Chapter 21
Whole-Mount Immunostaining Methods to Study the Blood and Lymphatic Vasculature in the Embryonic Mouse Skin and Adult Mouse Cornea

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21.1 Introduction

The blood and lymphatic vascular systems are vital units in mammals that carry blood and interstitial fluid. During development of an embryo, formation of these two vascular systems requires co-ordination of highly complex network of events. Development of blood vessels begins de-novo by formation of a primitive vascular network or primary plexus composed of mesoderm-derived endothelial progenitor cells by a process termed 'vasculogenesis'[1, 2]. Subsequently the primary plexus leads to formation of the mature vasculature by 'angiogenesis.' Shortly after the development of blood vessels, some of the blood endothelial cells differentiate to lymphatic endothelial cells by expression of *PROX-1*. These *PROX-1* positive endothelial cells then form the primitive lymph sacs leading to the formation of lymphatic vasculature [3]. Although both the vascular systems arise as primitive capillary plexus they are further reorganized into a highly complex network of vasculature in various organs through the process of pruning, reshaping, and vessel

AF & TK contributed towards development of the embryonic DS staining protocol and compilation/writing of the chapter.
KS, SS and TK contributed to development of the corneal flat mount staining protocol. KS contributed in writing the corneal flat mount part of the chapter.
FC and AC contributed to developing and writing the Matlab code for vessel width measurement.
All the above work was done under the supervision of TK.

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fusion [4]. Under pathological conditions such as tumor formation and injuries due to trauma, the existing blood/lymphatic vessels give rise to new vessels, in a similar fashion of complex vessel branching and morphogenesis as mentioned above [5, 6]. To study in-vivo angiogenesis and lymphangiogenesis in pathological and also normal physiological conditions, immunohistochemical techniques are employed wherein the blood/lymphatic vessels are detected with primary antibodies that interact with specific markers such as PECAM-1 (blood vessels) and Lyve-1 (lymphatic vessels), respectively, and second antibodies conjugated with a flouorophore/enzyme/biotin. Traditionally the tissue/organ wherein the vessel formation is intended to study is sectioned for immunohistochemical techniques. This method allows getting information on presence, increase/decrease of blood and lymphatic vessels in a particular tissue; however, it does not provide any information of the complex branching pattern of vessels. Additionally any quantification attempts from immunostained sections only give results based on the small area, so that one has to be very cautious to extrapolate the results to entire organ/tissue. Accordingly, it is necessary to stain whole tissue (whole mount) to better understand the processes of angiogenesis and lymphangiogenesis [7]. In this chapter we present detailed staining techniques using whole mount DS from mouse embryos and adult corneal tissues [8]. In addition we describe a new Matlab program specifically coded for purpose of vessel-width measurements following immunostaining. This method is more user-friendly and time-efficient compared to more traditional measuring tools such as Image-J (Fatima et al unpublished).

21.2 Part A: Whole Mount DS Immunostaining from Mouse Embryos

21.2.1 Materials

(a) Dulbecco's Phosphate-Buffered Saline (DPBS; Cellgro, Cat No: 21-030-CM)
(b) 4 % paraformaldehyde in PBS (PFA; Affymetrix, Cat No: 19943 1 LT)
(c) 100 % Methanol (EMD Millipore, Cat No: MX0475)
(d) 12-well tissue culture plate (Becton Dickinson, Cat No: 353043)
(e) Petri dish (Becton Dickinson, Cat No: 351029)
(f) Normal donkey serum (NDS; Sigma, Cat No: D9663)
(g) Triton X-100 (Sigma, Cat No: T8787)
(h) Rabbit anti-Lyve-1 (Abcam, Cat No: ab14917)
(i) AlexaFluor 488 donkey anti-rabbit IgG (Invitrogen, Cat No: A21206)
(j) R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD31 (BD Pharmingen, Cat No: 553373)
(k) Superfrost Plus microscope slides (VWR, Cat No:48311–703)
(l) Coverslips (VWR, Cat No:48393081)
(m) Vectashield mounting medium (Sigma, Cat No: F4680)

21.2.2 General Equipment

(a) Dissection microscope (Olympus)
(b) Fine forceps
(c) Small scissors
(d) Micropipettes and tips (10, 20, 100, 200, 1,000 μl)
(e) Pasteur-pipettes
(f) Refrigerator 4 °C and freezer −20 °C
(g) Tabletop centrifuge
(h) Nutator/rotating bench top shaker
(i) Confocal microscope (Zeiss LSM 510 Meta)

21.2.3 Methods

21.2.3.1 Preparation of Solutions/Buffers

(a) **0.2 % PBST:** To prepare 1,000 mL of 0.2 % PBST add 2 mL triton X-100 to 998 mL PBS. Stir until triton X-100 is completely dissolved using a magnetic stirrer.
(b) **Graded MeOH-PBST:** To prepare 100 mL of graded MeOH-PBST, add 75 mL MeOH to 25 mL PBST (to make 75 % MeOH-PBST), add 50 mL MeOH to 50 mL PBST (to make 50 % MeOH-PBST), add 25 mL MeOH to 75 mL PBST (to make 25 % MeOH-PBST).
(c) **Blocking buffer:** To prepare 10 mL of blocking buffer, add 1 mL normal donkey serum to 9 mL PBS (10 %).

