  
 a. Repeat this process from the pLN ideally all the way down to the ankle or at least down to the vascular bend till the majority of the vessel is detached from the underlying tissue (Figure 3C).  
 b. Perform step 27b in areas along the vessels that have high amounts of fat (they usually are towards the bend of the vascular branch).

**Note:** Around the vascular bend, a network of blood vessels can be intertwined with the PLVs, making the extraction more challenging. The region of PLVs that extend to the ankle area are amenable to separation once step 27a has been performed in the upper areas of the hind limb.

29. Once one of the PLVs has been separated from the fat and the BV, repeat steps 27 and 28 with the other PLV in the hindlimb.  
 a. Rotate the Petri dish to work more comfortably on the other LV.  
 b. Detach the BV before the PLV.



**Figure 3. Separation of PLV from fat and BV**

(A) Separation of PLV from BV by pulling on the PLV and subsequently using the tip of the scalpel in a downward motion to separate from BV. Blue arrow points to PLV. Red arrow points to blood vessel.  
 (B) Progressive side to side cutting with scalpel removes fat (encircled) surrounding the LV. See also [Methods video S1](#).  
 (C) PLV being pulled to show separation from underlying tissue from top of limb to the bend of the vascular branch (separation also present from bend to ankle but not shown). Arrow points to where to make the first cut to remove the PLV.  
 (D) Removal of PLV from upper to lower left hind limb by cutting with micro scissors. See also [Methods video S2](#).

30. Once the three vessels are detached, cut the PLV with micro scissors and place in a 35 mm TC dish containing HBSS on ice.
  - a. Make the initial cut proximal to the pLN and then pull towards the ankle. Since the vessel is detached from the tissue, the vessel should not resist pulling unless some regions remain attached to fat. These areas can be cut with micro scissors ([Figures 3C and 3D](#) and [Methods video S2](#)).
  - b. Separate the BV from the fat in the same way.

⚠ **CRITICAL:** When the BV is cut, an EtOH pad should be applied to stop the bleeding. See [troubleshooting 1](#).

31. Put instruments in ethanol, change gloves, and repeat steps 6–11 and 27–30 on the other limb, if desired.
32. After extraction is complete, euthanize the mouse.

### Generation of single cell suspensions from lymphatic and blood vessels

⌚ Timing: 1 h 20 min

Perform this section if a single cell suspension is required for downstream analysis such as cell culture, flow cytometry, and RNA sequencing. For cell culture, all steps must be performed in a biological safety cabinet.

33. Take the enzymatic solution from 37°C incubation and pipette 200  $\mu$ L to the lid of the 35 mm TC plate.
34. Using fine forceps, transport the extracted LVs to the 200  $\mu$ L of enzymatic solution.

**Note:** Since PLVs are extremely small, a stereomicroscope can be used to confirm that all PLVs (maximum 4 per animal) have been transferred to the enzymatic solution.

**△ CRITICAL:** Do not mince PLVs since vessel fragments can easily stick to the instruments, resulting in sample loss. See [troubleshooting 5](#).

35. With a P1000 pipette, transport the enzymatic solution containing the vessels to a 15 mL conical centrifuge tube containing the remainder of the enzymatic solution.
  - a. If the vessels do not dislodge easily, the pipette should be taken out of the solution and the forceps and micro scissors used to detach the vessels and transported to the lid once again. If vessels remain, repeat this step.
  - b. If possible, avoid clumping of the vessels. In our experience this leads to poor cell yield (most likely because the solution cannot reach the entirety of the vessels, see [troubleshooting 5](#)).

**Note:** Ensure that all PLVs are transferred to the solution. The pieces can be very sticky and attach to the tip of the pipette. The pieces can be dislodged by pipetting vigorously up and down. However, this should not be done more than 10 times as our experience indicates that it leads to poor cell yield.

36. Repeat steps 33–36 for the median blood vessels (maximum 2 per animal) if desired.
37. In 15 mL conical centrifuge tube, incubate enzyme solution with vessels at 37°C, 5% CO<sub>2</sub> for 60 min.
38. After 60 min, add 10 mL of DMEM with 10% FBS to neutralize enzymatic activity and centrifuge at 300 g for 5 min. Repeat twice.

