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## Imaging Blood Vessels and Lymphatics in Mouse Trachea Wholemounts

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### Abstract

Changes in blood vessels and lymphatics in health and disease are easier to understand and interpret when studied microscopically in three dimensions. The mouse trachea is a simple, yet powerful, and versatile model system in which to achieve this. We describe practical immunohistochemical methods for fluorescence and confocal microscopy of wholemounts of the mouse trachea to achieve this purpose in which the entire vasculature can be visualized from the organ level to the cellular and subcellular level. Blood vessels and lymphatics have highly stereotyped vascular architectures that repeat in arcades between the tracheal cartilages. Arterioles, capillaries, and venules can be easily identified for the blood vessels, while the lymphatics consist of initial lymphatics and collecting lymphatics. Even small abnormalities in either blood vessels or lymphatics can be noticed and evaluated in three dimensions. We and others have used the mouse trachea for examining in situ angiogenesis and lymphangiogenesis, vascular development and regression, vessel patency, differences in transgenic mice, and pathological changes, such as increased vascular permeability induced by inflammatory mediators.

### Keywords

Blood vessels; Lymphatic vessels; Endothelial cells; Angiogenesis; Lymphangiogenesis; Vascular regression; Immunohistochemistry; Confocal microscopy

## 1 Introduction

The increasing availability of transgenic mice, specific antibodies, and confocal microscopes has made wholemount preparations more attractive for studies of angiogenesis and lymphangiogenesis. To date, numerous tissues have been examined as wholemounts, including the retina, cornea, choroid, meninges, skin, intestine and mesentery but the

attributes of the mouse trachea are not well known. Yet, wholemounts of the trachea yield vastly more three-dimensional information than histological sections. The purpose of this chapter is to present practical step-by-step immunohistochemical methods for preparing the mouse trachea for studies of blood and lymphatic vessels by fluorescence and confocal microscopy.

This model system has been used by our laboratory and others for examining angiogenesis [1] and lymphangiogenesis [2], many other features including vascular development, remodeling and regression [3], testing therapeutic drugs [4], blood vessel patency [5, 6], vessel differences in transgenic mice, and changes in pathological conditions, such as increased vascular permeability in response to inflammatory mediators [7, 8] or infectious organisms [2].

## 2 Materials

### 2.1 Mice for Studying Blood and Lymphatic Vessels in Mouse Trachea

1. Tracheal blood vessels and lymphatics have been studied in wild-type and in several transgenic strains of mice. Rat tracheas can also be processed in a similar way but are thicker and more difficult to make flat. We have used doxycycline-regulated double-transgenic mice where the expression of a growth factor, e.g., VEGF-A [1] or VEGF-C [1] or marker of interest is driven by a doxycycline-responsive promoter gene *Scgb1a1* that is expressed in secretory Club cells found in the epithelium of trachea and large bronchioles (JAX strain).
2. Prospero homeobox 1-enhanced green fluorescence protein (Prox1-EGFP) transgenic mice have been particularly useful for studies of lymphatics and are available from the Mutant Mouse Resource Center at the University of California Davis as strain MMRC and from laboratories that use them [9], Although the endogenous green fluorescence of lymphatics in these mice fades rapidly after paraformaldehyde fixation, it can be amplified and made permanent by immunohistochemical staining for EGFP. Prox1-EGFP mice can be easily genotyped by the presence of green fluorescence of Prox1-EGFP in lens cells after a bright light is shined into their eyes.
3. Mice should be housed under pathogen-free conditions in filter-top cages to avoid accidental respiratory tract infections, which can have profound remodelling effects on airway blood vessels and lymphatics.

### 2.2 Anesthetics

Ketamine (87 mg/kg) plus xylazine (13 mg/kg) are injected intraperitoneally together in the same syringe (*see* Note 1).

### 2.3 Perfusion Apparatus

1. 1-L aspirator bottle with a hose outlet at the bottom (VWR/Kimax) is used for perfusion of fixative.

2. An aneroid sphygmomanometer (Medline) is used to generate a fixed pressure for perfusion of fixative from the aspirator bottle.
3. Silicone tubing and rubber bung are used to connect the sphygmomanometer and perfusion tubing to the aspirator bottle.
4. Male Luer-to-Luer adapters (Cole Palmer Cadence) attach the silicone perfusion tubing to an 18-gauge plastic catheter (Becton Dickinson), 30 mm in length that serves as the perfusion cannula.
5. A plastic tray similar to a restaurant dinner tray serves as a perfusion table that collects fixative during the perfusion.
6. The perfusion board is made from cork or a thick sheet of silicone or polystyrene foam (Styrofoam).
7. Surgical tape (e.g., 3 M) tape is wrapped around the front and hind paws of anesthetized mice and push-pins are used to fix the tape to the perfusion board.
8. A rubber band attached to front teeth and perfusion board is used to keep the neck and trachea straight during the perfusion.
9. A shadow-free surgical lamp is used for illumination during surgery, perfusion, and dissection.
10. 70% ethanol is used to moisten mouse fur before surgery and dissection.

#### 2.4 Instruments for Surgery and Dissection

1. 140-mm long coarse scissors are used to open the chest.
2. 90-mm long fine scissors from Fine Science Tools #14060–09 are used to cut the heart and trachea.
3. Hemostat (locking clamp) with curved smooth tips is used to clamp tubing from perfusion bottle.
4. Hemostat (locking clamp) with straight serrated tips is used to clamp the rib cage after opening the chest wall.
5. Dumont 11-cm straight forceps from Fine Science Tools are used for dissection.
6. Coarse straight forceps (10 mm long with serrated tips from Fine Science Tools are used for surgery and dissection.
7. Scalpel handle and rounded or straight scalpel blades are used for surgery and dissection.
8. Fine scissors from Fine Science Tools are used for fine dissection.
9. A shadowless surgical light provides ideal illumination during the perfusion.
10. A binocular dissecting microscope is useful for fine dissection.