21.2.3.2 Harvest of Mouse Embryonic DS

(a) Euthanize plugged pregnant dams (E14–E17) according to methods approved by institutional animal facility. All procedures were approved by Northwestern University's Institutional Animal Care and Use Committee (IACUC).
(b) Dissect intact uteri using scissors and place in cold PBS in a Petri dish.
(c) Carefully dissect out individual embryos in a six-well culture plate filled with cold PBS.
(d) Wash the embryos to remove residual blood.
(e) Fix in cold 4 % paraformaldehyde (PFA), overnight at 4 °C keeping the dish shaking on a nutator. Make sure to completely immerse embryos in PFA.
(f) Wash the embryos once in PBS for 5 min, transfer into cold 100 % methanol, and leave overnight at −20 °C.
(g) Carefully dissect the DS with help of fine forceps and scissors under the dissecting microscope (see note-1).

21.2.3.3 Immunostaining of Mouse Embryonic DS

(a) Following dissection rehydrate DS tissues in series of graded methanol (MeOH)/PBST (PBS + 0.2 % Triton X-100). Use 75 %, 50 %, 25 % MeOH/PBST for 5 min each (see note-2). Finally wash the DS tissues in PBST, two times for 5 min each.
(b) Block the DS tissues in blocking buffer, for 2 h at room temperature on a nutator.
(c) Depending on requirement, incubate the DS tissues in appropriate primary antibodies for overnight at 4 °C (see note-3). For dilutions see note-4.
(d) Following overnight incubation of the tissues in primary antibodies, wash the tissues four times (10 min each) in PBST at room temperature on a nutator.
(e) Incubate the tissues in secondary antibody and/or a fluorescent conjugated second primary antibody for 1.5 h at room temperature on a nutator (see note-5). For dilutions see note-4.
(f) Wash tissues four times (10 min each) in PBST. Avoid exposure of tissue specimen to light during washing. For counter staining of nuclei see note-5.

21.2.3.4 Mounting the DS Tissues on Glass Slides

(a) Transfer tissues on glass slides. Under the dissecting microscope, remove residual artifacts using fine forceps. Keep the inner sides of the DS facing upwards.
(b) For mounting, blot dry excess PBST using a kimwipe. Add 2–4 drops of vectashield mounting media, carefully layover coverslip taking care not to create bubbles. The slides are then cured overnight before viewing under the microscope (see note-6).

21.2.3.5 Confocal Microscopy

(a) Following immunostaining, the tissues can be viewed under a fluorescent microscope. For documentation of data, the tissues can be subjected to Z-section imaging under a confocal microscope.
(b) We have used a Zeiss UV-LSM 510 Meta for acquiring data presented in this chapter (see Sample Fig. 21.1). To begin we set-up appropriate lasers and excitation for each fluorophore. Z-sectioning parameters were set-up depending on the thickness where fluorescence was observed. For further details refer to Zeiss manual.

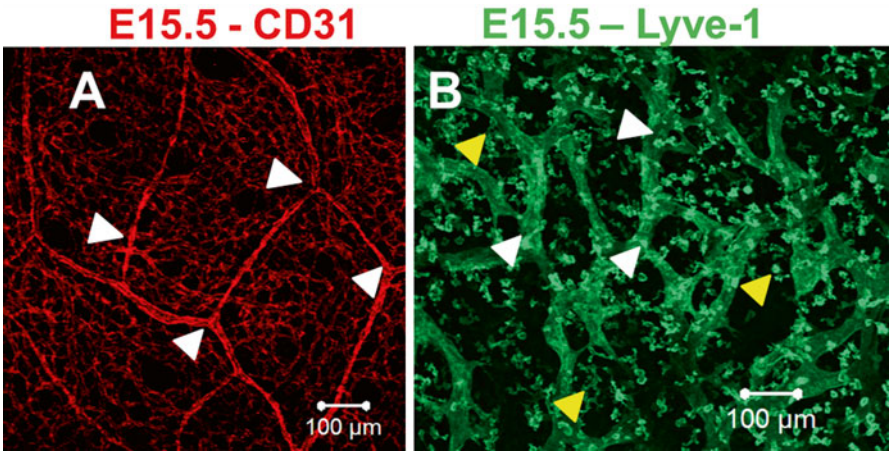

Fig. 21.1 Whole mount DS staining identifying blood/lymphatic vessels. (**a**), DS from E15.5 mouse embryos stained for blood vessel marker CD31. (**b**), DS from E15.5 mouse embryo stained for lymphatic vessel marker Lyve-1. Arrow heads (*white*) indicating the vessel branching pattern, arrow heads (*yellow*) indicate Lyve-1 positive macrophages in the skin (Scale bar = 100 µm)

