Video Article

Whole-mount Immunohistochemical Analysis for Embryonic Limb Skin Vasculature: a Model System to Study Vascular Branching Morphogenesis in Embryo

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Abstract

Whole-mount immunohistochemical analysis for imaging the entire vasculature is pivotal for understanding the cellular mechanisms of branching morphogenesis. We have developed the limb skin vasculature model to study vascular development in which a pre-existing primitive capillary plexus is reorganized into a hierarchically branched vascular network. Whole-mount confocal microscopy with multiple labelling allows for robust imaging of intact blood vessels as well as their cellular components including endothelial cells, pericytes and smooth muscle cells, using specific fluorescent markers. Advances in this limb skin vasculature model with genetic studies have improved understanding molecular mechanisms of vascular development and patterning. The limb skin vasculature model has been used to study how peripheral nerves provide a spatial template for the differentiation and patterning of arteries. This video article describes a simple and robust protocol to stain intact blood vessels with vascular specific antibodies and fluorescent secondary antibodies, which is applicable for vascularized embryonic organs where we are able to follow the process of vascular development.

Video Link

The video component of this article can be found at https://www.jove.com/video/2620/

Protocol

1. Collecting Mouse Embryonic Limb Skin (E13.5~E17.5)

1. Euthanize plugged females by approved procedure. According to our approved animal protocol, the females are euthanized by CO_2 exposure and then assured by cervical dislocation. Lay the animal on its absorbent paper towel and soak it thoroughly in 70% EtOH/H_2O from a squeeze bottle.
2. Dissect the uterus intact and place it in a 100 x 15 mm Petri dish containing ice-cold Hanks' Balanced Salt Solution (HBSS) to wash out blood.
3. Separate and dissect the embryo. Remove the very thin amnion from the embryo.
4. (Option) Dissect a single embryo in a 35 x 10 mm Petri dish if each embryo needs to be genotyped. Dissected tail is transferred to a 0.2 ml PCR tube for genotyping.
5. Cut off the forelimbs of embryo and transfer forelimbs by a ring forceps into 24 well plate containing 2 ml of ice-cold fresh 4% Paraformaldehyde (PFA) in Phosphate Buffer Saline (PBS).
6. Fix the forelimbs with gentle mixing on the Nutator Mixer at 4°C overnight.
7. On the following day, remove the PFA and wash three times for 5 min in 2 ml of PBS with gentle mixing on the Nutator Mixer at room temperature.
8. Transfer the forelimbs in 100% methanol (MeOH) and store them at -20°C enzyme freezer (the freezer with critical temperature control and without automatic defrost function). Primary antibodies listed in Table 1 work after the 100% MeOH treatment.
9. Peel off skin from the forelimb using fine tweezers. Limb skin, when dehydrated, should separate easily from the limb. First place the limb with ventral side (palm side) facing up. Then using fine tweezers, cut the skin as shown in the video. Next dissect around the entire limb, peeling the skin off gently without any damage.

2. Whole-mount Immunohistochemical Staining of Limb Skins

1. Rehydrate the limb skins in 5ml polypropylene round-bottom tube by incubating through the graded series of MeOH/PBT (PBS + 0.2%Triton X-100) (75%, 50%, 25%) for 5 min each. Note that exchanging solutions should be careful to avoid damaging the limb skins.