## 2.5 Fixatives, Buffers, Reagents, Incubation Solutions, Embedding Medium, Etc.

- 1.** Fixative: 1% paraformaldehyde in 1× phosphate-buffered saline (PBS)  
Fixation is with 1% paraformaldehyde. See Making 1% paraformaldehyde.
- 2.** Calcium and magnesium-free PBS and PBS-Triton  
The composition of 1× PBS is 0.2 g/L  $\text{KH}_2\text{PO}_4$ , 2.16 g/L  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g/L KCl, 8.0 g/L NaCl. Store at room temperature. 1× PBS is conveniently prepared by diluting PBS 10× concentrate (GenClone) with water 1:10. Make PBS-Triton by adding 3 mL of Triton-X 100 (Fisher) to 1 L of 1× PBS. Stir with magnetic stirrer until dissolved.
- 3.** “Immunomix” antibody incubation medium  
“Immunomix,” consisting of 1× PBS containing 0.3% Triton-X 100, 0.2% bovine serum albumin (BSA, Sigma), and 0.1% sodium azide as an antibacterial agent (Sigma), is used for primary antibody incubations for immunohistochemistry. Can be stored at 4 °C for many months. When doing immunostaining using primary antibodies raised in goat or sheep, omit the 0.2% bovine serum albumin, as it can cross-react with the goat or sheep antibodies.
- 4.** “SuperDAPI” mounting medium  
“SuperDAPI” consists of VECTASHIELD with DAPI mounting (Vector Labs) with additional DAPI (Sigma) at a final concentration of 50 µg/mL. See Subheading 3 on how to prepare (see Note 2).
- 5.** Coverslip sealing resin  
Tissues are mounted on microscope slides in Vectashield with SuperDAPI and the coverslips are sealed with Cytoseal (Thermo-Fisher) or nail varnish.
- 6.** Sylgard silicone resin for making dishes to pin wholemounts  
Sylgard 184 silicone elastomer resin is available as a kit (Fisher Scientific).
- 7.** Stainless steel pins for tracheal wholemounts  
10-mm long headless stainless-steel pins (“Austerlitz Minutien” insect pins: 0.20 mm diameter, Fine Science Tools) are used to pin tracheal wholemounts to the layer of Sylgard in 6-well dishes (any brand). 5–6 tracheas per well in a 6-well dish can be stained simultaneously in a diluted antibody volume of 1–2 mL.
- 8.** Bradykinin for inducing leakage from tracheal blood vessels  
Bradykinin acetate (Sigma) is more effective at inducing vascular leakage in the mouse trachea than substance P or histamine.
- 9.** Fluorescent microspheres for microscopic imaging of vascular leakage  
Bright red or green-fluorescent latex microspheres are available in a range of sizes from 20 to 1000 nm from several vendors. We use Fluospheres from Molecular Probes/Invitrogen (see Note 3).

## Immunohistochemical reagents

1. Antibodies and other reagents used for immunohistochemical staining of lymphatics and other types of cells in mouse trachea are listed in Table 1. Combinations of antibodies that stain lymphatics together with other cell types can be very informative.
2. Primary antibodies, and fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch), are typically used at a working concentration of 0.5–2.5 µg/mL (1:100–1:500 dilution) (*see* Note 4).

Pipettes and tips (10 µL, 200 µL and 1000 µL) for making antibody solutions.

Glass slides and coverslips (Fisher).

Fluorescence and/or confocal microscope.

It should have ×5, ×10, and ×20 dry objective lenses and ×40 and ×100 oil-immersion lenses and digital camera (*see* Note 5).

Micrometer slide with 1 mm scale (Ted Pella).

Microscope accessories:

Immersion oil for oil-immersion objective lenses (e.g., Zeiss Immersol 518 N (Fisher Scientific), lens paper (Fisher Scientific) and lens cleaning liquid (EM Sciences) for cleaning lenses after use.

Computer with image capture software, ImageJ (<https://imagej.nih.gov/ij/ImageJ>) and Photoshop (Adobe) for processing data.

## 3 Methods

### 3.1 Making 1% Paraformaldehyde Fixative

1. Formaldehyde vapor is toxic! Work in a fume hood or at ducted ventilation (*see* Note 6). To make 1 L of 1% paraformaldehyde (PFA) solution, add 10 g of powdered paraformaldehyde (Fisher) to a 1-L Pyrex flask with a magnetic stirring bar inside and a thermometer (be careful not to break it).
2. Add approximately one-third of the total final volume of distilled or de-ionized water. Stir with a magnetic stirrer. The result will be a cloudy white suspension.
3. Heat the suspension carefully to 55–60 °C on a hotplate with magnetic stirrer in a fume hood. Slowly and carefully add 1 N NaOH drop by drop until the suspension changes from cloudy white to clear. Only a few drops are needed. The suspension will suddenly turn almost clear but will contain some suspended fine white precipitate. If the temperature rises above 65 °C, discard the suspension and start over. Allow the suspension to cool and then filter it with coarse filter paper in a funnel to get a clear solution.
4. Add sufficient 10× PBS to achieve a final PBS concentration of 1× and allow the suspension to cool. When the solution reaches room-temperature adjust the pH to

approximately 7.4 by adding HCl or NaOH drop by drop. Adjust the volume to the correct final value by adding distilled or de-ionized water.

