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TECHNICAL NOTES

Protocol for Detecting the Primo Vascular System in the Lymph Ducts of Mice

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Abstract
The primo vascular system (PVS), which is the proposed conduit for the acupuncture *Qi*, is a complex network distributed throughout an animal's body. However, even with a microscope, it is not easily detectable because of its transparency. Thus, its existence is largely unknown in current anatomy. A convincing demonstration of its existence is needed. The lymph-primo vessel (PV), which is a subsystem of the PVS, is a very effective visual demonstration of the PVS. The lymph-PVS is a mobile threadlike structure floating in lymph ducts that has been observed in rabbits, rats, and mice by several independent teams. The involved techniques are novel and rather complicated; therefore, we have already provided detailed protocols for the surgery; for the injection of the staining dye; and for the detection, extraction, and identification of the PVS in rabbits and rats. However, the mouse is one of the most important laboratory animals used for various biomedical research purposes. For the convenience of researchers who wish to initiate the PVS experiments in mice, we provide a shortened version of the protocol, despite many similarities with previously published protocols. Thus, researcher can easily obtain the samples of the lymph-PVS of mice.

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1. Introduction

The primo vascular system (PVS) is being established as a new circulatory system that is distributed throughout an animal's body, including humans [1]. It was first discovered in the 1960s by Bong-Han Kim as an anatomical structure that corresponded to acupuncture meridians and is the conduit of the so called *Qi* of Traditional Chinese Medicine [2]. It was not confirmed until the year 2002 when serious investigations on the PVS began. The main reason for the difficulty of detecting the PVS in an animal's body is its transparency and small size. The PVS is composed of primo vessels (which are approximately 20—50 μm thick) and primo nodes (which are oval and approximately several hundred micrometers). The fluid flowing in the PVS is called the primo fluid. The primo vessels, nodes, and fluids are all transparent and therefore very difficult to detect without special techniques and experimental skills [3].

The PVS has been detected in various internal organs of mice, rats, rabbits, dogs, pigs, and humans [4]. Most visual and direct confirmations of the PVS has been by the aid of staining agents such as fluorescent nanoparticles [5] or Alcian blue [6] to observe the mobile threadlike PVS floating in the lymph. The PVS is observable *in vivo* and *in situ* in lymph vessels such as the thoracic ducts and vessels between the inguinal and axillary lymph nodes.

According to Kim [2], important functions of the PVS are hematopoiesis and regeneration of damaged tissues. This idea is supported by the recent detection of hematopoietic stem cells [7] and, more importantly, small embryonic-like stem cells in the PVS [8,9].

For researchers who want to reproduce the experiments, we have already presented a series of protocols for observing the PVS in the lymph vessels of rabbits [10] and rats [11,12]. Because of the importance of stem cells in the PVS, we present the current protocol for observing the PVS in the lymph vessels of mice. We used mice because stem cell research is mostly performed with mice rather than rats or rabbits. This protocol would be convenient for potential start-up researchers, even though it largely overlaps with previously published protocols.

2. Materials

2.1. Equipment and setup

2.1.1. Microscopes and light source

The following microscopes and light source are used: a stereomicroscope (SZX12; Olympus, Tokyo, Japan) with a charge-coupled device (CCD) camera (DP70; Olympus, Tokyo, Japan); a phase contrast microscope (BX51; Olympus, Tokyo, Japan) with a CCD camera (Infinity 3; Lumenera Corporation, Nepean, Canada); and a halogen lamp (KLS-100H-LS-150P; Kwangwoo Co, Ltd, Pohang, South Korea) for the light source and optical fiber illuminator (KLS-100H-LS-150P; Kwangwoo Co, Ltd, Pohang, South Korea).

2.1.2. Surgical instruments

Surgical instruments and ophthalmic surgical instruments by Tumed (Rotwildstraße, Germany) are used. The following equipment are also used: electric surgical unit (Surgitor, Korea); electrocautery (Umeco, Seoul, South Korea); Pet Specialty cordless trimmer (Oster Professional, Burns, USA); disposable Gentax latex gloves (Geneall Biotechnology, Seoul, Korea); masking tape (Scitech Korea Inc., Seoul, Korea); gauze (Scitech Korea Inc., Seoul, Korea); surgical drapes (Scitech Korea Inc., Seoul, Korea); and an electrical heating pad (30 mm × 30 cm; Woojin Tech, Seoul, Korea).

