공학석사학위논문

Protocol Development for Observation of the Primo Vascular System in Lymphatic System

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2013년 8월

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Protocol Development for Obervation of the Primo Vascular System in Lymphatic System.

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A Dissertation in Nano science and technology

Submitted to the Faculties of Seoul National University
in Partial Fulfillment of the Requirements for the Degree of
Master of Engineering

June 2013

Department of Transdisciplinary,
Program in Nano Science and Technology
The Graduate School of Convergence Science and Technology
Seoul National University

Abstract

The lymphatic primo vascular system (PVS) is the sub-system of the PVS that forms a network throughout an animal body as a third circulatory system in addition to the blood and lymph systems. In this study, the detailed protocol to observe the PVS in the lymph vessels near the caudal vena cava of a rat using the Alcian blue staining method was established. We investigated and analyzed the characteristics of the primo vessels and the inner wall surface of a lymph duct by atomic force microscopy. These data were compared with phase contrast x-ray microscope images of a primo vessel.

The PVS floating inside lymphatic ducts was observed by injecting the Alcian blue into the lumbar lymph nodes near the caudal vena cava in rabbits and rats. . In previous studies, PVS was only observed within the abdominal lymph system but techniques were extended to the thoracic duct area in this thesis. After extracting the primo vessel, the morphological features were examined by staining them with Phalloidin and DAPI with the confocal laser scanning microscopy.

For histological study of the PVS DiI, H&E and immune-histochemistry were performed. The DiI staining showed the outer membrane of the primo vessel and the H&E showed the distribution of cells and nuclei. The immuno staining with EMP-3 and vWF revealed that PVS had the EMP-3-positive epithelial layer and an inner vWF- positive endothelial layer, but not for CD31 or LYVE-1. These histological data provided the distinguishing features between lymph vessel and primo vessel.

The ultra-structures of the primo vessel and the surface of lymph vessel's inner wall were investigated using Atomic Force Microscope to find the differences between these two. Lymphatic endothelium layer were attached to filaments that were formed of many elastic fibers by interstitial collagen. On the other hand, the surface of a primo vessel (PV) was relatively smooth and had characteristic holes. These holes are considered to be size selective channels connecting outside and inside.

As a conclusion, the detailed protocol was developed for the anatomical observations and histological analysis of the PVS. We investigated the primo vessel and the inner wall of lymph vessel using an atomic force microscope and found that they are distinctively different. We observed the holes on the surface of primo vessels which was consistent with the phase contrast x-ray microscopy.

Key words: Primo Vascular System, Lymph, Alcian blue, Protocol, Atomic Force Microscopy, Holes, X-ray microscope.

Student Number: 2011-24240

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Chapter 1. Introduction

1.1 The Primo Vascular System

Acupuncture points(AP) are locations on the body and several hundred acupuncture points are considered to be located along Acupuncture meridians (AM). This theoretical basis of traditional oriental medicine has been well-known in East Asian civilization for a long time. However, many people wondered whether these acupuncture system is imaginary or real. Until now, there are many criticisms arises by modern medical doctors and scientists.

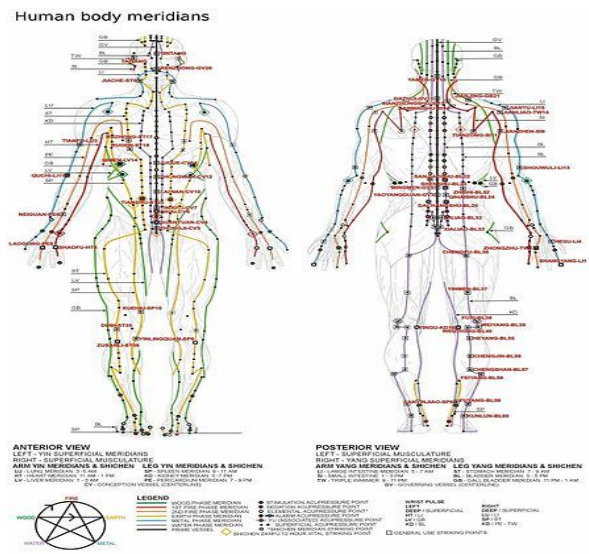

Figure 1.1 A diagram of Acupuncture Meridian System.