## Cell culture

⌚ **Timing:** 10 min

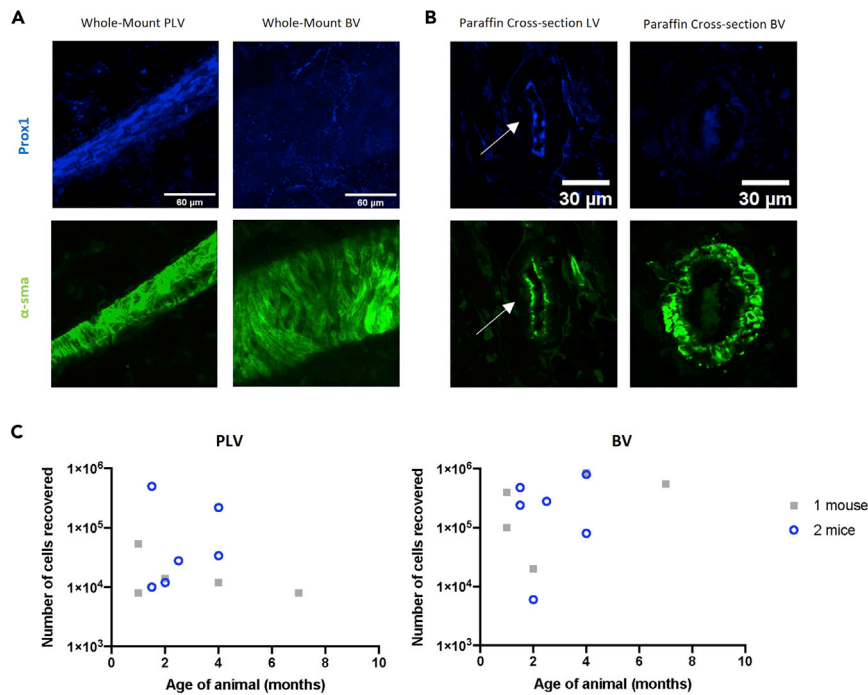
This section describes how to culture a PLV and BV derived single cell suspension. The following steps must be performed in a biological safety cabinet.

39. Resuspend cell pellet (obtained from step 38) in cell culture media (DMEM, 20% FBS, 1% P/S).
  - a. Take 10  $\mu$ L of each cell solution for counting and assessing cell viability.

**Note:** We recommend resuspending cells from PLVs and BVs in 100  $\mu$ L and 200  $\mu$ L cell culture media, respectively, based on typical cell yields. In our hands, cell viability is consistently above 90% (determined by trypan blue). For low cell yield see [troubleshooting 5](#).

40. Add cell suspension from LVs and BVs to respective collagen-coated wells.
  - a. We recommend using collagen-coated wells of a 48 well plate and a final volume of 500  $\mu$ L.
41. Allow cells to grow in the cell culture incubator for 7 days.

**Note:** After 1 day, a few cells should adhere. If around 10<sup>4</sup> total cells were plated, the well should reach 100% confluency within 7 days. To stimulate growth, media can be replaced after



**Figure 4. Expected outcomes for whole-mount and enzymatic digestion**

(A) Z-Stack compilation of whole-mount stained PLVs and BVs. Scale bars are 60  $\mu$ m.

(B) Paraffin cross-section images of PLVs and BVs. White arrow is pointing to LV. Scale bars are 30  $\mu$ m.