2.1.3. Syringes and filters

Syringes and filters used are the following: hypodermic syringe (Kovax-Syringe; Korea Vaccine Co., Seoul, Korea); BD ultrafine insulin syringe, 31G (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); BD filter syringe, 5 mL (Becton Dickinson Medicals Ltd., Singapore); BD filter syringe, 10 mL (Becton Dickinson Medicals Ltd.); glass microfiber filters, 110 mm (GE Healthcare Co., Buckinghamshire, UK, cat. no. 1820-110); and hydrophilic minisart syringe filter (Sartorius Stedim Biotech, Göttingen, Germany).

2.1.4. Staining and histology instruments

The staining and histology instruments used are the following: pH meter (Thermo Electron Corporation, Waltham, MA USA); glass funnel (Dongsung Science, Seoul, Korea); round bottom test tube (5 mL; BD Falcon, San Jose, CA, USA); Coplin jar (Fisher Scientific, Hampton, NH, USA); PAP pen (Invitrogen, Waltham, MA, USA); Vortex-2 Genie mixer (Scientific Industries, Bohemia, NY, USA); from 5-μL to 10-μL Finnpipette (Sartorius Korea Biotech Co. Ltd., Seong-Nam, Korea); disposable transfer pipette (Lappia, Korea); microslides (silane coated; 76 mm × 26 mm; Mutopure Chemicals Co, Ltd, Tokyo, Japan); 100 deckglaser cover slips (24 mm × 50 mm; Knittel Glass, Brunswick, Germany); and Leica CM1800 cryostat (Leica, Nussloch, Germany).

2.1.5. Dissecting instruments

The following dissecting instruments are used: two large scissors; a small microscissor; two large forceps; two microdissecting tweezers; small forceps; one pair of fine straight forceps; one pair of curved forceps; one microdissecting straight forceps; one pair of angular microdissecting forceps; and one 31 G insulin syringe (Fig. 1).

Caution!

1. All instruments and other equipment must be sterilized before use.
2. To reduce the chances of contamination when proceeding through the tissue layers, use separate sets for the skin and the peritoneal wall, and for dissecting and extracting the primo system in the lymph vessels.

2.2. Reagents and setup

2.2.1. Experimental animals

Seven-week-old ICR male mice (30—32 g) were purchased from DooYeol Laboratory Animal Company (Seoul, Korea). Males are preferred because they develop less abdominal fat, which makes the surgery easier. The animals are housed in a constant temperature-controlled environment (23°C) with 60% relative humidity. All animals are exposed to a 12-hour/12-hour light-dark cycle and have *ad libitum* access to food and water. Procedures involving animals and

