Video Article

Whole-mount Confocal Microscopy for Adult Ear Skin: A Model System to Study Neuro-vascular Branching Morphogenesis and Immune Cell Distribution

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Abstract

Here, we present a protocol of a whole-mount adult ear skin imaging technique to study comprehensive three-dimensional neuro-vascular branching morphogenesis and patterning, as well as immune cell distribution at a cellular level. The analysis of peripheral nerve and blood vessel anatomical structures in adult tissues provides some insights into the understanding of functional neuro-vascular wiring and neuro-vascular degeneration in pathological conditions such as wound healing. As a highly informative model system, we have focused our studies on adult ear skin, which is readily accessible for dissection. Our simple and reproducible protocol provides an accurate depiction of the cellular components in the entire skin, such as peripheral nerves (sensory axons, sympathetic axons, and Schwann cells), blood vessels (endothelial cells and vascular smooth muscle cells), and inflammatory cells. We believe this protocol will pave the way to investigate morphological abnormalities in peripheral nerves and blood vessels as well as the inflammation in the adult ear skin under different pathological conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57406/

Introduction

Skin is comprised of three layers: the epidermis, dermis and hypodermis. It has been used as a model system to study stem cell maintenance, differentiation, and morphogenesis in development as well as the regeneration, tumorigenesis, and inflammation in adult. Skin is richly vascularized and innervated such that the development of the peripheral nervous system and vascular system is well-coordinated.

We have previously demonstrated a whole-mount embryonic skin imaging technique with multiple labeling to study intact peripheral nerves and blood vessels including their cellular components[1,2,3,4]: sensory axons, sympathetic axons, Schwann cells in nerves, endothelial cells, pericytes, and vascular smooth muscle cells (VSMCs) in blood vessels. During the angiogenesis, a primary capillary network undergoes intensive vascular remodeling and develops into a hierarchical vascular branching network. In the developing dermis/hypodermis, arteries branch alongside peripheral sensory nerves and veins then form adjacent to the arteries. After the hierarchical vascular network is thoroughly covered with VSMCs, sympathetic nerves extend along and innervate large-diameter blood vessels[1,5,6]. Despite the significance in the close association between the developing nervous and vascular systems, a major question has been to address what happens to the neuro-vascular networks in various pathological situations in adults. A three-dimensional high-resolution imaging is necessary to appreciate the pathogenesis, along with anatomically recognizable branching morphogenesis and patterning.

Neuronal and vascular morphogenesis in adult mouse skin is commonly analyzed by tissue section staining. Other studies have used whole-mount imaging of the skin to visualize the peripheral nerves and blood vessels, in addition to the hair follicles, sebaceous glands, and arrector pili muscles[7,8,9]. However, the thickness of adult skin has made it difficult to analyze the skin over its entire depth.

In the present study, we developed a novel high-resolution whole-mount imaging of adult ear skin to overcome these challenges. Ear skin is readily accessible for dissection and subsequent whole-mount imaging of the skin over its entire depth. Thus, it is a straightforward and highly reproducible method that can be applied to compare three-dimensional architecture of peripheral nervous and vascular systems in the skin, with comprehensive quantification measurements. We demonstrated that the alignment of peripheral sensory and sympathetic nerves with large-diameter blood vessels is preserved in the adult skin. The goal of this protocol is to visualize branching morphogenesis, and the patterning of peripheral nerves and blood vessels, as well as the immune cell distribution at a cellular level in adult mouse models in various conditions such as inflammation and regeneration.

Protocol

All experiments in this section were performed under approval from the National Heart, Lung, and Blood Institute (NHLBI) Animal Care and Use Committee.

