Video Article

A Method for Labeling Vasculature in Embryonic Mice

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Abstract

The establishment of a functional blood vessel network is an essential part of organogenesis, and is required for optimal organ function. For example, in the thymus proper vasculature formation and patterning is essential for thymocyte entry into the organ and mature T-cell exit to the periphery. The spatial arrangement of blood vessels in the thymus is dependent upon signals from the local microenvironment, namely thymic epithelial cells (TEC). Several recent reports suggest that disruption of these signals results in thymus blood vessel defects^{1,2}. Previous studies have described techniques used to label the neonatal and adult thymus vasculature^{1,2}. We demonstrate here a technique for labeling blood vessels in the embryonic thymus. This method combines the use of FITC-dextran or *Griffonia (Bandeiraea) Simplicifolia* Lectin I (GSL 1 - isolectin B₄) facial vein injections and CD31 antibody staining to identify thymus vascular structures and PDGFR- β to label thymic perivascular mesenchyme³⁻⁵. The option of using cryosections or vibratome sections is also provided. This protocol can be used to identify thymus vascular defects, which is critical for defining the roles of TEC-derived molecules in thymus blood vessel formation. As the method labels the entire vasculature, it can also be used to analyze the vascular networks in multiple organs and tissues throughout the embryo including skin and heart⁶⁻¹⁰.

Video Link

The video component of this article can be found at <http://www.jove.com/details.php?id=3267>

Protocol

1. Fluorescein labeled dextran and GSL I-isolectin B₄ facial vein injections to label embryonic vasculature

1. Prepare FITC-dextran (50ug/mL) in Phosphate Buffered Saline (PBS) or GSL 1 - isolectin B₄ (20ug/200uL) in PBS in a 1.5mL Eppendorf tube and warm to 37°C. Add 100uL of stock 1.25mM Fast Green/PBS to the FITC-dextran solution (total volume 1mL) and 180uL of stock 1.25mM Fast Green/PBS to the GSL 1 - isolectin B₄ (total volume 200uL), so that the solution is visibly blue.
2. Dissect E14.5-E18.5 embryos and yolk sac together, leaving the allantoic stalk (umbilical artery and vein) intact.
3. Transfer embryos to a new petrie dish (60 X 15 mm) and immerse them in PBS at room temperature.
4. Position the embryo to provide a sagittal view of the head/face. Use micro dissecting forceps to gently grasp the embryo at the head.
5. Using a 30G needle, inject 50uL FITC-dextran (50ug/mL) or GSL 1 - isolectin B₄ (20ug in 200uL PBS) into the facial vein pointing the needle toward the back of the head.
6. When the dye is visible in the umbilical vein, remove the needle and separate the embryo from the allantoic stalk (umbilical artery and vein).
7. Following injections, allow embryo to remain in PBS at room temperature for 2-3 minutes so that the dye circulates throughout the embryo.

2. Whole-mount analysis of skin vasculature

1. After allowing the dye to circulate throughout the embryo, remove skin samples from regions of the limbs, back, and stomach, etc.^{8,9}.
2. Wash skin sample in cold PBS, and fix in 4% PFA/PBS for 2 hours^{8,9}. Wash 3 times for 10 minutes each in 4mL clear vial with 2mL cold PBS.
3. Place skin sample on a microscope slide and add 100 μ L of mounting media to each slide and a cover glass.
4. Allow slides to dry in a dark storage area.
5. Proceed to Step 2 'Image Acquisition' section.

3. Multi-color labeling of thymus and heart vasculature and perivascular cells for cryosections (Continue from Section 1, Step 7)

1. 'Flash freeze' whole embryo in liquid nitrogen. Embryos can be and stored at -80°C until analysis.
2. Alternatively, dissect out thymus, rinse in 4°C PBS, and fix in 2mL 4% Paraformaldehyde (PFA)/PBS for 2 hours. Wash 3 times for 10 minutes in cold PBS, place thymi in OCT, and freeze and store until use at -80°C.

3. For cryosectioning, spread OCT on a section 'block' and mount the embryo or dissected organs/tissues for sectioning.
4. Cut frozen tissue into 10 μ m thick sections and collect on slides.
5. Fix sections in acetone for 5-10 minutes. Wash 3 times in cold TBS.
6. Block in 10% donkey serum/TBS in a humidity chamber at room temperature.
7. Incubate sections for 1 hour-overnight with 100 μ l of primary antibodies in a humidity chamber at 4°C: in this example, we use rat anti-mouse CD31 (1:100) to label endothelium, and goat anti-mouse PDGFR- β (1:100) to label perivascular cells. It is useful to cover slides with individually cut Parafilm strips to ensure that the antibody is uniformly spread across the section.
8. Following incubation with primary antibody, wash sections 3 times in cold TBS. Incubate with 100 μ l of appropriate secondary antibodies for 30 minutes minimum.
9. Wash 3 times in cold TBS. Add 100 μ l of mounting media to each slide and a cover glass.
10. Allow slides to dry in a dark storage area.
11. Proceed to 'Image Acquisition' section.