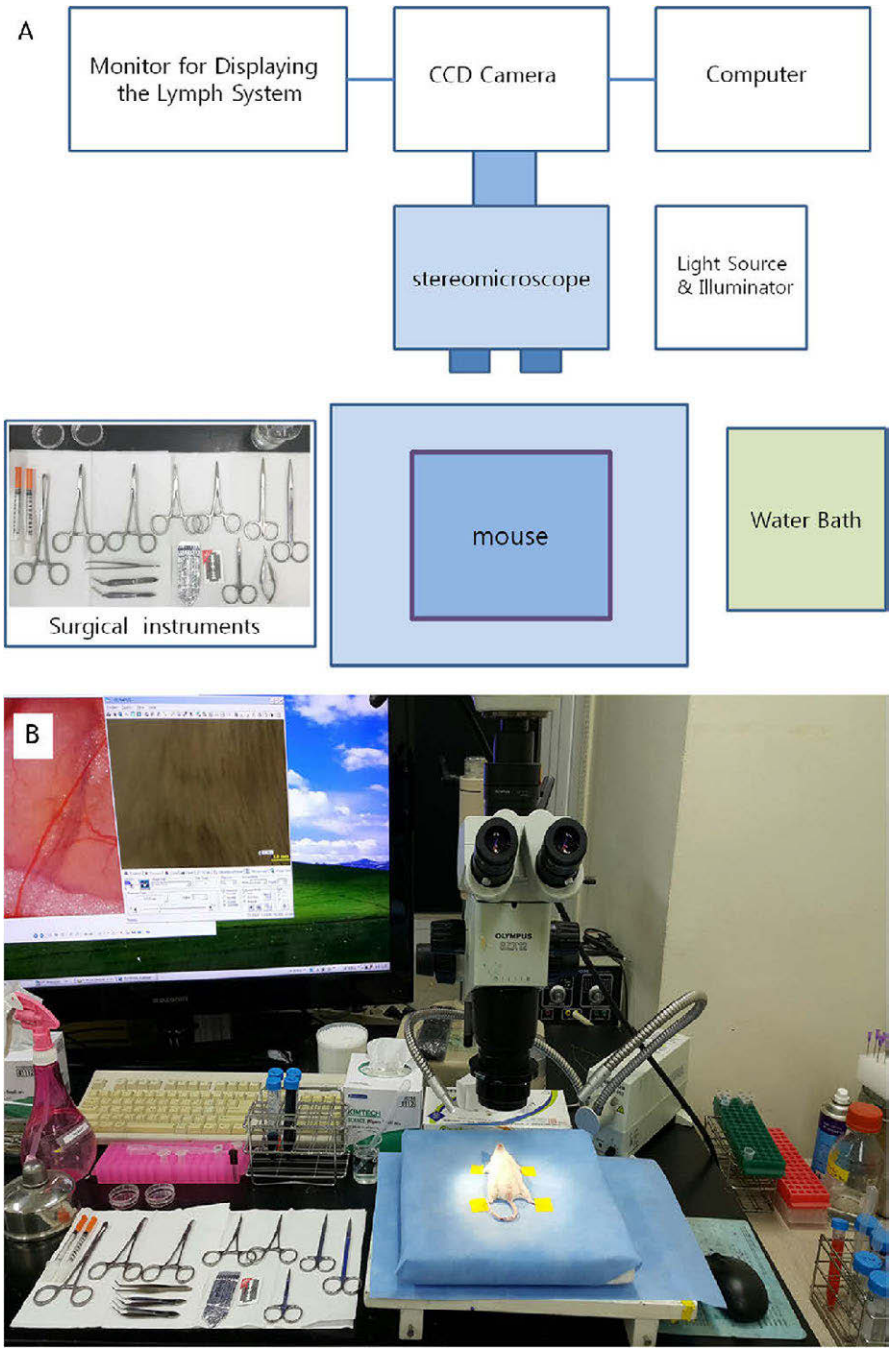

Figure 1 Arrangement of the experimental system. (A) The box diagram of the modules of the system. (B) A photograph of the arrangement. CCD = charge-coupled device.

their care conformed to the Institutional Ethics Committee of the Advanced Institute of Convergence Technology guidelines (approval number WJIACUC20140807-03-07).

2.2.2. Anesthesia

The anesthesia used is urethane (Xylazine; Bayer, Seoul, Korea).

2.2.3. Phosphate-buffered saline solution

Eight grams of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium phosphate (Na_2HPO_4), 0.24 g of monopotassium phosphate (KH_2PO_4), and 800 mL of distilled water are mixed to create the phosphate-buffered saline solution. Its pH is set to 7.4 by using a pH meter. An additional 200 mL of distilled water is added to the previously mixed solution to form 1 L (1,000 mL) of 1× PBS solution. It is stored at room temperature.

2.2.4. Alcian blue staining dye (0.5%)

Alcian blue (AB) 8GX (Sigma, St. Louis, MO, USA) and PBS pH 7.2 (i.e., 1× PBS) (Life Technology Corp, Waltham, MA, USA) are used. To make 0.5% AB staining dye, 0.015 g of AB powder is combined with 3 mL of 1× PBS solution. After mixing these ingredients, the solution is applied to the

vortex machine. This solution is placed in a heating cabinet (−61°C) for approximately 30 minutes to dissolve the AB powder completely. This solution is filtered with a filter paper in a funnel. A second filtration is then performed by using a 0.22-μm syringe filter attached in a 10-mL syringe. After the second filtration, the AB solution is loaded in a 1-mL insulin syringe (31 gauge) while using a warm bath to keep it at a constant temperature of 38—40°C before injecting it into the lymph system.