From 1961 to 1965, some scientists started to claim that the acupuncture points and meridians are existing and have anatomical structures. Among these researchers, a scientist in North Korea named Bonghan Kim published five papers about scientific studies on the anatomical structure and physiology of AM and AP [1-4]. He strongly insisted Bonghan theory which is that an AM and AP correspond to a primo vessel (PV, Bonghan duct) and primo node (PN, Bonghan corpuscle), respectively. Also in his paper, which the Primo vascular system(PVs) is composed of Bonghan duct(PV), Bonghan corpuscle(PN), Bonghan liquid(PL) and these are distributed in the dense networks which formed a novel circulatory

system throughout an animal's body. PVs are linked to either to one end of a PN or to both ends. Bonghan liquid is made in the Bonghan corpuscle, and flowing in the Bonghan duct. The key component of Bonghan liquid is sanal (Primo Microcell). Sanal means 'live egg' literally, and is a kind of embryonic-like stem cell. He argued that these networks were the third circulatory system in addition to the vascular and lymphatic circulatory system.

To be more specific, the Bonghan circulatory system is composed of several sub-networks located at various sites inside the body. These sub-networks can be categorized as: (1) a superficial Bonghan system located in the skin, (2) an intravascular Bonghan system that runs along the interior of the large veins, arteries, and lymphatic vessels and is afloat in the blood/lymph stream, not adhering to the vessel wall, (3) an extra-vascular Bonghan system that runs along the exterior of large blood vessels, (4) an organ-surface Bonghan system that spreads on various internal organ surfaces, (5) an intra-organ Bonghan system located inside various internal organs, and (6) a neural Bonghan system that exists inside the brain and spinal cord and runs along the exterior of peripheral nerves [6].

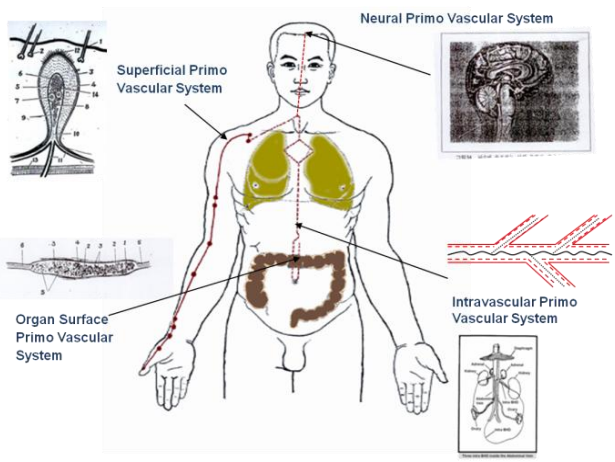

Figure 1.2 A schematic drawing of PVS. A brown colored line represents lung meridian. Part of PVS at various sites is showed for an example. This drawing was illustrated by Biomedical Physics Laboratory, Seoul National University.

However, despite of many attempts to disclose his method of observing the PVS by several research teams, no exact confirmation of his work was ever

accomplished. So they thought that the Bonghan theory was false and was rejected as false until now. Because of that, the research was forgotten for about forty years due to lack of records about experiment methods.

In 2002, the Bonghan theory was rediscovered by the research team of Biomedical Physics Laboatoy for Korean Medicine in Seoul National University. This team has been made great progress in proving Kim's theory. PVS has been reported to exist in a blood vessel [7-9],lymph vessel[10-12], brain ventricle and spinal cord[13], and on internal organ surface[14-17].

To prove his theory, many modern techniques and instruments such as various types o electron microscopy, confocal laser scanning microscopy, atomic force microscopy, fluorescent nanoparticles, immunohistochemistry, proteomic analysis, and electrophysiological method have been employed to re-establish Bonghan Kim's work.

1.1.1 Morphology of primo vascular system

Primo vascular system is composed of primo vessels, primo nodes, and primo fluid is flowing in the primo vascular system. Primo vessel is composed of many sub-vessels due to encircled by endothelial cells. A primo vessel exhibits special rod-shaped nuclei (10−20 μm in length) that were clearly visible by phase-contrast microscopy. A lumen of a primo sub-vessel is about 6−10 μm in diameter [18].