4. Multi-color labeling of thymus vasculature and perivascular cells for vibratome sections (Continue from Section 1, Step 7)

1. Dissect out thymus lobes from embryo and rinse in cold PBS.
2. Fix thymus in 4% PFA/PBS at room temperature for 2 hours.
3. Wash in PBS-Triton X (0.15%) 3 times, 10 minutes and place thymi in a small plastic cartridge and submerge in 4% low melt agarose/PBS (~4°C). The thymus should be in contact with the bottom of the cartridge.
4. Allow agarose to solidify on ice (3-5 minutes). Use a razor blade to cut off excess agarose. Add glue to the vibratome block and adhere sample to the block.
5. Add cold PBS to vibratome water bath until the sample and blade are immersed.
6. Set speed and amplitude (high amplitude and low-moderate speed is ideal for soft thymus sections). The amplitude should be reduced if sections break up due to excess agitation.
7. Cut 50 μ m sections.
8. Using a paintbrush, collect sections in a 24-well microplate in cold PBS.
9. Block sections in 500 μ l of 10% donkey serum in PBS-Triton X (0.15%) for 30 minutes.
10. Incubate sections for 8 hours to overnight with primary antibody, such as anti-CD31 and anti-PDGFR- β , in a covered 24-well microplate.
11. Wash 3 times in PBS-Triton X (0.15%) over a total of 8 hours at 4°C.
12. Block sections in 10% donkey serum in PBS-Triton X (0.15%) for 30 minutes.
13. Incubate sections for 8 hours to overnight at 4°C with appropriate secondary antibodies.
14. Wash 3 times in PBS-Triton X (0.15%) over a total of 8 hours at 4°C.
15. Re-fix samples in 4% PFA/PBS for 30 minutes on ice.
16. Wash 3 times in PBS-Triton X (0.15%) over 30 minutes on ice.
17. Dehydrate samples through a graded MeOH/PBS-Triton X series: 25% MeOH, 50% MeOH, 75% MeOH, and 100% MeOH at 10 minutes for each step. Replace 100% MeOH with fresh MeOH after 10 minutes and incubate for 1 hour at room temperature.
18. In a glass container, mix BABB (Benzyl Alcohol: Benzyl Benzoate) in a 1:2 ratio. Combine BABB with MeOH for a final concentration of 50% BABB and 50% MeOH. Incubate the sample in BABB:MeOH for 10-15 minutes.
19. Transfer sample to a glass container with 100% BABB and incubate for 10-15 minutes or until cleared, at room temperature.
20. Fill depression slide (0.7mm depth) with fresh 100% BABB and transfer sample to the slide. Add cover glass (No. 1.5) and seal with 2-3 coats of nail polish. Allow nail polish to harden in the dark at room temperature, then store sample at 4°C.

Note: Slides must be completely sealed prior to confocal image acquisition. Images should be acquired within 12-24 hours, as fluorescent dyes can fade in BABB.

21. Proceed to 'Image Acquisition' section.

5. Image acquisition

1. Image 10 μ m frozen sections with a confocal microscope using the Plan-Apochromat 20X/0.8 objective (512 X 512 Pixels) with 488- (FITC-dextran/GSL 1 - isolectin B₄), 543-, and 633-nm laser lines.
2. Acquire confocal z-sections of whole mount skin and 50 μ m agarose-embedded sections using the Plan-Apochromat 10X/0.4 objective (512 X 512 Pixels) with 488- (FITC-dextran/GSL 1 - isolectin B₄), 543-, and 633-nm laser lines. Serial Z-sections should be collected sequentially at 1-micron for each respective channel.
3. Reconstruct serial Z-sections using Zeiss Axiovision 4.6 or other image analysis software.

6. Representative Results:

Efficient labeling of the embryonic vasculature is critical for assessing blood vessel defects in embryonic mice. Figure 1 shows specific labeling of E16.5 thymus blood vessels (1A-B) and co-labeling with CD31 (1B), in addition to staining of the right and left ventricles (1E-F), respectively. The GSL I-isolectin B₄ protocol for cryosections as described in sections 1, 3, and 5 was used in these experiments. Whole-mount labeling of the skin blood vessel network on E16.5 mice, using the protocols described in sections 1, 2, and 5 is shown in Figure 1C-D.

Figure 1 Legend. FITC GSL I - isolectin B₄ facial vein injections into E16.5 mouse embryos. **a.** Cryosection of embryonic thymus following injection. **b.** Merge of CD31 co-labeling with isolectin B₄. **c.** and **d.** Whole-mount of embryonic skin vasculature following injection. **e.** and **f.** Cryosection of embryonic heart **e.** (right ventricle) **f.** (left ventricle) following injection.