2.2.5. Histology

For histology, the following are used: Tissue-Tek OCT freezing compound (Sakura Finetek, Tokyo, Japan), base molds (15 mm × 15 mm × 5 mm, Fisher Scientific), and a gel/mount medium (Biomeda Corp, San Francisco, CA USA; No. M01).

2.2.6. Preparation of the 4′,6-diamidino-2-phenylindole stock solution

The molecular probe 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen Molecular Probes, Cat. No. D1306; 1:10,000) is used for a stock solution. To make the 5 mg/mL DAPI stock solution (14.3 mM), the contents of one vial (10 mg) are dissolved in 2 mL of deionized water or dimethylformamide (DMF). However, DAPI is not very soluble in PBS. For long-term storage, the stock solution can be aliquoted and stored at < −20°C. For short-term storage, the solution can be maintained at 2—6°C and protected from light. When handled properly, DAPI solutions are stable for at least 6 months.

2.2.7. Preparation of the DAPI working solution

The DAPI stock solution is diluted to 300nM in PBS. Add approximately 300 μL of this dilute DAPI staining solution.

2.2.8. The overview and time distribution of the entire procedure

- Steps 1—4: Prepare the animal before the surgical operation (time, 20—25 minutes).
- Steps 5—9: Locate the lumbar lymph nodes (time: 90 minutes).
- Steps 10—14: Visualize and observe the PVS (time: 40 minutes).
- Steps 15—16: PVS tissue harvest (time: 60 minutes).
- Steps 17—19: Analyze the PVS with DAPI (time: 30 minutes).

3. Surgical procedures

3.1. Animal preparation (time: 20—25 minutes)

(1) For 48 hours, fast the mice to reduce fats. This helps to obtain a clear view of the lymphatic system in fat tissues. Lean mice are needed to avoid bleeding during surgery and to allow the precise injection of AB.
(2) Anesthetize the mouse with an intramuscular injection of a mixture of urethane (0.18 mL) and xylazine (0.02 mL) by using a 1 mL hypodermic syringe.
(3) Shave the abdominal skin by using the Pet Specialty cordless trimmer. Fix the mouse with its head away from the operator by taping its feet to the operating table. Cover the mouse's eyes with gauze to prevent exposure to light.
(4) Adjust the illuminators, the stereomicroscope, and the monitoring system for optimal observation.

3.2. Locating the lumbar lymph node (time: 1 hour 30 minutes)

Surgical procedure setup notes:

(1) Sterilize surgical supplies and instruments before the surgery.
(2) One drape is to be laid out as a sterile surface for the placement of instruments and another drape is to be laid out with a precut hole that will show where the incision is to be placed on the mouse.
(3) One standard pair of scissors is to be used to cut suture material; two forceps and a fine needle holder are used for handling and gripping the suture, and a scalpel with a blade is used.
(4) Preheat to 39°C the PBS and AB solution (pH 7.4), which is loaded in a syringe.

3.3. Laparectomy

Caution!

Be careful not to damage blood vessels and other fine structures. Wear sterile gloves and do not allow instruments and supplies to come into contact with nonsterile surfaces, other than the mouse's tissues.

(5) Position the anesthetized mouse on its back on a heating pad to maintain its body temperature. To disinfect the abdominal skin, wipe the skin with a cotton-tipped applicator and aseptic tissue paper soaked in 70% (v/v) ethanol.
(6) Grip the middle skin of the abdomen with toothed forceps and incise the outermost skin along the middle line (linea alba) down to the symphysis pubis and up to the ensisternum with surgical scissors. The depth of incision should be shallow to view the muscular layer.
(7) Hold the abdominal muscle upward. Using small scissors, make a small slit along the linea alba in the straight muscle of the abdomen at the lower one-third of the line from the ensisternum to the symphysis pubis. Insert the scissors into the slit and cut the abdominal muscle along the linea alba to the xiphoid process. Avoid bleeding during this cutting. The

incision must be on or around the linea alba because blood vessels are sparse in this area.