21.3 Part B: Adult Mouse Corneal Flat Mount Immunostaining

21.3.1 Materials

(a) Dulbecco's Phosphate-Buffered Saline (DPBS; Cellgro, Cat No: 21-030-CM)
(b) 4 % paraformaldehyde in PBS (PFA; Affymetrix, Cat No: 19943 1 LT)
(c) Bovine serum albumin (BSA; Sigma, Cat No: A2153)
(d) 100 % Methanol (EMD Millipore, Cat No: MX0475)
(e) 12-well tissue culture plate (Becton Dickinson, Cat No: 353043)
(f) 48-well tissue culture plate (Becton Dickinson, Cat No: 353078)
(g) Petri dish (Becton Dickinson, Cat No: 351029)
(h) Nutator
(i) Normal donkey serum (NDS; Sigma, Cat No: D9663)
(j) Triton X-100 (Sigma, Cat No: T8787)
(k) Tween-20 (Sigma, Cat No: P9416)
(l) Rabbit anti-Lyve-1 (Abcam, Cat No: ab14917)
(m) AlexaFluor 488 donkey anti-rabbit IgG (Invitrogen, Cat No: A21206)
(n) R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD31 (BD Pharmingen, Cat No: 553373)
(o) Superfrost Plus microscope slides (VWR, Cat No: 483117032A)
(p) Coverslips (VWR, Cat No: 48393081)
(q) Fluoromount aqueous mounting medium (Sigma, Cat No: F4680)

21.3.2 Methods

21.3.2.1 Preparation of Solutions/Buffers

(a) **Dissection buffer:** To prepare 100 mL of dissection buffer, combine 100 mL of DPBS with 0.3 g of BSA (0.3 %). Store at 4 °C.
(b) **Permeabilization buffer:** To prepare 100 mL of permeabilization buffer, combine 100 mL of DPBS with 0.1 mL of Triton X-100 (0.1 %). Store at 4 °C.
(c) **Blocking buffer:** To prepare 10 mL of blocking buffer, combine 9.5 mL of DPBS with 0.5 mL of NDS (5 %).
(d) **Wash buffer:** To prepare 500 mL of wash buffer, combine 500 mL of DPBS with 0.5 mL of Tween-20 (0.1 %).

21.3.2.2 Dissection of Corneal Tissue

(a) Fill each well of a 12-well plate with 1.5 ml of DPBS.
(b) Enucleate (remove) the entire eye and place in one well of the plate. Swirl to rinse any blood away from the eye.
(c) Repeat for each eye.
(d) Remove DPBS from wells and replace with cold 4 % PFA.
(e) Place the plate on the nutator at 4 °C for 20 min.
(f) Wash the eyes with DPBS for 5 min, three times.
(g) Fill the Petri dish with dissection buffer.
(h) Transfer an eye to the Petri dish. Dissect the cornea by cutting the eye in half at the border of the limbus and conjunctiva. See note-8
(i) Cut the cornea four times from the outer to inner cornea to allow flat mounting onto the slide.
(j) Replace the DPBS in the 12-well plate with 100 % methanol. Put the plate at −20 °C for 20–30 min.

21.3.2.3 Immunostaining of the Corneal Tissue

(a) Incubate corneas in permeabilization buffer for 15 min at 4 °C, two times.
(b) Incubate in blocking buffer for at least 20 min at RT.
(c) Transfer corneas to the 48-well plate. Incubate overnight in primary antibody (150 μL) solution in each well of a 48-well plate at 4 °C. See note-4 for antibody dilution.
(d) Transfer the corneas back to a 12-well plate and wash with wash buffer for 5 min, four times.
(e) Incubate the corneal tissues in secondary antibody and/or a fluorescent conjugated second primary antibody for 1.5 h at room temperature on a nutator. For dilutions see note-4.

(f) Wash the corneas in a 12-well plate with wash buffer for 5 min, 4 times each.
(g) At this point DAPI can be used for counterstaining the nuclei. See note-6.

21.3.2.4 Mounting the Corneal Tissue

(h) Gently transfer each cornea to a microscope slide. Avoid bubbles.
(i) Use a kimwipe to wick away most of the liquid on the slide without touching the tissue.
(j) Apply two drops of fluoromount and carefully place a coverslip over the tissue.