Discussion

Whole-mount and PECAM-1 (CD31) staining on sections are the conventional methods for labeling the vasculature in embryonic mice. These methods require the use of direct and/or indirect immunofluorescence, and detergents to permeabilize mouse tissue. This proves to be a rather timely process. Here, we have employed FITC-dextran or isolectin B₄ facial vein injections to directly label the embryonic vasculature, thereby eliminating the requirement for antibody labeling steps. Furthermore, this method allows the assessment of the functional status of the vasculature, as 'leaky' phenotypes will be revealed by this method but not by conventional antibody staining methods.

We have validated the efficiency of this technique by analyzing sections of embryonic thymus, and heart, as well as whole mounts of skin vasculature shortly after facial vein injections. Additionally, we have stained thymus sections for CD31 following FITC-dextran or isolectin B₄ injections to demonstrate that FITC specifically labels embryonic vascular structures. Therefore FITC-dextran or isolectin B₄ facial vein injections in embryos can be utilized to rapidly assess vascular phenotypes in multiple organs throughout development. In addition, this method allows researchers to determine the specific time during development in which the peripheral embryonic vasculature connects to an organ of interest, via angiogenesis. We have included an antibody labeling protocol that is compatible with this FITC-dextran or isolectin B₄ protocol. Using this protocol, PDGFR- β , SMA- α , and other antibodies, which label structural components of blood vessels, may be employed to further assess the nature of embryonic vascular defects.

Important Notes: A dye such as Fast Green can be added to the FITC-dextran/GSL 1 - isolectin B₄ solution in order to visualize the mixture as it is injected into the facial vein. Shortly after FITC-dextran injection, the dye can be observed in the umbilical vein and in blood vessels throughout the yolk sac and placenta. At this point, the umbilical cord should be removed from the embryo. In the event that dye is not detected in the allantoic stalk (umbilical artery and vein) the technique likely failed. We have observed that if the needle does not directly enter the facial vein, FITC-dextran/ Fluorescein labeled GSL 1 - isolectin B₄ accumulates in adjacent areas of the face. We have also observed that FITC-dextran labels the entire vasculature, while GSL 1 - isolectin B₄ labels most embryonic vascular structures, but also non-vascular cells in the liver. A fluorescence-dissecting microscope can be used to test whether the injection was successful.

Mice

C57Bl6/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Embryos were generated via wild type, timed matings. Experiments were approved by the University of Georgia's Institutional Animal Care and Use Committee.

Disclosures

No conflicts of interest declared.

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References

1. Cuddihy, A.R., *et al.* VEGF-mediated cross-talk within the neonatal murine thymus. *Blood*. **113**, 2723-2731, doi: 10.1182/blood-2008-06-162040 (2009).
2. Muller, S.M., *et al.* Gene targeting of VEGF-A in thymus epithelium disrupts thymus blood vessel architecture. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10587-10592, doi: 050275210210.1073/pnas.0502752102 (2005).
3. Muller, S.M., *et al.* Neural crest origin of perivascular mesenchyme in the adult thymus. *J. Immunol.* **180**, 5344-5351, doi: 180/8/5344 (2008).
4. Foster, K., *et al.* Contribution of neural crest-derived cells in the embryonic and adult thymus. *J. Immunol.* **180**, 3183-3189, doi: 180/5/3183 (2008).
5. Liu, C., *et al.* Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood*. **108**, 2531-2539, doi: blood-2006-05-024190 10.1182/blood-2006-05-024190 (2006).
6. Lavine, K.J., *et al.* Fibroblast growth factor signals regulate a wave of Hedgehog activation that is essential for coronary vascular development. *Genes Dev.* **20**, 1651-1666, doi: 20/12/1651 10.1101/gad.1411406 (2006).
7. Lavine, K.J., Kovacs, A., & Ornitz, D.M. Hedgehog signaling is critical for maintenance of the adult coronary vasculature in mice. *J. Clin. Invest.* **118**, 2404-2414, doi: 10.1172/JCI34561 (2008).
8. Mukoyama, Y.S., Gerber, H.P., Ferrara, N., Gu, C. & Anderson, D.J. Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development*. **132**, 941-952, doi: dev.01675 10.1242/dev.01675 (2005).
9. Mukoyama, Y.S., Shin, D., Britsch, S., Taniguchi, M. & Anderson, D.J. Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell*. **109**, 693-705, doi: S0092867402007572 (2002).
10. Murphy, P.A., *et al.* Endothelial Notch4 signaling induces hallmarks of brain arteriovenous malformations in mice. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 10901-10906, doi: 10.1073/pnas.0802743105 (2008).