Caution!

Be careful to avoid inserting the scissors too deeply into the peritoneal cavity and keep the tip of scissors pointed upwards to avoid damaging the underlying structures.

3.4. Locating lumbar nodes

(8) Expose the area of the caudal vena cava by moving the intestines to the animal's right side and cover them with gauze soaked in saline. Spray warm saline solution over the gauze to avoid dryness.
(9) By removing adipose tissues with the curved forceps, expose a lumbar lymph node. Grasp the node and tease away the surrounding connective tissues with a cotton bud before injecting AB into the node.

Critical steps!

1. First locate the caudal vena cava and search around it for lumbar lymph nodes. In some mice, the lymph systems are covered with thick adipose tissue and layers of membranes. Remove the adipose tissues and membranes to secure a clear view of the lymph systems.
2. Use curved forceps and a cotton bud to expose the lymph nodes. Choose the largest node among the two or more nodes at the lumbar lymph node position.
3. Large blood vessels must not be damaged. If necessary, maintain hemostasis by pressing them with a cotton bud. In the advent of massive bleeding, use electrocautery to stop bleeding. The blood capillaries spread in the adipose tissues can be removed by using a cotton swab.

Caution!

Use a disposable transfer pipette to add warm saline solution drop by drop over the lymphatic vessel to avoid dryness.

4. Visualization and observation of the PVS (time: 40 minutes)

4.1. Visualization of the PVS with AB injection

(10) By using a 31-gauge ultrafine insulin syringe, inject a small amount of preloaded 0.5% AB solution (0.1—0.2 mL on each side), which has been preheated to 37°C in a warm bath, into the lumbar node on both sides. Injecting the dye at a slow rate is essential; otherwise, it will leak out and stain the surrounding area. After the injection, the lymph ducts will become blue because the AB solution flows upward with the lymph fluid (Fig. 2). The AB will also stain many small branches of the lymph ducts. Drop warm saline often on the lymphatic ducts to avoid their drying out.

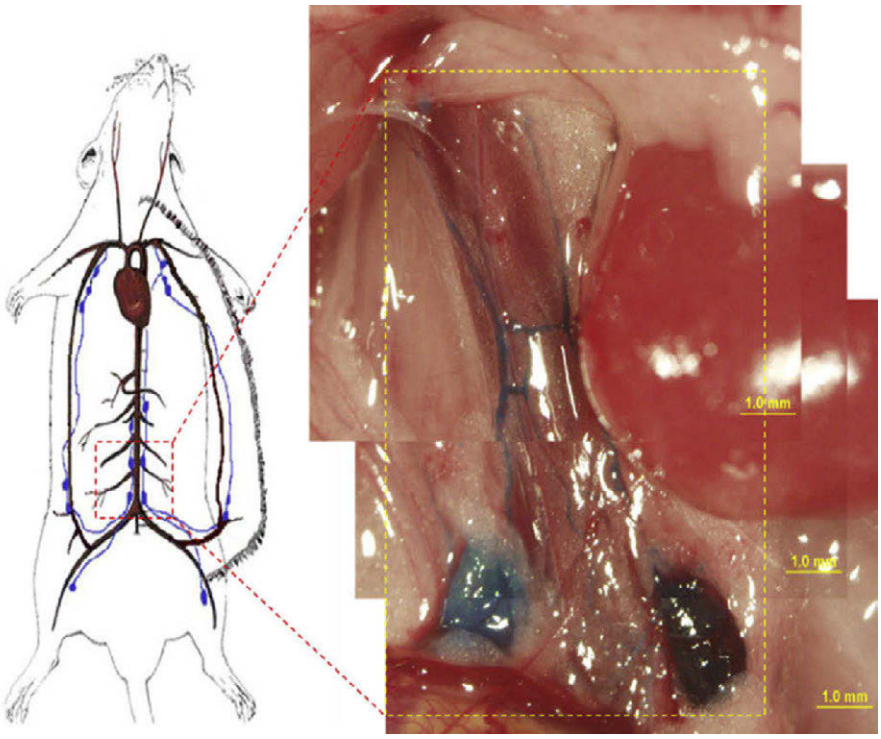

Figure 2 The schematic diagram shows the locations of the major lymph ducts and lymph nodes around the caudal vena cava. The magnified view is a stereomicroscopic image of the network of lymph ducts stained with Alcian blue. Two lumbar nodes are at the bottom.