21.3.2.5 Fluorescent Microscopy

1. We have used Zeiss Axiovision fluorescent microscope, for image documentation.
2. Lower magnification images (2.5×) are documented to quantify the entire blood/lymphatic vascular area (Sample Fig. 21.2).
3. Higher magnification images (10×) should be documented to study the branching pattern of the vasculature.
4. We used open source Image J software from NIH to quantify the vascular area. In this protocol, we have not provided details of quantifying vascular branching pattern for corneal flat mount.

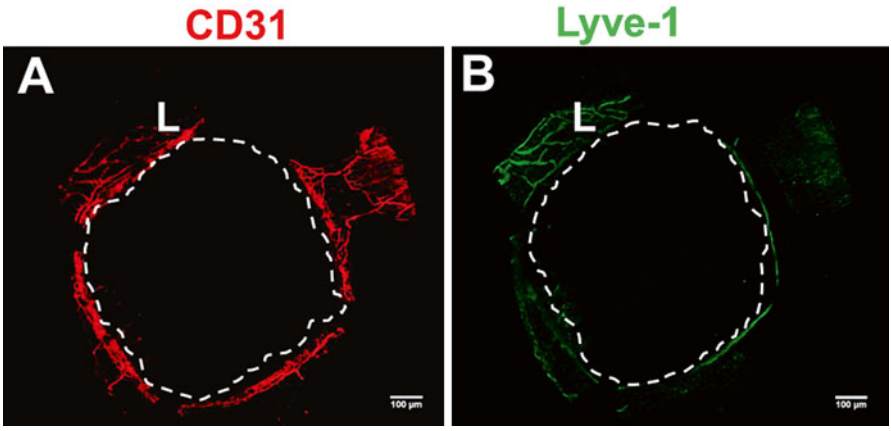

Fig. 21.2 Adult mouse corneal flatmount identifying blood/lymphatic vessels. (**a**), Adult mouse cornea stained for blood vessel marker CD31. (**b**), Adult mouse cornea stained for lymphatic vessel marker Lyve-1. Area within *dotted line*, avascular cornea, *L* Limbus (Scale bar = 100 µm)

21.4 Part C: Quantification of Lymphatic Vessel Width via Digital Imaging Software

Traditionally, vessel width (VW) has been measured manually via ImageJ or similar software, which requires the user to load each image file individually and a use a "drag and drop" approach to linear width measurement. ImageJ exports values in terms of pixels, which then need to be converted to SI units by the user, after obtaining an appropriate scaling factor from the microscope software that captured the initial image. With a large volume of images, the amount of work for a single user can become prohibitive. In our experience, having a single, preferably blinded user of the software allows for the most accurate measu rements of LVW, as it eliminates interoperator variability and systemic bias, so we created a more streamlined method of LVW measurement to cut down on the time required for analysis. Matlab is a highly versatile development package which includes a number of helpful scientific function libraries, which suited our needs. Our software was based on the same fundamental algorithm for distance determination as ImageJ, namely recording the vertical and horizontal displacement between the two selected points on each edge of the lymphatic vessel of interest and applying the Pythagorean theorem to determine the diagonal distance. However, we built in batch image processing, which allows the user to queue a number of images for analysis by designating a directory in which they are located. With the batch processing feature, the user sets a given number of vessels to measure in each image, and once they have completed their measurement of an image, the software pulls up the subsequent image, which significantly streamlines the workflow. The data is output to an Excel file, with averages and standard deviations calculated automatically, as well as SI unit conversion once a scaling factor is provided. We also opted for a simpler "click and click" approach to the actual measurement, which reduces the burden of precise mouse movements on the user and allows for more rapid measurement. When utilizing the Matlab software, we found no difference between previously and newly recorded VWs and users noted significantly easier use and decreased time expenditure.

21.5 Notes

1. After dissection of the embryonic DS, examine it under the dissecting microscope and carefully remove muscle tissue without disturbing the visible blood vessels. If excess muscle tissue is left unattended, staining and visualization of lymphatic vessels will be hampered.
2. Always make fresh Triton X-100, just before the start of the experiment.
3. Make sure the DS tissues are completely immersed in antibody solution. Use 1.5 ml tube or 24-well tissue culture dish for overnight incubations with 800 μL of antibody.

4. Antibody dilutions: Rabbit anti-Lyve-1 (10 μg/ml), Anti-CD31 (1:100), Donkey anti-rabbit Alexa 488 (1:500). Dilute primary antibodies in blocking buffer, the Alexa secondary antibodies should be diluted in PBST.
5. Fluorescent conjugated primary and secondary antibodies should be protected from light. All incubations should be carried out in dark.
6. The nuclei in DS/corneal tissues could be counterstained with DAPI (10 μg/mL in PBS). We have not used DAPI counterstaining in this protocol.
7. Store the slides with stained tissues at 4 °C, until ready for documentation.
8. Remove any other ocular tissues (lens, retina, and iris) and discard. Very carefully remove the iris from behind the cornea.

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