2. Wash twice for 5 min in PBT with gentle mixing on the Nutator Mixer at room temperature.
3. Block the limb skins with either 10%Goat serum/PBS+0.2%TX100 blocking buffer for goat secondary antibodies or 10%Donkey serum/PBS +0.2%TX100 blocking buffer for donkey secondary antibodies for 2 hours with gentle mixing on the Nutator Mixer at room temperature.
4. Place the limb skins on a 35 x 10 mm Petri dish and transfer by a ring forceps into 2ml-microcentrifuge tube with 800µl of primary antibodies (appropriate dilution as listed in Tables) in the blocking buffer (either 10%Goat serum/PBS+0.2%TX100 or 10%Donkey serum/PBS +0.2%TX100). Incubate the limb skins with gentle mixing on the Nutator Mixer at 4°C overnight. Note that multiple primary antibodies derived from different species (e.g., rat monoclonal antibody+ rabbit polyclonal antibody) can be used simultaneously.
5. On the following day, place the limb skins on a 35 x 10 mm Petri dish and transfer by a ring forceps into 5ml polypropylene round-bottom tube with 4ml of the washing buffer (either 2%Goat serum/PBS+0.2%TX100 or 2%Donkey serum/PBS+0.2%TX100).
6. Wash five times for 15 min with gentle mixing on the Nutator Mixer at room temperature.
7. Place the limb skins on a 35 x 10 mm Petri dish and transfer by a ring forceps into 2ml-microcentrifuge tube with 800µl of secondary antibodies in the blocking buffer (either 10%Goat serum/PBS+0.2%TX100 or 10%Donkey serum/PBS+0.2%TX100). Typically we use secondary antibodies from Jackson ImmunoResearch (Cy3, Cy5, 1:300 dilutions) or Invitrogen (Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 633, 1:250 dilution). Filter the secondary antibody solution using 0.22µm PVDF membrane syringe filters to remove aggregated particles of the secondary antibodies. Incubate the limb skins in the dark or wrapped with aluminum foil for 1 hour with gentle mixing on the Nutator Mixer at room temperature. Note that different fluorescent conjugated secondary antibodies derived from different species can be used simultaneously.
8. Place the limb skins on a 35 x 10 mm Petri dish and transfer by a ring forceps into 5ml polypropylene round-bottom tube with 4ml of the washing buffer (either 2%Goat serum/PBS+0.2%TX100 or 2%Donkey serum/PBS+0.2%TX100). Wash five times for 15 min in the dark or wrapped with aluminum foil with gentle mixing on the Nutator Mixer at room temperature.
9. (Option for counterstaining against nucleus) Incubate the limb skins with 4ml of the washing buffer (either 2%Goat serum/PBS+0.2%TX100 or 2%Donkey serum/PBS+0.2%TX100) with To-Pro-3 (Invitrogen T3605, 1:3000 dilution) in the dark or wrapped with aluminum foil for 10 min with gentle mixing on the Nutator Mixer at room temperature. Then, wash three times for 5 min with 4ml of the washing buffer (either 2%Goat serum/PBS+0.2%TX100 or 2%Donkey serum/PBS+0.2%TX100) in the dark or wrapped by aluminum foil with gentle mixing on the Nutator Mixer at room temperature. Note that strong and specific staining for nuclei is observed for To-pro-3 in a specific emission (HeNe 633 nm excitation).

3. Mounting the Limb Skins on Slide

1. Place the limb skins on a 35 x 10 mm Petri dish. Remove dusts, crystals, fibers from the inner layer of the skins using fine tweezers under the stereomicroscope with low illumination to avoid extensive photo bleaching.
2. Transfer the limb skins to adhesive microscopic slide by a ring forceps. Place the skins with the inner layer lying upward on the slide (i.e. towards coverslip). Flatten the skins carefully using fine tweezers and remove carry-over washing buffer by Kimwipe.
3. Mount in anti-fade mounting media without air bubbles. We use 25"x25" coverslip. Cure on a flat surface in the dark (e.g., the samples mounted using ProLong Gold reagent are placed overnight in the dark at room temperature before viewing). For long-term storage, seal the coverslip to the slide and store at 4°C.