1. Adult Mouse Ear Skin Collection

1. Euthanize adult mice by carbon dioxide (CO_2) exposure in a closed chamber and then confirm the euthanasia by cervical dislocation.
   NOTE: The experiment follows the National Institutes of Health (NIH) guideline for the euthanasia method.
2. Dissect the ear from the base and place it in a 35 x 10 mm^2 Petri dish containing 2 mL of Hank's Balanced Salt Solution (HBSS). Briefly trim hairs with scissors.
3. Peel the posterior skin and anterior skin away carefully from the intervening cartilage.
   NOTE: Cartilage attaches to the anterior skin.
4. Transfer the posterior and anterior skin separately to a 24 well plate containing 1 mL of ice-cold fresh 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) per well. Flatten the posterior and anterior skin in the 4% PFA.
5. Fix the posterior and anterior skin with cartilage at 4 °C for 1 h.
6. Wash the posterior and anterior skin 3 times for 5 min in 1 mL of PBS with gentle mixing on a mixer at room temperature.
7. Transfer the posterior and anterior skin with cartilage to the bottom of a 35 x 10 mm^2 Petri dish. Cut off the base region, which is folded and has adipose and connective tissues. Peel off the cartilage from anterior skin using fine curved forceps.
8. Carefully remove the hairs, adipose tissues, and connective tissues from the inside of the posterior skin using fine curved tweezers. Keep the skin wet with PBS.

2. Whole-mount Immunohistochemical Staining of Mouse Ear Skin

NOTE: All experiments in the following sections were performed in accordance with the NIH laboratory safety guidelines.

1. Prepare the blocking buffer. Filter 10% heat inactivated goat serum (HIGS) diluted in PBS with 0.2% triton X-100 (TX-100) blocking buffer, or 10% donkey serum (DS) diluted in PBS with 0.2% TX-100 blocking buffer, using a 0.45 µm filter unit.
2. Transfer the posterior and anterior skin to a 24 well plate containing 1 mL of 10% HIGS blocking buffer or 10% DS blocking buffer per well. Incubate the skin for 30 min with gentle mixing on a mixer at room temperature.
3. Prepare the primary antibody solution by diluting the primary antibodies (**Table of Materials**) in blocking buffer (either 10% HIGS or 10% DS).
   NOTE: Whole-mout immunohistochemical analysis of adult ear skin with antibodies to pan-neuronal marker neuron-specific class III β-tubulin (Tuj1, rabbit polyclonal IgG or mouse monocloncal IgG2a, 1:500 dilution at the final concentration of 2 µg/mL), pan-endothelial cell marker platelet endothelial cell adhesion molecule 1 (PECAM-1, hamster monoclonal IgG, 1:300 dilution at the final concentration of 3.3 µg/mL), myelin sheath marker myelin basic protein (MBP, rabbit polyclonal IgG, 1:200 dilution at the final concentration of 5 µg/mL) and inflammatory myeloid cell marker CD11b (Rat monoclonal IgG2b, 1:50 dilution at the final concentration of 20 µg/mL) was shown in **Figure 1** and **Figure 2**. The skin was incubated with Cy3-conjugated antibody for vascular smooth muscle cell marker α smooth muscle actin (αSMA) together with secondary antibodies (2.6). The primary antibodies tested by ourselves were listed in **Table of Materials**. Multiple primary antibodies derived from different species can be mixed simultaneously.
4. Transfer the posterior and anterior skin to new well containing 150 µL of the primary antibodies solution. Incubate the skin with gentle mixing on a mixer at 4 °C overnight.
5. On the following day, transfer the posterior and anterior skin to new wells in the 24 well plate or aspirate the blocking buffer containing primary antibodies. Add 1 mL of either 2% HIGS diluted in PBS with 0.2% TX-100 washing buffer or 2% DS diluted in PBS with 0.2% TX-100 washing buffer. Wash the skin with 3 changes of the washing buffer every 15 min with gentle mixing on a mixer at room temperature.
6. Prepare the secondary antibody solution (**Table of Materials**). Dilute secondary antibodies in the blocking buffer (either 10% HIGS or 10% DS) and filter the blocking buffer containing secondary antibodies through a 0.22 µm polyvinylidene difluoride (PVDF) membrane syringe filter connected to a 1 mL syringe.
   NOTE: Alexa 488 or 633-conjugated goat anti-rabbit IgG (H+L) or mouse IgG2a for Tuj1, Alexa 647-conjugated goat anti-hamster IgG (H+L) for PECAM-1, Alexa 488-conjugated goat anti-rabbit IgG (H+L) for MBP, and Alexa 594-conjugated rat IgG (H+L) for CD11b were used with 1:250 dilution at the final concentration of 8 µg/mL. The skin was incubated with Cy3-conjugated αSMA antibody (1:500 dilution at the final concentration of 2-3 µg/mL) in combination with these secondary antibodies.
7. Centrifuge the solution at 13,000 x g for 10 min to remove aggregated particles of the secondary antibodies from the blocking buffer.
   NOTE: Different fluorescent conjugated secondary antibodies derived from different species can be mixed simultaneously.
8. Transfer the posterior and anterior skin to well containing 150 µL of the secondary antibodies solution. Wrap the 24 well plate in aluminium foil to avoid light and incubate the skin for 1 h with gentle mixing on a mixer at room temperature.
9. Transfer the posterior and anterior skin to new wells in the 24 well plate or aspirate the secondary antibody solution by pipet completely. Add 1 mL of either 2 % HIGS washing buffer or 2% DS washing buffer.
10. Wrap the 24 well plate in aluminium foil and wash with 3 changes of the washing buffer every 15 min with gentle mixing on a mixer at room temperature.