5. Store 1% paraformaldehyde at room temperature in a tightly capped bottle for up to 2 weeks. Do not freeze. Discard if a precipitate is formed (*see Note 7*).

### 3.2 Preparation of Antibody Incubation Medium

Add 3 mL of Triton-X 100, 2 grams of BSA, and 10 mL of 10% sodium azide solution to a 1-L bottle and fill to 1 L with 1× PBS. Add a magnetic stirring bar and stir slowly to dissolve the BSA. If you attempt to dissolve the BSA quickly, it will clump and take significantly longer to completely dissolve (*see Note 8*).

### 3.3 Preparation of Sylgard Resin Dishes

Instructions for polymerizing Sylgard184 silicone resin are included in the 1-lb kit. Wear gloves. Add 1 part catalyst by weight to 10 parts resin in a plastic beaker and mix gently, trying not to introduce too many air bubbles into the mixture. If available, use a desiccator bell jar connected to a vacuum source to remove remaining air bubbles. Pour the resin into shallow plastic 6-well dishes or similar containers to a depth of 5–10 mm. Allow the viscous liquid resin to polymerize overnight on a flat surface.

### 3.4 Preparation of SuperDAPI Mounting Medium

To prepare SuperDAPI, make DAPI stock solution by dissolving DAPI dilactate (Sigma) at 1 mg/mL in deionized water. Store this DAPI stock bottle in the dark at 4 °C. Next, take a fresh 10 mL dropper bottle of Vectashield mounting medium (H-1000). Pipette 500 µL of DAPI stock solution into an empty glass dropper bottle. Add Vectashield drop by drop and swirl to mix it. Repeat a few times to dilute the DAPI solution with the entire 10 mL volume of Vectashield and mix well. Label the bottle as SuperDAPI and keep it at 4 °C in the dark.

### 3.5 Fixation, Cardiac Perfusion, and Trachea Dissection

1. For studies of vascular permeability, inject bradykinin (10 mg/kg iv) and fluorescent microspheres (20–50 µL) mixed in the same 28-gauge insulin syringe via tail vein immediately before anesthesia and perfusion.
2. After anesthesia, position the mouse ventral side up on the dissection board. Pinch rear paws to ensure adequate anesthesia by lack of reflexes. Secure the mouse to the dissection board with tape around limbs and push pins. Hold the head flat with a rubber band placed between the teeth and two push-pins to keep the trachea straight.
3. Moisten the fur with 70% ethanol and cut and remove the skin to expose the ribs and ventral chest wall.
4. Using coarse scissors, cut the chest open to make a U-shaped flap. The base of the U should be at the level of the eighth rib and across the sternum. Avoid cutting the lungs by gently moving them out of the way of the scissors. Also avoid cutting the diaphragm to prevent the abdominal contents from getting in

the way. The vertical portion of the U should end at the level of the fourth rib. Reflect the chest flap and clamp it with the serrated-tipped hemostat.

5. Hold the beating heart with coarse forceps and, using the fine scissors, make a shallow cut about 2 mm long at the tip of the left ventricle. Gently tear open the left and right atria with fine forceps (don't use scissors) to create an exit route for blood and perfused fixative (*see* Note 9).
6. Insert the perfusion catheter into the aorta through the opening in the left ventricle and perfuse the systemic vasculature with fixative for 2 min at a pressure of 120 mmHg (*see* Note 10).
7. When the perfusate coming out from the right atrium is cleared of blood, finish the perfusion by clamping the perfusion line. Rinse away any blood remaining in the thoracic cavity with tap water from a hose or a squirt bottle.
8. Lift up the heart by the apex, cut the main vessels with scissors and remove the heart.
9. Using medium straight scissors cut the trachea in the order shown in Fig. 1. Cutting the trachea neatly while it is still stretched under tension in situ in the mouse is much easier than after it has been removed. Cut the trachea along the ventral midline with fine scissors to make a flat whole-mount. At this point keep the larynx and the bronchi attached to the trachea and use these regions as handles to manipulate the tissue with fine forceps or for securing with pins. Avoid damaging the cylindrical part of the trachea. The larynx and bronchi cannot be made into successful flat mounts, so they are cut off at the final step before mounting.
10. With fine forceps carefully remove the thyroid glands, recurrent laryngeal nerves at either side of the trachea and any major accumulations of adipose tissue on the adventitial surface. But don't worry too much about perfect cleaning, because the structures can be omitted from the final stack of confocal images.
11. Wash trachea with several changes of PBS for about 1 h at room temperature to thoroughly rinse out the fixative.

### 3.6 Incubation Procedure for Immunohistochemistry

1. Stretch the tracheas and pin them by the corners in the Sylgard dish, with up to 6 tracheas per well. Incubate with gentle rotation or agitation at room temperature. Mount the tracheas with a gap between them and the silicone layer so that incubation solutions can circulate and reach both sides of whole mounts and be readily exchanged when washing.
2. Rinse fixative from specimens with several washes of PBS. Time is not critical, provided all of the fixative is rinsed out; more changes, and longer is better. Usually,  $6 \times 10$  min is enough.
3. Mouse tracheas can be processed in a small volume (1–2 mL) of incubation medium. Incubate tracheas in antibody incubation medium containing one or



more primary antibodies AND 5–10% blocking serum from the host species in which the secondary antibodies are raised (usually goat or donkey). The blocking step with normal serum reduces nonspecific staining by the primary antibodies. Combinations of primary antibodies that are raised in different species can be used, e.g., rabbit + goat + rat with appropriately colored species-specific secondary antibodies. Incubate tissues at room temperature for at least 6 h (overnight is better) in a 6-well dish on a mechanical rotator.