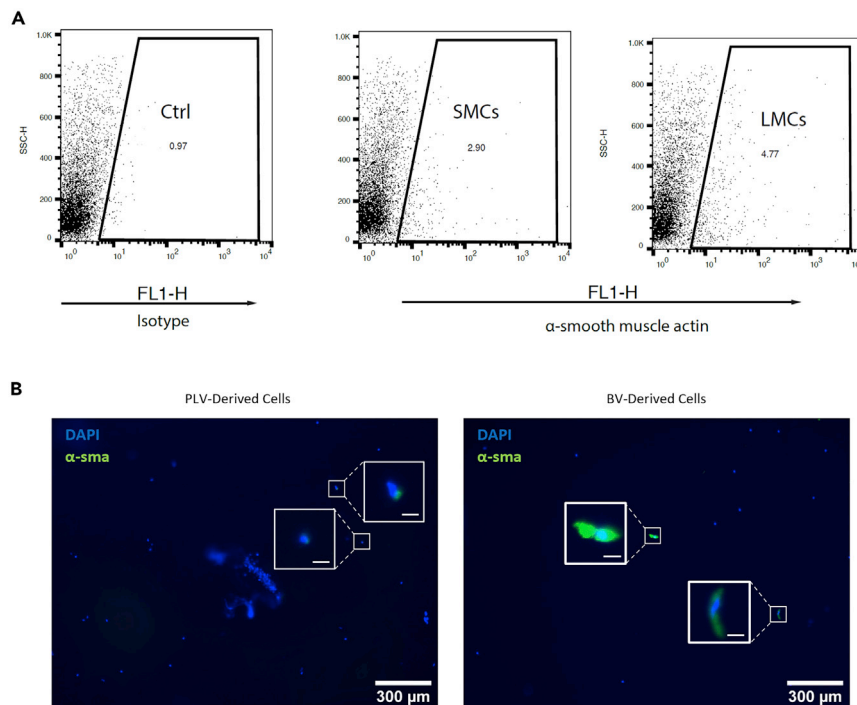
(C) Cell yield of PLVs and BVs from groups of 1 or 2 mice at different ages.

5 days of plating to replenish nutrients. It is recommended to check the cells every day to assess growth and potential microbial contamination. For suboptimal growth see [troubleshooting 6](#).

## EXPECTED OUTCOMES

We describe a reliable method to isolate popliteal blood and lymphatic vessels that enables the study of their cellular composition and structure by whole-mount staining and sectioning (Figures 4A and 4B). Moreover, we also describe how to obtain viable single cells from the popliteal vessels for downstream applications such as cell culture, flow cytometry and RNA sequencing. The cellular yield per mouse from the enzymatic digestion varies from  $8 \times 10^3$  to  $1.4 \times 10^5$  total cells for PLVs (Figure 4C) and  $2 \times 10^4$  to  $8.5 \times 10^5$  total cells for BVs. This yield is sufficient for performing downstream applications such as tissue culture, flow cytometry and single cell sequencing (data not shown). Our method of vessel isolation allows for the retrieval of PLVs in mice. This approach can also be used to collect other collecting lymphatic vessels in mice, including the collecting lymphatic vessel that runs from the inguinal to axillary lymph node. Similarly, protocols have been published on how to make single cell suspensions from PLVs, but these either use lengthier digestions than ours (Jones et al., 2018) or include cells from BVs (Kenney et al., 2020, 2022). Our method describes how to obtain single cell suspensions from PLVs within 1 h of digestion.

The described isolation and digestion protocol was designed to study LMCs, which are  $\alpha$ -sma+ cells that are responsible for lymphatic vessel contractility. Using this protocol, it can be expected that few cells recovered are  $\alpha$ -sma+ (less than 5% as determined by flow cytometry, Figure 5). However, the described culture method enriches and allows for the expansion of these  $\alpha$ -sma+ cells as determined by immunofluorescent staining (Figure 6). After a week of growth, the yield from a single well (of a 48 well plate) is  $1$ – $2 \times 10^4$  total BV or PLV derived cells.



**Figure 5. Expected outcomes after digestion for  $\alpha$ -sma<sup>+</sup> cells**

(A) Flow cytometry analysis of cells from PLVs and BVs, respectively, stained for  $\alpha$ -sma.

(B) Composite images of unfiltered cells derived from digestion of PLV and BVs and stained for  $\alpha$ -sma. Scale bar of of full image is 300  $\mu$ m and 20  $\mu$ m for enlarged areas.

## LIMITATIONS

The popliteal lymphatic vessels (PLVs) are small and fragile. As a result, the cell yield from the described surgical and digestion procedures is lower than that of major blood vessels such as the aorta. The cell yield is also variable because it is susceptible to factors including the age and anatomy of the mouse and the speed and precision with which the procedure is performed. For the latter factors, the protocol requires practice and/or surgical expertise to obtain viable cells when generating single cell suspensions from multiple mice. Additionally, although our digestion method is shorter or equally lengthy than those described elsewhere, it can significantly impact gene expression as it is over 30 min.