4. Confocal Microscopy

1. Set up appropriate lasers for fluorophores. We use Leica TCS SP5 confocal microscope with three laser sources including Argon 488nm (for Alexa Fluor 488 and GFP), DPSS 561nm (for Alexa Fluor 568 and Cy3)and HeNe 633nm (for Alexa Fluor 633, Cy5 and To-Pro-3).
2. Use sequential scan tool to avoid or reduce crosstalk in which all dyes in double or triple-stained samples will be excited at the same time. In the sequential scan mode, images will be recorded in a sequential order.
3. More general information about fluorescent dyes and lasers for excitation may be founded in "Confocal Microscopy for Biologists" by Hibbs (2004)

5. Representative Results

Whole-mount triple-label confocal immnofluorescence microscopy in mouse forelimb skin at E15.5 with antibodies to the pan-endothelial cell marker PECAM-1 (Figure 1A-C, D blue), the neurofilament marker 2H3 (Figure 1A green), and the smooth muscle cell marker αSMA (Figure 1A, B red) revealed a characteristic branching pattern of αSMA^+ arteries, aligned with $2H3^+$ peripheral nerves in the limb skin. In addition to blood vessel branching, this limb skin vasculature model is used to study patterning of lymphatic vessel branching using antibodies to the lymphatic endothelial cell marker LYVE-1 (Figure 1D, E red) and Neuropilin2 (NRP2) (Figure 1D, F green).

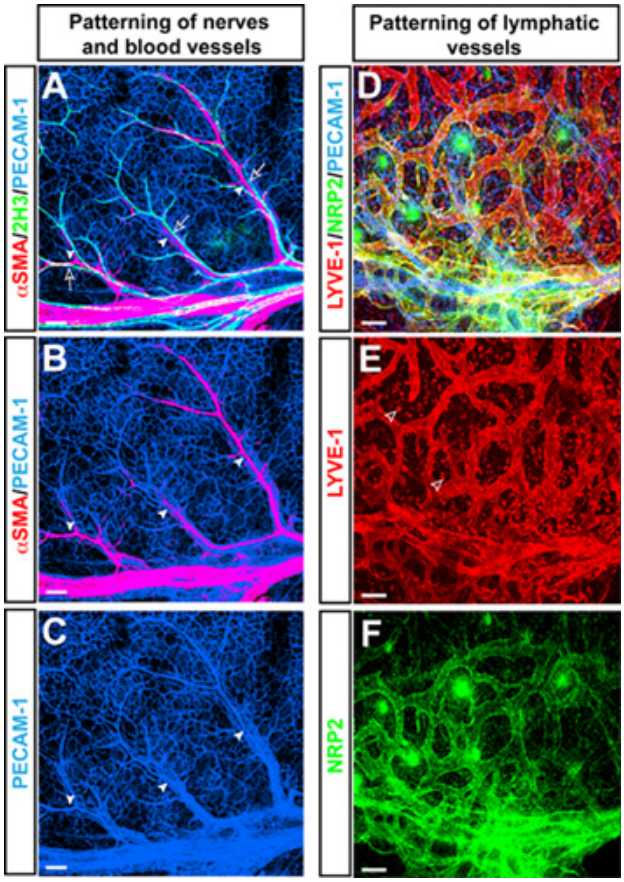

Figure 1. (A-C) Arteries align with peripheral nerves in the embryonic limb skin. Whole-mount triple-label confocal immnofluorescence microscopy with antibodies to the pan-endothelial cell marker PECAM-1 (A-C, blue), the neurofilament marker 2H3 (A, green), and the smooth muscle cell marker αSMA (A and B, red) is shown. At E15.5, $2H3^{+}$ peripheral nerves (open arrows) associate with arteries (arrowheads) which are covered by $αSMA^{+}$ smooth muscle cells. (D-F) Lymphatic vasculature in the embryonic limb skin. Triple-label confocal immnofluorescence microscopy with antibodies to the pan-endothelial cell marker PECAM-1 (D, blue), the lymphatic endothelial cell marker LYVE-1 (D and E, red) and Neuropilin2 (NRP2) (D and F, green) is shown. Lymphatic vessels are visualized by both LYVE-1 and NRP2, whereas LYVE-1 is also expressed by a subset of macrophages (open arrowheads). Scale bar: 100μm.