4. After the primary antibody incubation step, wash the tracheas with several changes of PBS-Triton for several hours. Wash whole mounts with PBS-Triton for a minimum of 6 h or overnight at room temperature.
5. Incubate for 1–4 h or longer (overnight is best) for whole mounts in fluorescent secondary antibodies diluted 1:500 in PBS-Triton. Do not use serum in this step. Secondary antibodies conjugated to Cy3 can be used for the red channel, conjugated to Alexa 488 for the green channel, and conjugated to Alexa 647 for the far-red channel of fluorescence or confocal microscopy.
6. Repeat the wash step with PBS/Triton.
7. After this penultimate washing step, fix the tissues again briefly for 2 min in 1% PFA. This important step helps to prevent fading and preserves the fluorescence by fixing the labeled antibodies. Wash briefly ( $3 \times 2$  min) with PBS to remove the fixative.
8. If you prefer, the whole process is easily adapted permanent specimens for viewing by transmitted light microscopy, by using HRP-labeled secondary antibodies or streptavidin/biotin procedures.
9. Fluorescent polystyrene microspheres can be used to mark sites of vascular leakage. *See ref. 7 for dose, duration, etc.*
10. Transfer the trachea wholemounts into a small volume of SuperDAPI in a watch glass or depression slide (VWR). The tracheas will become clear and almost invisible as the SuperDAPI medium infiltrates them.
11. After the final washing step, immerse the tracheas in Vectashield + SuperDAPI for 2–5 min in a few drops of VectaShield in a depression slide or watch glass. This step gives time for the mounting medium to infiltrate into the tracheal whole mount, which as a result, becomes more transparent.
12. Mount the tracheal whole mounts in SuperDAPI medium on glass slides, using as little mounting medium as possible. For most purposes, it is best to mount the trachea with the epithelial side up, as the blood vessels are closer to this surface and will be closer to the objective lens. Cover the wholemount with coverslips. If you have used too much mounting medium and the coverslips are floating, absorb the excess by blotting with paper towels.
13. Apply gentle pressure with forceps to the outside of the coverslip and a longitudinal stretching motion to squish and elongate the trachea. This part requires some practice.

- Carefully paint the edges of the coverslips with Cytoseal 60 (Fisher Scientific #831016) or nail varnish to seal them to the glass slides. This step permits any immersion oil to be wiped off the coverslip. Use a fume hood for this step, as Cytoseal 60 contains volatile and noxious solvents (toluene and butyl benzyl phthalate.) Allow the Cytoseal to harden for about 1 h in a fume hood at room temperature before viewing the slides on a microscope (*see* Note 11). Store slides in a slide box or plastic slide tray (VWR) in the dark in a refrigerator. Because of the final fixation, the fluorescence should last for up to 1 year.

### 3.7 Microscopy, Photography, and Tiling in Photoshop

- Prior to observation with a fluorescence or confocal microscope, take the slides out of the refrigerator and let them come to room temperature for a few minutes so any condensation can evaporate.
- Photograph the sections in appropriate color channels with camera exposure adjusted for brightness, contrast, and resolution. For conventional fluorescence microscopy, it is better to photograph red and green channels separately, rather than using dual band red/green fluorescence filters. Some bright fluorescence from one channel can bleed into the other channel. If this occurs, the bleed through image needs to be blackened in Photoshop (Window menu > Channels > select channel with bleed-through image > Edit > Fill > Black 100% > File > Save). Red and green channels can be pasted into R and G channels and separately adjusted and viewed in the Channels window (*see* Note 12).
- Low magnification montages or panoramas of entire tracheas can be made by stitching together multiple photographs taken at a higher magnification and resolution. This “photo-tiling” can be done with Photoshop or other applications. For example, 10–20 images at 5× are usually sufficient to make a panorama of an entire section of mouse trachea. Images at 5× have a great depth of field, so almost all blood vessels and lymphatics are in focus. Photograph individual images so that they have about 30% horizontal and vertical overlap with adjacent images.
- In Photoshop, open all of the individual images > File > Automate > Photomerge > select “Auto Layout” (if not already clicked) and “Blend Images Together” and click “Add Open Files”. Click OK. After processing, Photoshop will assemble the images into a seamless whole. If there are gaps in the image, the original photos have not had enough overlap with their neighbors and need to be re-photographed. Sometimes individual stray images can be dragged manually into the correct position in the panorama. The panorama will be in multiple Photoshop layers and will have a large file size. When you are sure the panorama is correct, flatten the multiple merged images (Layers > Flatten Image > Save as JPG).
- It is useful to take a photo of a calibrated micrometer scale slide to calculate the final magnification of the panorama. An easy and accurate way to put a scale bar on your final micrograph is to take a photo of the micrometer slide at the same

magnification as your image. Put the photo of the micrometer slide into a new layer in PhotoShop, use it to make a scale bar of a suitable length, then delete it, leaving only the scale bar.