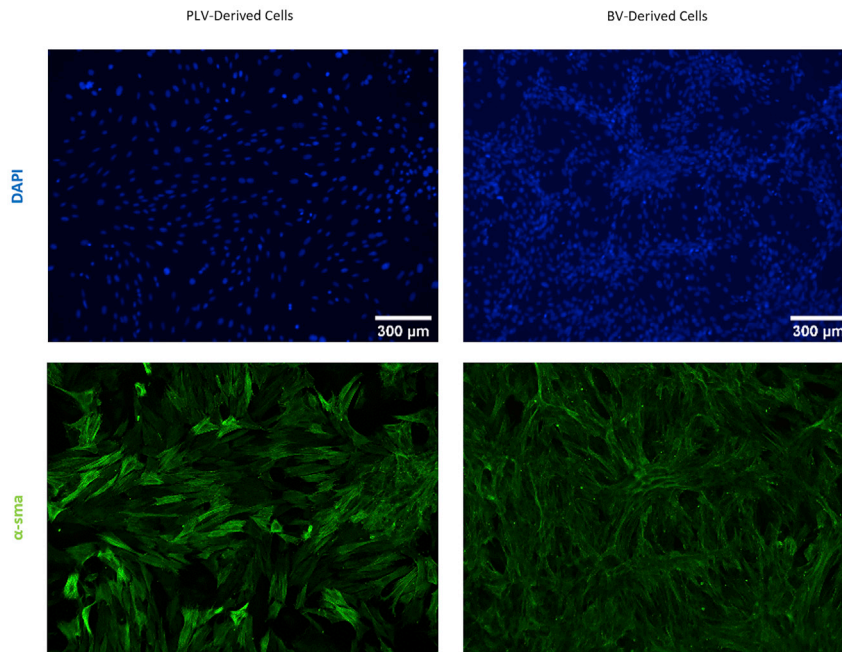
The *in vitro* expansion of the PLV-derived cells depends highly on the initial number of cells plated and after one week in culture, confluency will be only obtained if the initial culture consists of  $1 \times 10^4$  cells or more. Moreover, the described culture method enriches  $\alpha$ -sma<sup>+</sup> cells and is not intended to study lymphatic endothelial cells. Also, compared to other methods that could be used to enrich the  $\alpha$ -sma<sup>+</sup> cell population (such as FACS), the *in vitro* expansion of  $\alpha$ -sma<sup>+</sup> cells will alter their gene expression profile.

Finally, the whole-mount staining of PLVs allows visualization of the intact three-dimensional structure of lymphatics and the distribution of LMC coverage through epifluorescence or confocal microscopy. However, the most inner layers of fat-buried PLVs or the thicker blood vessels (usually imaged for comparison with PLVs) are harder to image by whole-mount staining.

## TROUBLESHOOTING

### Problem 1

Bleeding (steps 8, 13, 27b).



**Figure 6. Expected outcomes of cells after culture**

Tissue culture stain of PLV and BV derived cells after a week of growth. Scale bar is 300 µm.

#### **Potential solution**

Soak blood with EtOH pad for small capillaries or small cuts of bigger vessels. For deep cuts of the main vessels, euthanasia is the only effective method to stop the bleeding. In all cases, if the tape immobilizing the paw is tight, the flow of blood returning to the heart through veins is reduced and the bleeding due to accidental cutting of vessels (mostly veins) surrounding the lymphatics will be minimized.

#### **Problem 2**

PLVs break or Evans blue becomes difficult to see (steps 7a, 12a, 27b).

#### **Potential solution**

The PLVs can be pulled with forceps, but are delicate. A balance between exerting enough force for detaching them from the fat and not breaking them should be found. If excessive pulling is needed to detach the PLVs from the underlying tissue, it is likely that additional cutting with the microscalpel or micro scissors is needed. Once a PLV is broken, it should be retrieved as soon as possible because the Evans blue will leak out and the vessel will become hard to visualize.

In addition, when the PLVs are pulled with forceps, the flow of the vessels becomes interrupted and Evans blue can become dimmer. Usually, pressing gently on the footpad or ankle of the mouse forces additional Evans blue dye into the PLV.

#### **Problem 3**

Whole-mount PLVs are hard to image with confocal (step 26).