The ultrastructure of primo sub-vessels in a primo vessel shared some common properties with blood and lymphatic capillaries, but possessed their own distinct structural features [19]. A primo sub-vessel was made up of a single, nonfenestrated endothelial cell layer and had a relatively small, regular lumen, ~6 μm in diameter, similar to a blood capillary, while a lymphatic capillary possessed a more irregular and wider lumen than a primo sub-vessel or a blood capillary. The abluminal wall of the ductule was not lined by an enveloping membrane, which is surrounded blood and lymphatic capillaries. And a primo sub-vessel is surrounded by fibrin-like fibers, which were randomly distributed in the stroma of a primo vessel [18].

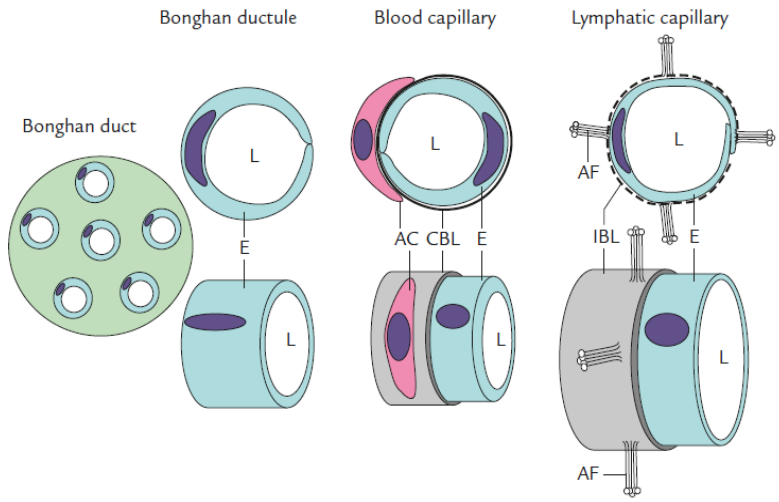

Figure 1.3 Structural properties of primo sub-vessel (formerly Bonghan ductule) and of blood and lymphatic capillary. E = endothelium; L = lumen; AC = accessory cell; CBL = complete basal lamina; IBL = incomplete basal lamina.
[*J Acupunct Meridian Stud* 2009;2(2):107−117]

When using DAPI and phalloidin staining method, it is more obvious to describe the difference between them.

There are two distinctive characteristic in a primo vessel compared to artery and vein. The first difference to the other circulatory vessels, the primo vessel shows longitudinally arranged rod-shaped nuclei which are nuclei of endothelial cells. The nuclei of artery shows both longitudinally arranged rod-shaped nuclei and transversely arranged nuclei which are nuclei of smooth muscle, while nuclei of vein arranged irregularly.

The second characteristic in a primo vessel is parallel lines which are walls of primo sub-vessels.