### 3.8 Pixel Intensity Analysis, Surface Plots, and Morphometry with ImageJ

1. Morphometric parameters such as the size, area density, fluorescence intensity, etc. of immunostained blood vessels and lymphatics in tracheal wholemounts can be measured with ImageJ) after image optimization in Photoshop.
2. Camera settings and Photoshop adjustments must be the same for all images in the batch being analyzed, because the measurement of area density is based on the percentage of pixels above a certain brightness threshold.
3. In Photoshop adjust the contrast and brightness of the color channel containing vessels of interest, such that the background where lymphatics are absent is almost black and the vessels are bright.
4. Launch ImageJ and open the image (File > Open > select the image of interest).
5. In the “Image” menu of ImageJ scroll down to “Color” then across to “Split Channels”. Keep the color channel containing the vessels of interest and discard the others.
6. Surface plots in ImageJ are a useful way to compare the intensity of staining in images. Arrange the images side by side, open in ImageJ and choose the color channel to be analyzed as in **steps 4 and 5** above. In ImageJ Analyze menu select “Surface Plot”. A topographic image with mountains and valleys representing fluorescence intensity should be produced. To make a color-coded heat map of the fluorescence intensities, in the ImageJ Image menu (third from left in ImageJ menu) select “Lookup Tables” at bottom and choose one that works for you, e.g., “16 colors”. For an example, see Fig. 3c.
7. For additional analysis of your image, select the region of interest using the “Freehand selection” lasso tool (fourth from the left in the ImageJ icon menu).
8. In the “Analyze” menu of ImageJ scroll down to “Histogram” describing the number of pixels in the image with a brightness of 0 to 225. Click “Copy”.
9. Paste the data into an Excel spreadsheet, and calculate the total number of pixels with intensity 0 to 225 in the region of interest bounded by the yellow line.
10. Also in Excel, calculate the total number of pixels above the chosen brightness threshold, and calculate the percentage they represent of the total number of pixels. The absolute value of the brightness threshold is arbitrary but must be less than the brightness of the structures identified as blood vessels or lymphatics and must be the same for all images being analyzed.
11. A plugin called Image-based tool for Counting Nuclei (ICTN) for ImageJ, which can be downloaded from <https://imagej.nih.gov/ij/plugins/itcn.html>, can be used for counting nuclei of Prox1-stained lymphatic endothelial cells.

12. Morphometric point counting can be used as an alternative to ImageJ for measuring area densities. For this approach, a rectangular test grid is overlaid on the image, and points of intersection are counted manually. A plugin to create such a grid in ImageJ can be accessed by clicking Analyze > scroll down to “Tools” > “Grid”.
13. The whole process takes 3 days from mouse to image analysis. Day 1: perfusion washing, mounting in Sylgard dishes and primary antibody incubation overnight; Day 2: washing and secondary antibody incubation overnight; Day 3: washing, final fixation and mounting on glass slides, imaging and analysis. A low magnification confocal image of the architecture of mouse tracheal blood and lymphatic vessels is shown in Fig. 2. Examples of various useful immunohistochemical markers related to blood vessels and lymphatics are shown in Fig. 3.

## 4 Notes

1. Written records must be kept on the use of ketamine, as for other controlled substances, and made available for inspection by your institution’s Environmental Health and Safety (EH&S) department.
2. Vectashield with DAPI mounting medium (Vector Labs product) does not contain enough DAPI to properly stain tracheal whole mounts. However, it does contain a useful proprietary anti-fade agent than prolongs fluorescence and minimizes bleaching.
3. A detailed product description of fluorescent microspheres from Molecular Probes/Invitrogen is available at: <https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fmp05000.pdf&title=Rmx1b1NwaGVyZXMgRmx1b3Jlc2NlbnQgTWljcm9zcGhlcmVz>
4. Secondary antibodies (e.g., from Jackson ImmunoResearch Laboratories) that are pre-absorbed for other species are superior to unabsorbed ones. They are more expensive but result in lower background staining.
5. Any brand of microscope from a reliable maker is suitable. Low magnification lenses ( $\times 5$ ,  $\times 10$ ,  $\times 20$ ) are useful for making panoramas and images with a great focal depth. For fluorescence microscopy, oil-immersion lenses  $\times 40$  and  $\times 100$  provide a brighter image with more resolution, but they require more care in their use and have a shallower focal depth. Ordinary paper towels or tissues can be used for wiping and cleaning glass slides and coverslips, but NOT for objective lenses. Carefully clean oil immersion lenses with purpose-made lens paper, which can scratch and damage the lens surface. Keep the microscope stage clean and dry; wipe up any spilled liquid with tissue paper.
6. The Occupational Safety Health Administration (OSHA) limit for formaldehyde in the workplace is 0.75 parts formaldehyde per million parts of air (0.75 ppm)

measured as an 8-h time-weighted average, or a short-term exposure limit of 2 ppm during a 15-min period. Check your local EH&S regulations for conditions for performing perfusion with paraformaldehyde solution and take any necessary training courses.

7. Do not freeze aliquots of paraformaldehyde in solution, as the activity will be lost. Do not use formalin (37% paraformaldehyde +15% methanol) as the fixative. Local safety regulations may require bottles to have warning labels of “Carcinogen” or similar. Dispose of waste paraformaldehyde in accord with local safety regulations.
8. Sodium azide is toxic and should be handled with care. Read the safety data sheet before handling it and check with local regulations and authorities for policies governing its use and disposal. Wear gloves, mask, and protective clothing. For safety, sodium azide is best stored and handled as a solution in a break-proof bottle surrounded by a safety container. Make a 10% aqueous solution by carefully dissolving 10 g sodium azide powder in 100 mL of deionized water.
9. When creating a route for outflow of blood and fixative during the perfusion, use a toothed forceps and do not cut the heart atria with scissors, as there is a risk of accidental cutting of the aorta or other structures by mistake.
10. In our experience, 1% paraformaldehyde solution is just as good for fixing mouse tissues for immunohistochemistry with most antibodies and produces fewer fumes than 4%. Perfusion should be done in a well-ventilated place, e.g., a fume hood or perfusion board equipped with approved ventilation nearby to minimize the danger of breathing toxic formaldehyde fumes. For safety, wear gloves, mask, and personal protective clothing/equipment.
11. When using objective lenses with short working distances (20× and above), be careful to avoid bringing the lens into contact with Cytooseal that has not fully hardened. If that should accidentally happen, clean off the Cytooseal immediately with lens paper and lens cleaning fluid.
12. If mistakes are made in Photoshop, they can be reversed by using the History menu window or the Edit > Step Backwards command.