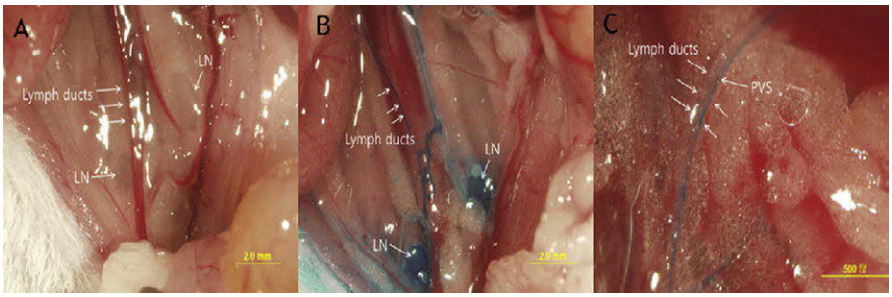

Figure 3 Stereomicroscopic images of the lymph ducts and two lymph nodes. (A) Transparent lymph ducts beside the caudal vena cava before the Alcian blue (AB) injection. (B) The lymph ducts become blue immediately after the AB injection into the lymph nodes. (C) The lymph ducts become transparent again after being washed for 2 hours by lymph flow. The thin blue PVS has become visible in the lymph ducts. LN = lymph node; PVS = primo vessel system.

Caution!

1. Remove air out of the syringe before beginning the injection. Insert the needle at an angle of $< 40^{\circ}$ relative to the lymph nodes. Wait several minutes after the insertion until the node becomes stabilized.
2. To avoid AB precipitation when it is dissolved in 1× PBS, boil 1× PBS in the hot plate before dissolving AB into it. To maintain the temperature of solution, store the solution in an oven (68—75°C) before use.
3. If blood vessels in a lumbar node are damaged during the injection of AB, blood will flow in the lymph ducts and will coagulate within the PVs, and cause a thickening of PVs because of adhered blood cells.

(11) To maintain the lymph flow for efficient washing of AB, move the internal organs back to their original positions, close the outermost skin, and cover the mouse's body with tissue paper 3—4 times to maintain body temperature. Avoid heating the mouse with infrared light. Let the mouse remain for 60 minutes to allow sufficient washing of AB so that the lymph ducts become clear again.

4.2. Observation of the PVS

(12) Open the abdomen, move the internal organs to the side, and repeat step 8.

(13) Using a stereomicroscope, search for the PVS in lymph vessels and trace the PVS up to the cisterna chyli near the diaphragm (Fig. 3). The lymph ducts near the caudal vena cava of a mouse form a complicated network and contain the PVS as mobile threadlike structures floating in the lymph flows. A movie showing the mobile primo vessel (PV) floating in the lymph ducts with branching is supplied as supplementary material (SI). The PV branches where the lymph duct branches are visible.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jams.2015.03.008

Critical steps!

1. A fine operation is required to avoid bleeding when tracing the PVS because the blood vessels are tangled in the complicated structures of membranes and fats.
2. Large lymph ducts are located regularly, but small lymph vessels are distributed irregularly. Because of this factor, search for as many of them as possible to avoid missing the PVS in them.

(14) Euthanize the mice by an intracardiac injection of 0.7 mL urethane (3.2 g/kg). Check the mouse's vital signs to confirm euthanasia.

Extra procedure

To search further for the PVS in the thoracic cavity, the following two steps are to be performed.