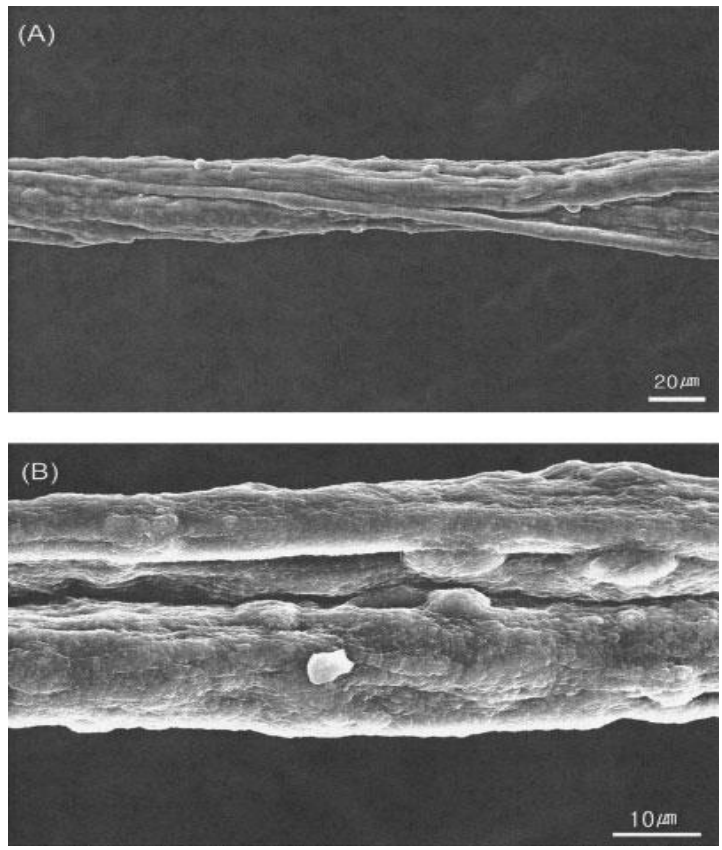

Figure 1.4 (A) Scanning electron micrograph of a novel threadlike structure (Bonghan duct) whose thickness is about 40 μm. It has a bundle structure consisting of many sub-ducts with a typical thickness of about 10 μm. This bundle structure is one of the characteristic features that distinguish Bonghan ducts from lymph vessels. (B) A more magnified view shows clearly the sub-ducts and their surface structures. [*MICROSCOPY RESEARCH AND TECHNIQUE* 70:34–43 (2007)]

A primo node is oval-shaped (spindle or cucumber shape) and 0.2 mm ~ 1 mm in size [19]. Both of its ends gradually reduce in size to become threadlike. A primo node contained various cell types, such as monocytes, granulocytes, and small and large lymphocytes, scattered randomly in the matrix as single cells or gathered in a follicle-like formation [18].

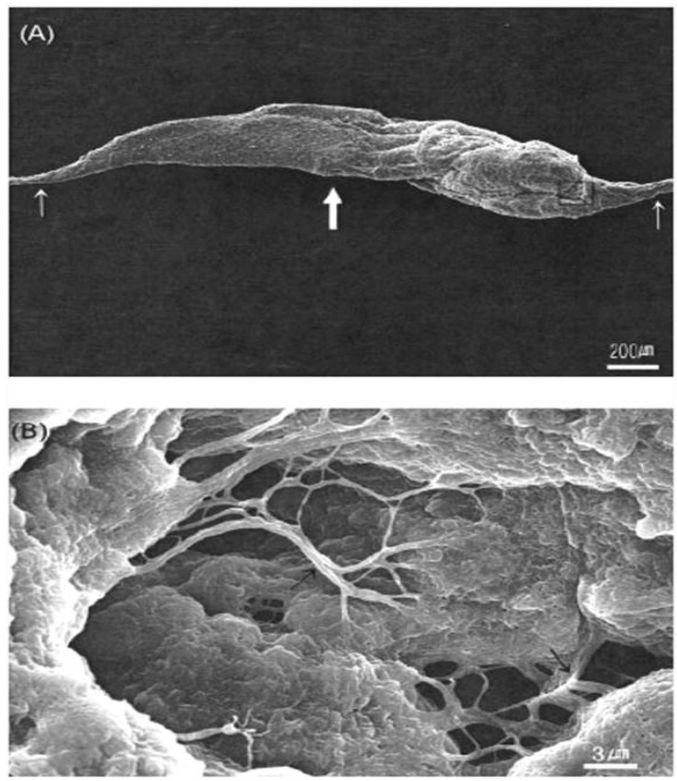

Figure 1.5 (A) Scanning electron micrograph of a typical Bonghan corpuscle (bold arrow) on the surface of an organ of a rat. It is of spindle or cucumber shape with a length 2 mm and a thickness 0.4 mm. Both of its ends gradually reduce in size to become threadlike (arrows). Its surface is rather coarse. This and the following micrographs are the first SEM images ever taken of the novel threadlike structures and corpuscles. (B) Magnified view of Figure 1.5A. The surface at the transition region from corpuscle to threadlike has a fenestra with collagenous fibers (arrows). [*MICROSCOPY RESEARCH AND TECHNIQUE* 70:34–43 (2007)]

1.1.2 Function of primo vascular system

Until these days, discovering the function of PVS in our body still remains as a problem for solution. Since Kim claimed that primo vascular system does regenerative function in organisms in 1960s [20], there have been several studies about function of primo vascular system going on.