3. Mounting the Ear Skin on Slide

1. Place the skin on the bottom of a 35 x 10 mm^2 Petri dish. Carefully remove the hairs, adipose tissues, connective tissues, dusts, and fibers from the inside of the skin using fine curved forceps under stereomicroscope with low illumination to minimize extensive photo bleaching. Keep the skin wet with PBS.

2. Transfer the skin to an adhesive microscope slide using forceps. Place the posterior and anterior skin with the inside facing up on the slide. Flatten the skin using forceps.
3. Mount the skin in a liquid anti-fade mounting medium to avoid photobleaching and preserve fluorescent signals. Make sure that no air bubbles are on or around the skin.
4. Cover with coverslip on the skin samples carefully and store the mounted skin sample slides in the dark overnight at room temperature to allow the mounting media get firm. Seal the coverslip to the slide with nail polish and store it at 4 °C for long-term storage.

4. Confocal Microscopy

1. Set up appropriate lasers for fluorophores. A confocal microscope with three laser sources (Argon 488 nm, DPSS 561 nm and HeNe 633 nm) is used in this experiment.
2. Use the sequential scan tool, which simultaneously excites triple-stained samples, to avoid and reduce any overlap.
   NOTE: Images will be taken in a sequential manner using the sequential scan mode.
3. Image under a 10X objective. Use the tile scan tool to capture the whole ear skin. Set Z-stack and make sure that the z-position covers the entire thickness of ear skin.

Representative Results

Adult mouse posterior ear skin (**Figure 1A**) and anterior ear skin (**Figure 1B**) were immunostained with antibodies to αSMA (red), Tuj1 (green), and PECAM-1 (blue). Posterior skin was immunostained to study neuro-immune distribution using antibodies to CD11b (red) and MBP (green), together with Tuj1 (blue) (**Figure 2A**). Distribution of $CD11b^{+}$ inflammatory cells, including macrophages was detected at a single cellular resolution (**Figure 2B**).