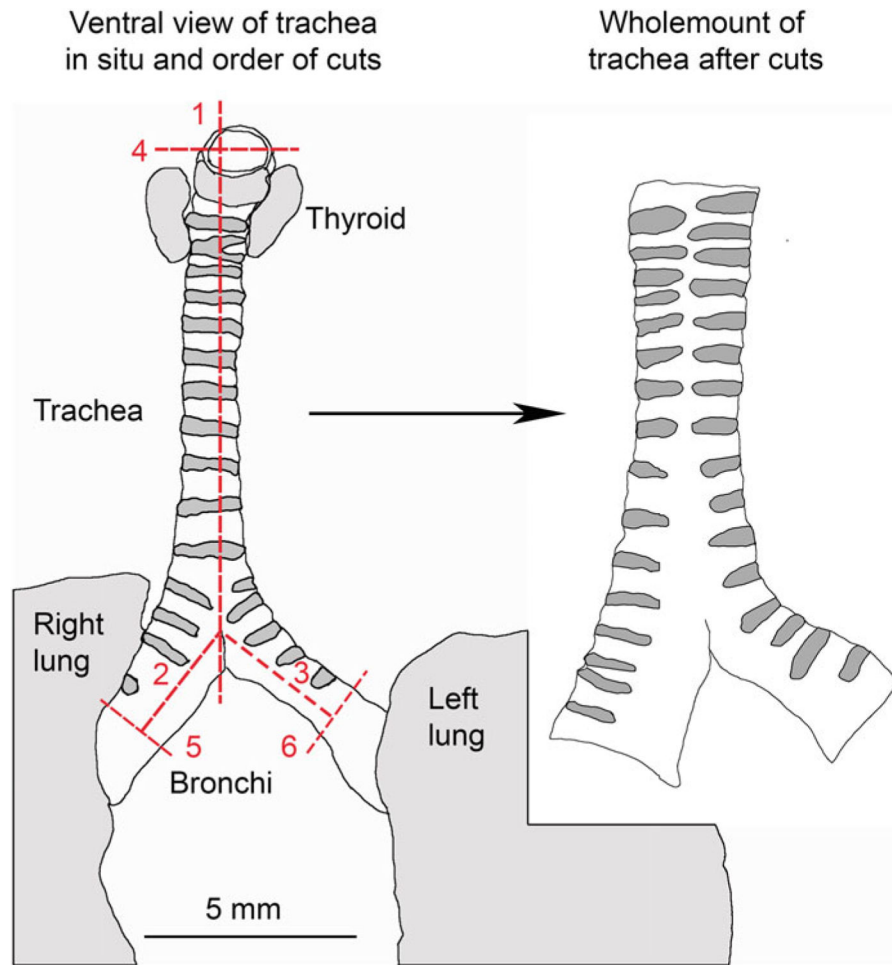
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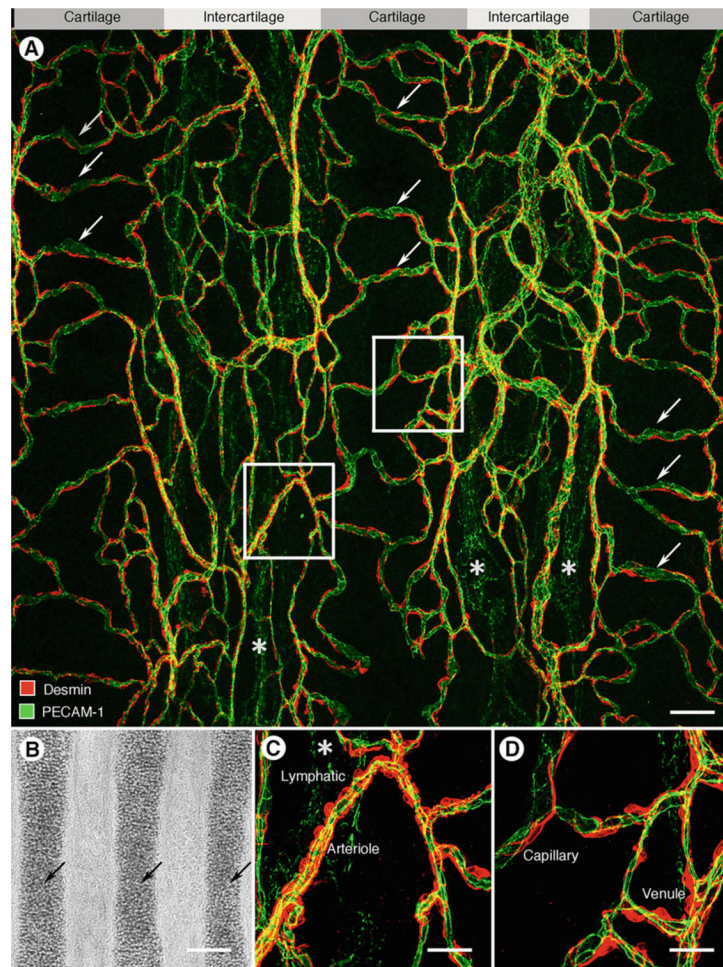
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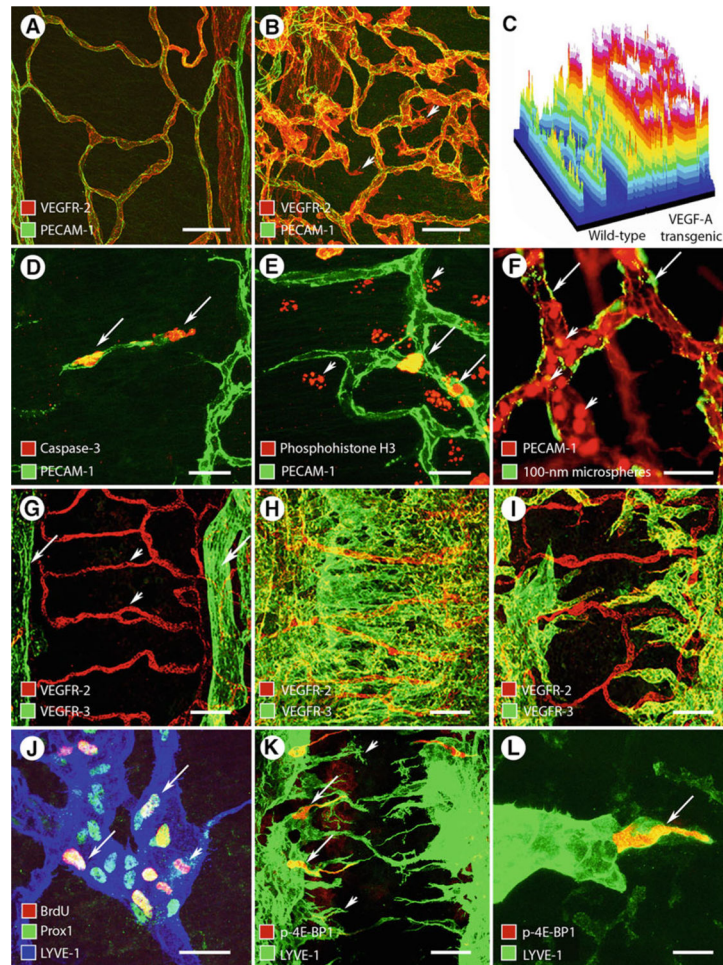