1. Open the diaphragm and note the lymphatic system's connection between the thorax and the abdomen. Cut the rib cage and pin the ribs outwardly open. The superior vena cava can be located by lifting the heart and separating the lobes of the lung. The thoracic duct is a thin transparent tube just beside the right side of the superior vena cava.
2. Search for the blue thin threadlike PVS floating inside the thoracic duct.

4.3. Primo vessel system tissue harvest (time: 1 hour)

(15) Using microscissors, cut the lymph vessels that contain the PVS and put the specimen on a slide (Fig. 4A). Incise the lymph vessel longitudinally by using microscissors. Pick up the PVS with microforceps, gently pull the PVS outward (i.e., toward the surgeon's body), and place it on a glass slide.

Critical steps!

1. Tear the lower end of the harvested lymph vessel with sharp-end forceps and expose the end part of the PVS in it. Pull the PV gently out.

(16) Fix the PVS specimens isolated from the lymph vessels with either 4% paraformaldehyde (PFA) solution or 10% neutral buffered formalin (NBF) solution and store them for 1—2 days at 4°C in a refrigerator for further analysis.

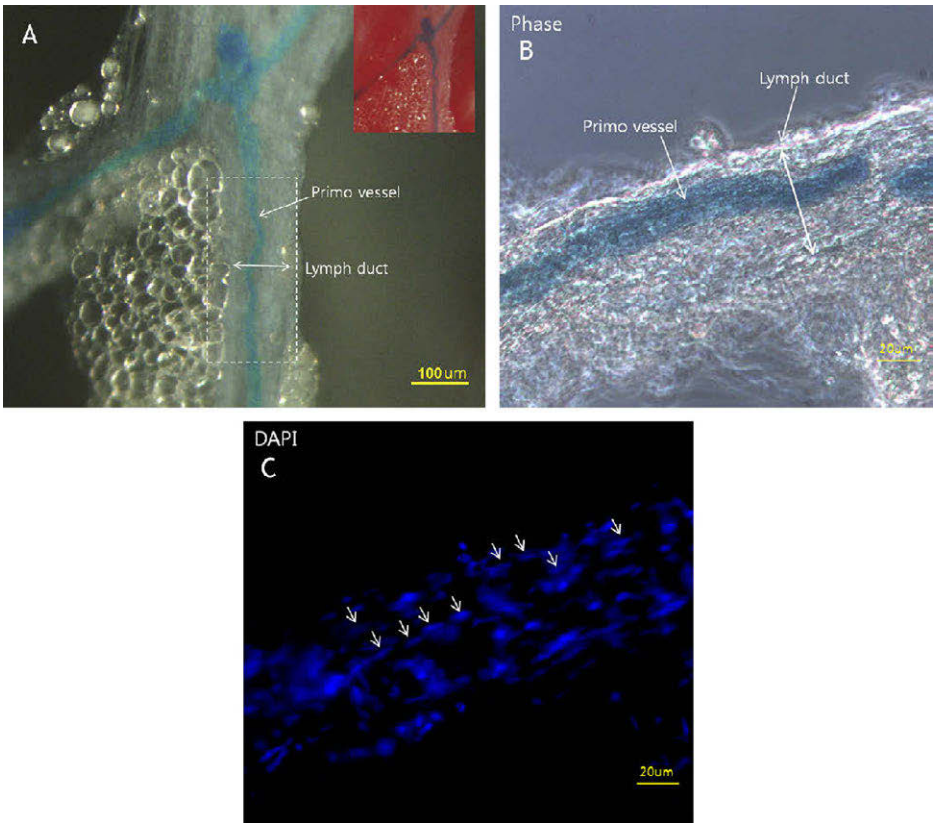

Figure 4 A harvested primo vessel (PV). (A) The stereomicroscopic image of a PV in a lymph duct after extracting it and fixing it with neutral buffered formalin on a slide. The branching of the lymph and primo vessels are clearly visible. The right inset is the stereomicroscopic image of the PV before extraction. (B) Phase contrast microscope image of the PV of the dotted box in (A) at higher magnification. The thickness of the PV is approximately 20 μm. (C) The 4′,6-diamidino-2-phenylindole (DAPI)-stained rod-shaped nuclei (arrows) are aligned along the PV. It is inside a lymph vessel.