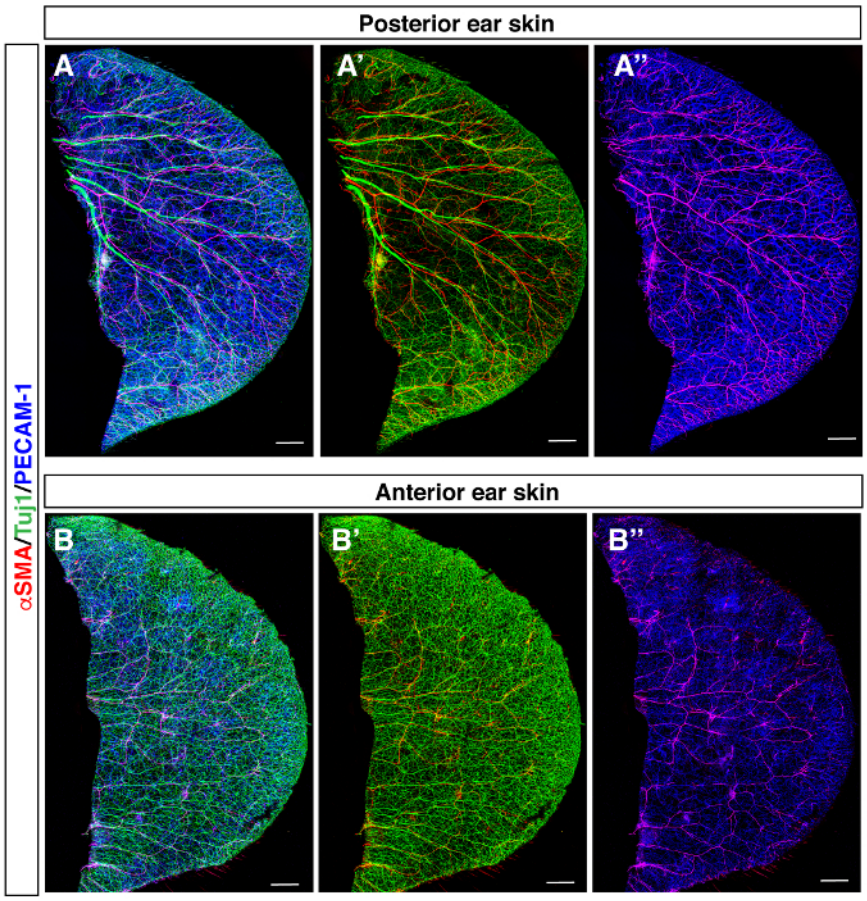

Figure 1: Alignment ofperipheral nerves and blood vessels in adult ear skin. Whole-mount triple immunofluorescence confocal microscopy of posterior and anterior ear skin with antibodies to αSMA (red), Tuj1 (green), and PECAM-1 (blue) is shown. (**A**) VSMC-covered large-diameter blood vessels align with peripheral nerves in the posterior ear skin. (**B**) Smaller-diameter blood vessels covered with VSMCs align with smaller-diameter peripheral nerve bundles in the anterior ear skin. Scale bar = 1 mm Please click here to view a larger version of this figure.