Discussion

The vascular system is crucial for organ development during embryogenesis as well as for organ maintenance and reproductive functions in the adults, because it supplies sufficient oxygen and nutrients to the organs. Proper vascular network is well-established with complex and multi-step processes by angiogenesis in which pre-existing capillary network is reorganized with highly branched and hierarchical structures. Although numerous works have been shown that a variety of molecules is involved in these processes, it has not been clear how organs or their components provide the signals to promote the stepwise process of vascular development including endothelial differentiation and patterning of vascular branching. To address this question, appropriate vascularized organs where we are able to follow the process of vascular development are required. The limb skin vasculature model with gain-of-function and loss-of-function genetic manipulations enables high-resolution analysis of vascular development.

We describe here a detailed protocol including dissection of embryonic mouse forelimbs, whole-mount immunohistochemical staining and confocal microscopy that is routinely used in our laboratory to analyze limb skin angiogenesis and lymphangiogenesis. The protocol provides reproducible results and intact vasculature for early as well as late embryonic limb skin is easily imaged by confocal microscopy. It is also applicable for adult ear skin vasculature (Y.M. and K. Perkins, unpublished). To further explore the imaging of the dynamics of blood vessel growth and remodeling, however, other animal model like zebrafish with fluorescently labelled blood vessels is useful for time-lapse imaging of the vasculature *in vivo* with high-resolution.

Whole-mount confocal microscopy with multiple labelling by vascular markers permitted us to image blood and lymphatic endothelial cells, and their neighbours including smooth muscle cells and pericytes in the skins. In addition to the vascular marker antibodies (Table 2), antibodies for reporter gene products (β-galactosidase, β-gal and green fluorescent protein, GFP, Table 2) that recapitulate the expression pattern of endogenous genes of your interest can be used. For example, we used forelimb skin from embryos carrying *lacZ* reporter targeted to the *ephrinB2* (Wang *et al.*, 1998) or *EphB4* locus (Gerety *et al.*, 1999), which provides a histochemical indicator of *ephrinB2* or *EphB4*. EphrinB2, a transmembrane ligand, is expressed by arteries but not veins, whereas its receptor, the tyrosine kinase EphB4 is preferentially expressed by veins (Wang *et al.*, 1998). Whole-mount immunohistochemical analysis in forelimb skins of E15.5 $ephrinB2^{lacZ/+}$ or $EphB4^{lacZ/+}$ heterozygous

embryos revealed that peripheral sensory nerves associated preferentially with arterial vessels (Mukouyama *et al.* 2002). These mice are available in the Jackson Laboratory (*Efnb2*^{tm1And}/*J*: stock#006039, *Ephb4*^{tm1And}/*J*: stock#006044).

The study of different stages of these vascular systems reveals the cellular dynamics of angiogenesis including vascular branching, arterial/venous differentiation, lymphatic vessel development and smooth muscle/pericyte coverage in the developing limb skin. By E13.5, primitive capillary plexus was established in the limb skin but no association between sensory nerves and blood vessels was observed. By E14.5, vascular remodelling occurred and nerve associated with remodelled branched vessels, which express arterial endothelial cell markers and have αSMA^{+} smooth muscle cells (Mukouyama *et al.* 2002). Genetic studies suggest that peripheral sensory nerves provide a template for determining the blood vessel branching patterns and arterial differentiation (Mukouyama *et al.* 2002). The analysis of nerve-*Vegf-A* conditional mutants revealed that nerve-derived VEGF-A is required for arterial differentiation in the limb skin vasculature (Mukouyama *et al.* 2005). Our results also showed that the nerve-*Vegf-A* conditional mutants exhibit less impairment of nerve-vessel alignment, indicating the involvement of additional angiogenic factor(s). This suggests that the limb skin vasculature model allows us to study different mechanisms that control arterial differentiation and patterning of vascular branching. Moreover, what controls venous differentiation and patterning of venous and lymphatic vessel branching still remains to be answered.

Disclosures

No conflicts of interest declared.

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