4.4. Identification with DAPI (time: 30 minutes)

(17) Wash the fixed samples with 1× PBS solution two or three times. Pay good attention to and maintain the branches of the PV. Place the fresh or the fixed PV sample on a glass slide with 1—2 drops of 1× PBS solution. Wash the samples in PBS and place them on slides. Slightly flatten the tissues to examine them under a microscope.

(18) Stain the same specimen with Prolong Gold Antifade reagent (Seoul, Korea) with DAPI again for 10 minutes to examine the nuclei in the endothelial cells of the PV. When applying DAPI, mix thoroughly, but be careful to avoid creating too many bubbles. Drain excess solution from the slide. Apply two separate drops of Gel Mounte (Merck KGaA, Darmstadt, Germany)—DAPI on the sections, and lower the cover slip onto the sections. Let the slide remain in darkness for a few minutes, and seal the cover slip with a transparent manicure.

Caution!

Avoid exposing the sample to light during the DAPI staining procedures.

(19) To observe the rod-shaped nuclei, examine the specimens with a fluorescence phase contrast microscope (Olympus BX51, Olympus, Tokyo, Japan; Fig. 4B) and a confocal laser scanning microscope (confocal laser scanning microscope — CLSM; C1 plus, Nikon, Tokyo, Japan; Fig. 4C).

5. Remarks

We have previously reported a series of protocols for the observation of the PVS in the lymph vessels of rabbits [10] and rats [11,12]. The rationale of presenting the current protocol, despite its similarity to previous protocols, is the importance of using the mouse as a laboratory animal because many biomedical data and tools have been accumulated; therefore, future work on the PVS in mice will be greatly needed. In particular, the recent observation of small embryonic-like stem cells in the primo node [7—9] makes the mouse protocol more worthwhile because antibodies for this kind of experiments are only well developed for mice. Therefore, it is convenient for future mice-PVS researchers to search for details of specific experimental procedures for the mouse.

The characteristic features of the PVS are mostly similar and the on-site identification criteria are nearly the same for different animals. To distinguish the PVS from artifacts such as coagulation of AB with lymph fluids in a string-like form, the following need to be checked: (1) the structure is a mobile threadlike structure that is not adhered to the lymph

walls; (2) the PVs are elastic, but AB coagulation is not and is therefore easily broken; a mobile blue threadlike structure should be tested for its elasticity so as not to confuse it with an artifact temporarily formed by staining dye aggregations, which are easily crushed to pieces by shaking the lymph duct. A PV is elastic, which can also be sensed when it is extracted from a lymph duct *ex vivo* on a slide; (3) the thickness of a PV is rather uniform and approximately 20—30 μm; (4) the PNs form the thicker parts of the PV, and their numbers, thicknesses, and lengths vary; they can be recognized as the cucumber-shaped thick parts of a PV; (5) the PVs pass through the lymph valves; (6) the PVs branch in the area where the lymph vessels branch; (7) if the thickness of a PV is $>$ 50 μm, the PV most likely contains adhered blood cells or lymphocytes; this coagulation is because of bleeding in the lymph node during injection; and (8) confounding fake string-like tissues can sometimes appear. We recommend that the DAPI-test be applied to the putative PV specimens because the characteristic distribution of the rod-shaped nuclei is a good criterion to discern the PV from other string-like tissues [10—12].

A PV is a bundle of several subvessels. This bundle structure is a key feature of a PV that uniquely distinguishes it from blood or lymph vessels. The PVS can be positively identified by observing the distributions of nuclei in the longitudinal sections to distinguish it from blood or lymph vessels. The nuclei of the endothelial cells of a PV are arranged in broken parallel lines; they are rod-shaped and approximately 10—20 μm long. This feature can be easily checked with DAPI staining (Fig. 4). These rod-shaped nuclei may be endothelial cells lining the subvessels. The endothelial nature of these cells has only been proven by the Von Willebrand factor (vWF) criterion and has not yet been confirmed by using other characteristics. The development of antibodies such as LYVE-1 will be a critical step toward the complete tracing of the whole PVS network.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

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