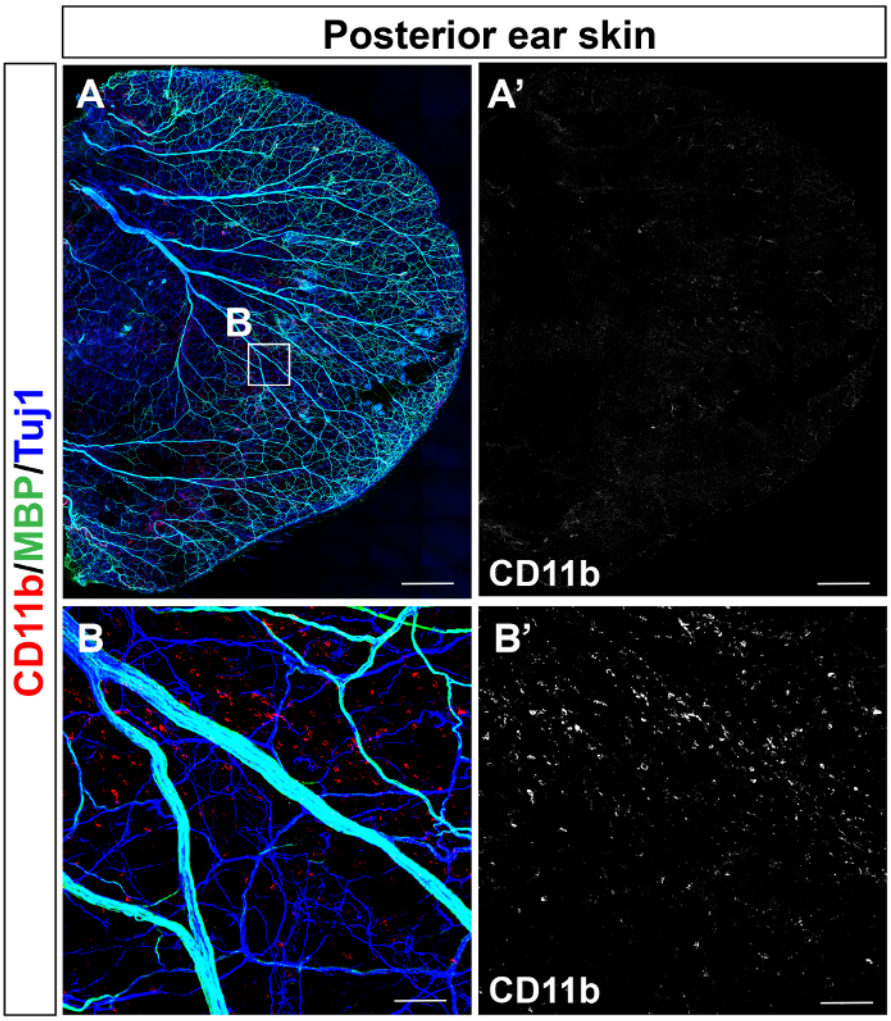

Figure 2: Myelination of peripheral nerves and CD11b$^+$ myeloid cell distribution in adult ear skin. Whole-mount triple immunofluorescence confocal microscopy of posterior ear skin with antibodies to CD11b (red) and MBP (green), together with Tuj1 (blue), is shown. (**A**) Medium-to-large diameter peripheral nerves are myelinated. (**B**) Close-up image in (A). CD11b$^+$ inflammatory cells distribute evenly in the posterior ear skin. Scale bar = 1 mm (A), 100 µm (B). Please click here to view a larger version of this figure.

Discussion

This protocol describes the whole-mount immunonohistochemical imaging of adult ear skin for the analysis of neuro-vascular structures and immune cell distribution. We believe this method has numerous experimental advantages for researchers to study branching morphogenesis and the patterning of peripheral nerves and blood vessels, as well as three-dimensional distribution of skin components including immune cells and hair follicles. The results of the imaging can be quantified using imaging softwares for further quantitative analysis.

Proper preparation of the ear skin is critical for the success of this protocol. First, the ear skin should be carefully dissected soon after euthanizing the mouse. The posterior part of the ear skin should be peeled away from the cartilage. Then, the cartilage should be peeled off from anterior skin before staining. Second, connective tissues, adipose tissues, and hairs should be removed gently and thoroughly before mounting. Due to the existence of peripheral nerves on the surface of the skin, careful removal is required to avoid damaging the nerves. Third, the ear skin should be unfolded with the removal of some thick tissues from the ear skin. Finally, the ear skin should be flat-mounted without air bubbles.

One limitation of this protocol is that ear-tagged ear skin is not appropriate for the analysis as ear tag causes a hole or a scar. Therefore, identification of mice by different methods other than ear tag such as labeling on the tail is necessary in case there are multiple mice to analyze.

The entire ear skin can be scanned by confocal microscopy with a tile scan tool, although a previous reports demonstrated that a region of interest can be imaged with a high resolution[10]. Interestingly, the whole-mount imaging of the entire ear skin reveals distinct branching morphogenesis and patterning of peripheral nerves and blood vessels between the posterior and anterior skin (**Figure 1**): the posterior skin has large-diameter nerve bundles (20–50 µm) aligned with remodeled large-diameter blood vessels covered with αSMA$^+$ VSMCs (20–60 µm), while the anterior skin has smaller-diameter nerve bundles (<20 µm) aligned with smaller-diameter but remodeled blood vessels covered with αSMA$^+$ VSMCs (<20 µm).

There are a remarkable number of mouse models[11] to elucidate the mechanism of human dermatological diseases such as atopic dermatitis[12], psoriasis[13], wound healing[14], and diabetic neuropathy[15]. We have applied this protocol to the ear skin of diet-induced obesity mice and type 2 diabetic mice to study diabetic neuropathy[16]. This protocol can be applied from juvenile to adult mouse skin in various pathological conditions.

Disclosures

The authors have nothing to disclose.

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