**Fig. 1.** Schematic diagram of mouse trachea in situ showing the order of cuts (dashed red lines) to make a wholemount preparation. 1: longitudinal cut along ventral aspect of trachea; 2 and 3: cuts along bronchi; 4: cut across larynx; 5 and 6: cuts across bronchi. The resulting wholemount is shown on the right



**Fig. 2.** Vasculature of adult mouse trachea. Whole mount preparation showing the vasculature of the adult trachea (endothelial cells, PECAM-1, green) with pericytes and smooth muscle cells (desmin, red) at P70. Long axis of the trachea oriented horizontally. **(a)** Confocal micrograph showing ladder-like arrangement of capillaries (arrows) that span three cartilage rings. Most arterioles, venules, and weakly stained lymphatic vessels (\*) are confined to regions between cartilages. Bar: 50  $\mu$ m. Boxed regions are enlarged in **c**, **d**. **(b)** Cartilages (arrows) of entire panel **a** are shown here in a brightfield image. **(c)** Arteriole with densely packed circumferentially oriented smooth muscle cells and a weakly stained lymphatic (\*). **(d)** Venule and capillaries with obliquely or longitudinally oriented pericytes stained for desmin. Scale bar in **d**: 50  $\mu$ m in **a**, 150  $\mu$ m in **b**, and 20  $\mu$ m in **c**, **d**. (Image reprinted with permission from Ref. [3])





**Fig. 3.** (a, b) Capillaries over tracheal cartilages of transgenic mice at (a) baseline and (b) 7 days after doxycycline-induced overexpression of VEGF-A overexpression in VEGF-A transgenic mouse. Abundance of angiogenic vessels and increase in VEGFR-2 expression. Bar: 50  $\mu$ m. (c) ImageJ surface plot showing increase in VEGFR-2 expression (fluorescence intensity) of A, B. (d, e) In mice, the tracheal vascular plexus is remodeled over the first week of neonatal life. (d) Endothelial cells first undergo apoptosis at P3, as shown by activated caspase-3 (arrows), and later (e) growth at P7 as shown by phosphohistone H3 (arrows). Arrowheads indicate other dividing cells. Bar: 20  $\mu$ m. (f) Increased vascular permeability shown by extravasation of 100-nm green-fluorescent microspheres (arrows) from tracheal venules 4 min after bradykinin challenge (10 mg/kg iv). Numerous intravascular leukocytes adherent in venules (arrowheads) are also stained by the PECAM-1 antibody. Bar: 50  $\mu$ m. (g-i) Tracheal lymphatics marked by VEGFR-3 staining at (g) baseline and (h) after overexpression of VEGF-C in CCSP/VEGF-C transgenic mouse. (h) Doxycycline administered P0-P7, and (i) from P70-P77. Much stronger lymphangiogenesis in neonatal mouse. Arrows: lymphatics. Arrowheads: blood vessels. Bar 50  $\mu$ m. (j) Tracheal lymphatic endothelial cell nuclei (arrows) labeled by BrdU staining 3 days after induction of VEGF-C in CCSP/VEGF-C transgenic mouse. Arrowhead marks mitotic cell. Bar: 20  $\mu$ m.