A recent research data showed about primo vessel and primo node's cell composition and there reported that the immune cells inside primo node consisted of mast cells (20%), eosinophils (16%), neutrophils (5%) and histiocytes (53%)

with a few lymphocytes (1%) as well as round immature cells, possibly stem cells (3%) [21]. It means primo vascular system may be deeply involved in immune system and more.

1.2 Previous researches on Primo vascular system in the Lymphatic system

The lymphatic system is part of the circulatory system, comprising a network of conduits called lymphatic vessels that carry a clear fluid called lymph.[22] It plays an essential role in immune responses by serving as a conduit for antigen-presenting cells to the lymph nodes. [23] Despite its vital roles in our body, our understanding of this system has been very limited compared to the other circulatory systems.

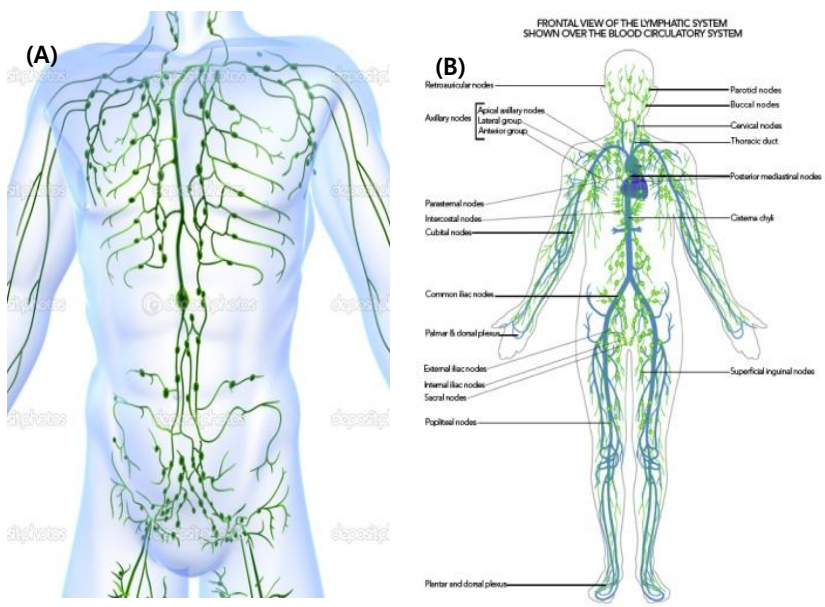

Figure 1.6 (A) Lymphatic System. (B) Frontal view of the lymphatic system shown over the blood circulatory system.

In modern medicine, the anatomical composition of lymphatic system has been known to the lymph nodes, lymph valves and lymph vessels. However, there were threadlike structures floating inside lymphatic vessels and researchers started to expose this new finding.

Bonghan Kim(1963) was the first founder of threadlike structures and is thought to be this subsystem of a new circulatory system in addition to already well –known vascular, nervous, and lymphatic systems. He claimed that they form a network throughout a body and stated that this structure existed not only in the lymphatic vessels, but also in the major vascular systems and in the skin. In addition to that he report that this system could resolve medical problems which cannot be overcome with modern medical science.

Kim published 5 articles about this new system, however, in his paper, he only claimed the observation of new system, but did not provide detailed information of how to find it. Even a tremendous development of sophisticated optical instruments no one was able to detect previously this novel structure by microscopic inspection of lymphatic vessels. Until now, no instrument and technique specifically designed for testing his theory has been developed.

Recently, by applying some staining method which are with Acridine-Orange, Janus Geen B, Alcian blue, and Tryphan blue allowed us to visualize the primo-vessels and nodes afloat inside the lymphatic vessels without adherence to the vessel wall. But still many of barriers has been existed in carrying out the experiments and reproducing the same result so felt keenly the necessity of writing protocol which describes in sufficiently precise detail all the steps so that another researcher can independently perform the experiment and expect to get results that are not different from our original experiment. At this time it is not known what the best protocol is, but in this thesis I am able to demonstrate a protocol with an improved false alarm rate.