#### **Potential solution**

If PLV tissues cannot be imaged with confocal after sample retrieval, it likely is because the sample is too thick. After staining, we recommend looking at the sample under an epifluorescence microscope or fluorescence stereomicroscope to verify staining and to guide additional removal of fat tissue (if

necessary). If the PLV is too deep within the fat, confocal images will yield images of lower quality or will not be possible at all. Additionally, flattening the sample in between the coverslip and the slide should help reduce the thickness.

#### **Problem 4**

PLVs are buried in a deep layer of fat and/or intersect with blood vessels (steps 28–30).

#### **Potential solution**

The PLVs of some mice are more covered in fat. Generally, this directly correlates with their body weight. Leaner mice (20 g for a female, 25 g for a male) have less fat covering the lymphatics compared to overweight and obese mice. We have also observed that younger mice (1.5–2 months) usually have less fat covering the PLVs and their lymphatics are more easily detached from the surrounding tissue compared to older (6 months and over, [Figure 4C](#)) or obese mice. Older mice also tend to have more intricate blood vessel patterns that make the extraction more complicated. For these reasons, if fat excess and blood vessel intricacy are a problem, the use of younger and leaner mice is recommended, particularly for the first times attempting this protocol.

#### **Problem 5**

The cell yield is lower than  $10^4$  total cells (steps 31, 35, 39a).

#### **Potential solution**

Since the vessels are extracted sequentially from the mouse, longer surgery times are associated with decreased viability of recovered cells. The procedure should be performed as quickly as possible (40 min to an hour per mouse). Early euthanasia or death of the mouse will also lead to fewer viable cells and administering the appropriate dose of K/X is critical to avoid this.

The integrity of the vessels at the time of the digestion is also important because it affects how the enzymatic solution interacts with the sample. It is best to have each PLV in one single piece and without fat rather than multiple fragments with fat that can aggregate. Multiple and sticky fragments also can contribute to sample loss when transporting the vessels from HBSS to the enzymatic solution.

If the cell yield of the blood vessel is low, adding 0.5 mg/mL of elastase can aid increasing cell release.

All these factors impact the success of the procedure and contribute to the cell yield variability.

#### **Problem 6**

Cells do not reach confluency after 7 days (step 41).

#### **Potential solution**

Plated cells will take longer to reach 100% confluency 7 days after plating if the initial cell yield is low ( $>10^4$  total cells). In most cases, replacing the media and keeping the cells in culture for an additional 2–3 days is sufficient for the well to reach 100% confluency. We do not recommend filtering the cell suspension if used for culture because we have observed that vessel fragments that remain from the digestion can still contain some  $\alpha$ -sma+ cells and that growth clusters tend to form around them.

## **RESOURCE AVAILABILITY**

### **Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dennis Jones ([djones1@bu.edu](mailto:djones1@bu.edu)).

### Materials availability

This protocol did not generate any new materials.

### Data and code availability

This protocol did not generate any data sets.

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101800>.

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### AUTHOR CONTRIBUTIONS

G.A.-A. performed the experiments and wrote the paper. D.J. supervised the study and reviewed and edited the paper.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### REFERENCES

Jones, D., Meijer, E.F.J., Blatter, C., Liao, S., Pereira, E.R., Bouta, E.M., Jung, K., Chin, S.M., Huang, P., Munn, L.L., and Vakoc, B.J. (2018). Methicillin-resistant *Staphylococcus aureus* causes sustained collecting lymphatic vessel dysfunction. *Sci. Trans. Med.* *10*, eaam7964.

Kenney, H.M., Bell, R.D., Masters, E.A., Xing, L., Ritchlin, C.T., and Schwarz, E.M. (2020). Lineage

tracing reveals evidence of a popliteal lymphatic muscle progenitor cell that is distinct from skeletal and vascular muscle progenitors. *Sci. Rep.* *10* (18088).

Kenney, H.M., Wu, C.-L., Loiselle, A.E., Xing, L., Ritchlin, C.T., and Schwarz, E.M. (2022). Single-cell transcriptomics of popliteal lymphatic vessels and peripheral veins reveals altered

lymphatic muscle and immune cell populations in the TNF-TG arthritis model. *Arthritis Res. Ther.* *24*, 64.

Liao, S., Jones, D., Cheng, G., and Padera, T.P. (2014). Method for the quantitative measurement of collecting lymphatic vessel contraction in mice. *J. Biol. Methods* *1*, e6.