**(k, l)** Proliferating lymphatic endothelial cells marked by staining for p-4E-BP1 (arrows). Arrowheads mark lymphatic sprouts. Bar: 50  $\mu\text{m}$  in K and 10  $\mu\text{m}$  in L. (Images reprinted with permission; **a–c** from Ref. [1], **d, e** from Ref. [3], **f** from Ref. [7], **g–i** from Ref. [10], and **j–l** from Ref. [5])

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**Table 1**

Markers of endothelial cells in blood vessels and lymphatics and other cells in mouse trachea

Cell type	Marker	Host	Vendor	Catalog #	Comments
<i>Vascular endothelial cells</i>					
	PECAM-1 (CD31)	Armenian Hamster Rat	Thermo BioLegend	MA3105 102502	Clone 2H8 Clone MEC13.3 Weaker expression also on lymphatic EC
	VE-cadherin (CD144)	Rabbit Goat Rat	Millipore/Sigma R&D Systems BD Biosciences	MABT886T AF1002 550548	Clone Vli37 Clone 11D4.1
	Endoglin (CD105)	Rat	eBioscience	14-1051	Clone MJ7/18
	Endomucin	Rat Goat	Invitrogen R&D Systems	14-5851-81 AF4666	Clone V.7C7 On venules, not arterioles
	Von Willebrand factor	Rabbit	Dako	0082	
	MECA-32 (PV-1)	Rat	BD Biosciences	550,563	Clone MECA-32
Endothelial glycocalyx	<i>Lycopersicon esculentum</i> lectin	Plant	Vector Laboratories	DL-1174-1	
Endothelial glycocalyx	<i>Griffonia simplicifolia</i> isolectin B4	Plant	Vector Laboratories	FL-1201-5	
Endothelial basement membrane	Collagen-IV	Rabbit	CosmoBio	LB-1403	Strong on blood vessels and collecting lymphatics
VEGF receptor	VEGFR-2	Goat	R&D Systems	AF644	Weaker expression also on lymphatic EC
Lumen of patent blood and lymphatic vessels	Podocalyxin	Goat	R&D Systems	AF1556	
High endothelial venules (HEV)	MECA-79	Rat (IgM)	BD Bioscience	553,863	Found in bronchoalveolar lymphoid tissue (BALT)
<i>Lymphatic endothelial cells</i>					
	EGFP in Prox1-EGFP mice	Chicken	Aves	GFP 1020	Overall best marker Strong signal. Marks cytoplasm and nucleus
	LYVE-1	Rabbit Rat Goat	AngioBio R&D Systems R&D Systems	11-034 MAB2125 AF2125	Good marker Also found on some macrophages
	Prox1	Rabbit Goat	AngioBio R&D Systems	11-002P AF2727	Nuclei of lymphatic EC and neuroendocrine cells
	VEGFR-3	Goat	R&D Systems	AF743	Weaker signal and higher background than Prox1
	CCL21	Goat	R&D Systems	AF469	In Golgi apparatus of lymphatic endothelial cells
	Neuropilin-2	Goat	R&D Systems	AF564	Endothelial cells of lymphatics and venules
	Podoplanin	Syrian hamster	AngioBio	11-033 Clone 8.1.1	Also expressed by some tracheal epithelial cells
<i>Activity markers</i>					
Dividing cells	Phospho-histone H3	Rabbit	Millipore	06-570	
Dividing cells	BrdU	Rat	Abcam	Ab567	Needs pre-injection of BrdU
Dying cells	Activated caspase-3	Rabbit	R&D Systems Cell Signaling	AF835 9579	Clone D3E9

Cell type	Marker	Host	Vendor	Catalog #	Comments
Adhesion molecule	ICAM (CD54)	Rat	eBioscience	14-1061-82	Clone 429
Adhesion molecule	VCAM (CD106)	Rat	Millipore/Sigma	MAB2627	Clone 6C7.1
Adhesion molecule	P-selectin	Rat	BD Biosciences	550,289	Clone RB40.34
mTOR activity	p-4E-BP1	Rabbit	Cell Signaling	2855	In lymphatic sprouts
mTOR activity	p-S6RP	Rabbit	Cell Signaling	4858	In lymphatic sprouts
Leakage marker	RCA1 lectin	Plant	Vector Laboratories	B-1085-5	Biotinylated
<i>Other cell types</i>					
Smooth muscle cells	$\alpha$ -smooth muscle actin	Mouse	Millipore/Sigma	F3777 (FITC) C6198 (Cy3)	Also marks airway smooth muscle cells
Pericytes	NG2	Rabbit	Millipore/Sigma	Ab5320	
Pan-epithelial cells	E-cadherin Pan-cytokeratin	Rat Rabbit	Thermo Dako	13-1900 Z0622	Clone ECCD-2
Club epithelial cells	CCSP	Rabbit	Millipore/Sigma	07-623	
Ciliated epithelial cells	Acetylated tubulin	Mouse	Millipore/Sigma	T7451	Clone 6-11B-1
T-lymphocytes	CD3e	Armenian hamster	BioLegend	100,302	Clone 145-2C11
B-lymphocytes	B220	Rat	BioLegend	103,202	Clone RA3-6B2
Neutrophils	S100A8 A100A9	Goat Rabbit	R&D Systems Abcam	AF3059 Ab105472	
Macrophages	Iba1 Mrc1/CD206 F4/80	Rabbit Rat Rat	WAKO BioRad BioLegend	019-19741 MCA2235GA 123102	Also known as Aif1 Clone BM8 Better in wax sections
Dendritic cells	CD11c	Rat	eBioscience	14-0114-81	Clone N418 Better in wax sections