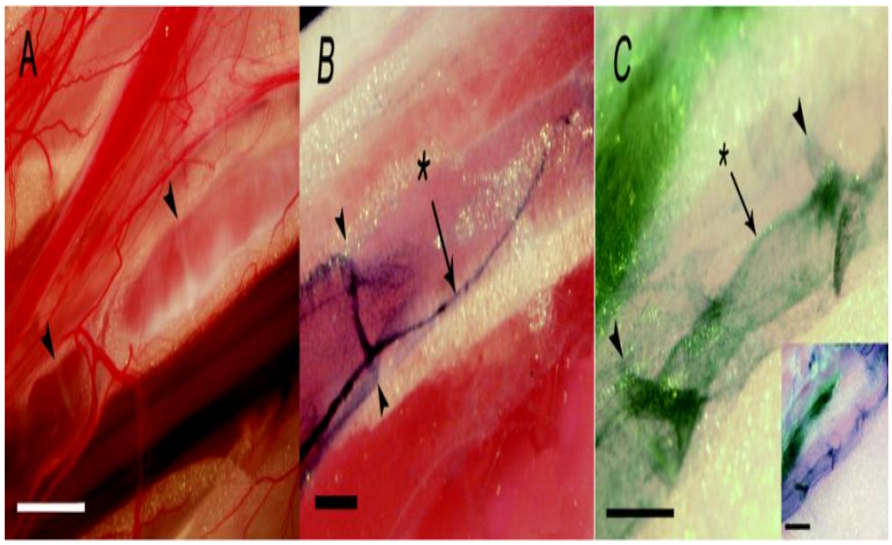

Figure 1.7 A stereoscopic image of lymphatic vessel around the inferior vena cava of a rabbit. Typical view without staining (A) and after injection of Janus Green B into the lymphatic vessel (B and C). [*Anatomical Record* 284B (2005) 35]

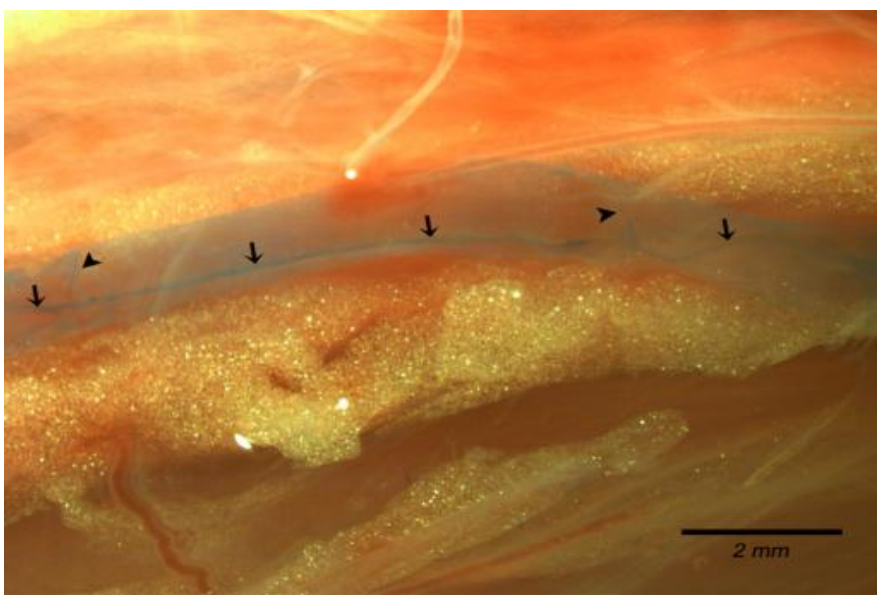

Figure 1.8 An in situ image of Alcian blue injected lymphatic vessel was captured. The hyaluronan-rich primo vessel (arrows) is prominently stained with Alcian blue, while the surrounding lymphatic vessel is weakly stained.
[*LYMPHATIC RESEARCH AND BIOLOGY* Vol 4: No 4, 2006]

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Chapter 2. Microscopic Devices

2.1. Atomic Force Microscopy

2.1.1 Basic Principle

Atomic force microscopy (**AFM**) is a very high-resolution type of scanning probe microscopy, with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The AFM is one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale. The information is gathered by "feeling" the surface with a mechanical probe. The AFM consists of a cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law.[1] Depending on the situation, forces that are measured in AFM include mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces, Casimir forces, solvation forces, etc. Along with force, additional quantities may simultaneously be measured through the use of specialized types of probes.

2.1.2 AFM Probe Deflection

The most AFMs use a laser beam deflection system, where a laser is reflected from the back of the reflective AFM lever and onto a position-sensitive detector. AFM tips and cantilevers are microfabricated from Si or Si_3N_4. Normally, the probe is a sharp tip, which is a 3-6 um tall pyramid with 15-40nm end radius.

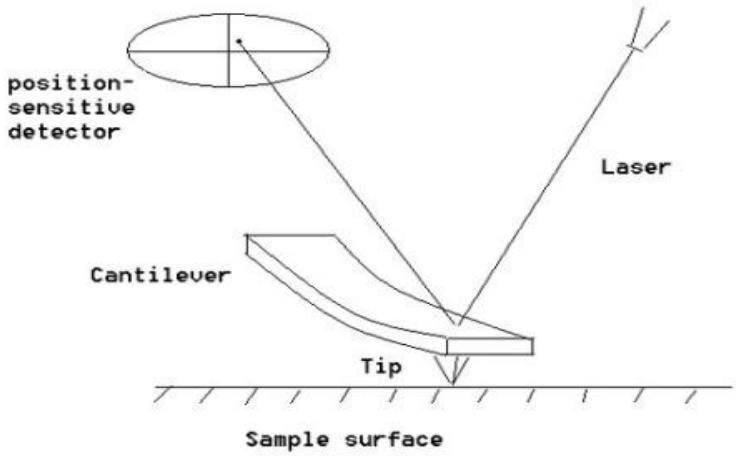

Figure 2.1 A block diagram of Atomic Force Microscope.

To acquire the image resolution, AFMs can generally measure the vertical and lateral deflections of the cantilever by using the optical lever. The optical lever operates by reflecting a laser beam off the cantilever. The reflected laser beam strikes a position-sensitive photo-detector consisting of four-segment photo-detector. The differences between the segments of photo-detector of signals indicate the position of the laser spot on the detector and thus the angular deflections of the cantilever [2].

2.1.3 Measuring Forces

Because the atomic force microscope relies on the forces between the tip and sample, knowing these forces is important for proper imaging. The force is not measured directly, but calculated by measuring the deflection of the lever, and knowing the stiffness of the cantilever. Hook's law gives $F = -kz$, where F is the force, k is the stiffness of the lever, and z is the distance the lever is bent.

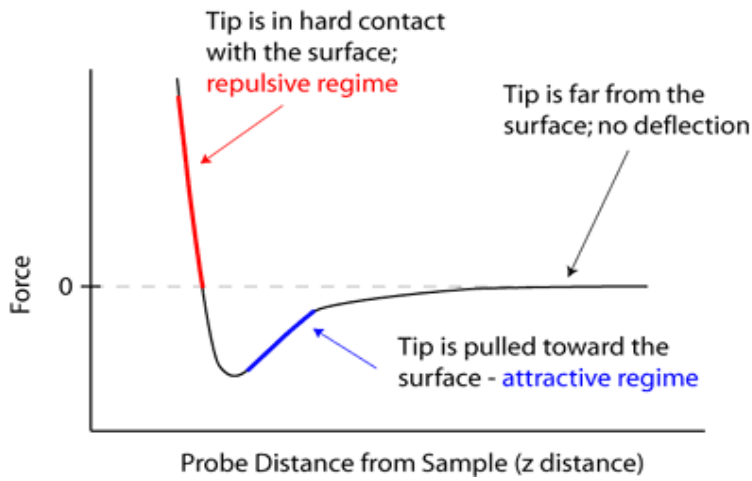

Figure 2.2 A diagram of measuring forces of AFM.

2.1.4 AFM Imaging Mode

Contact Mode

The first and foremost mode of operation, contact mode is widely used. In contact mode, AFMs use feedback to regulate the force on the sample. The AFM not only measures the force on the sample but also regulates it, allowing acquisition of images at very low forces. The feedback loop consists of the tube scanner that controls the height of the tip; the cantilever and optical lever, which measures the local height of the sample; and a feedback circuit that attempts to keep the cantilever deflection constant by adjusting the voltage applied to the scanner. A well-constructed feedback loop is essential to microscope performance. Because the tip is in hard contact with the surface, the stiffness of the lever needs to be less that the effective spring constant holding atoms together, which is on the order of 1 - 10 nN/nm. Most contact mode levers have a spring constant of < 1N/m [3].

Non-contact mode

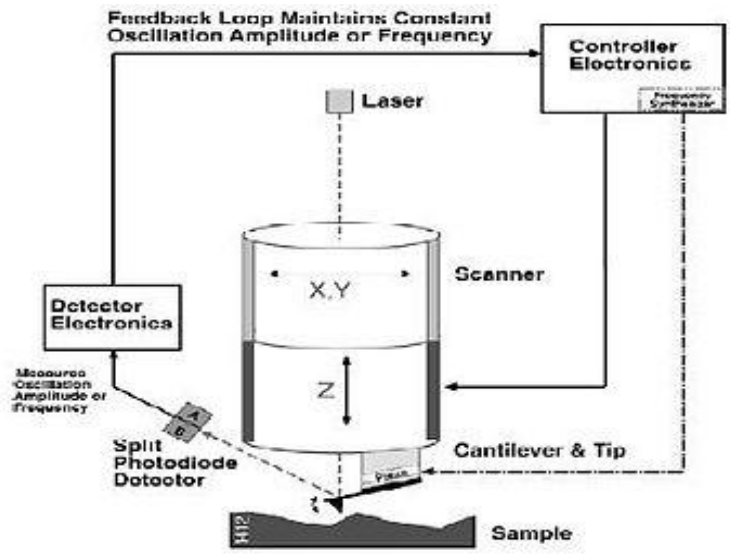

Figure 2.3 AFM : non-contact mode.

In this mode, the tip of the cantilever does not contact the sample surface. Noncontact mode belongs to a family of AC modes, which refers to the use of an oscillating cantilever. A stiff cantilever is oscillated in the attractive regime, meaning that the tip is quite close to the sample, but not touching it (hence, "noncontact"). The forces between the tip and sample are quite low, on the order of pN (10^{-12} N) . The detection scheme is based on measuring changes to the resonant frequency or amplitude of the cantilever. Non-contact mode AFM does not suffer from tip or sample degradation effects that are sometimes observed after taking numerous scans with contact AFM. This makes non-contact AFM preferable to contact AFM for measuring soft samples, e.g., biology sample and organic thin film. In the case of rigid samples, contact and non-contact images may look the same [4].

Intermittant-contact / “tapping mode” AFM

Commonly referred to as “tapping mode” it is also referred to as intermittent-contact or the more general term Dynamic Force Mode (DFM).

A stiff cantilever is oscillated closer to the sample than in noncontact mode. Part of the oscillation extends into the repulsive regime, so the tip intermittently touches or “taps” the surface. Very stiff cantilevers are typically used, as tips can get “stuck” in the water contamination layer. [5]

The advantage of tapping the surface is improved lateral resolution on soft samples.

The AFM can be used to image and manipulate variety of surfaces of living material such as tissue or cells. In biophysics, it allows to measure the mechanical properties of living cells. Most importantly, topography and error signal images of AFM was used to for morphological study of primo vessels. In the most of morphological study in this research, contact mode AFM was used.

2.2 Fluorescent staining

2.2.1 DAPI

DAPI or **4',6-diamidino-2-phenylindole** is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. DAPI can pass through an intact cell membrane therefore it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower.

When bound to double-stranded DNA DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue) (cited from tools. Invitrogen.com/contents/sfs/manuals/mp01306.pdf). Therefore for fluorescence microscopy DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. The emission peak is fairly broad [6]. DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA [7, 8].

DAPI's blue emission is convenient for microscopists who wish to use multiple fluorescent stains in a single sample. There is some fluorescence overlap between DAPI and green-fluorescent molecules like fluorescein and green fluorescent protein (GFP) but the effect of this is small. Use of spectral unmixing can account for this effect if extremely precise image analysis is required.

DAPI is a popular nuclear counterstain for use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures. When used according to our protocols, DAPI stains nuclei specifically, with little or no cytoplasmic labeling.

(cited from tools.invitrogen.com/contents/sfs/ manuals/mp01306.pdf).

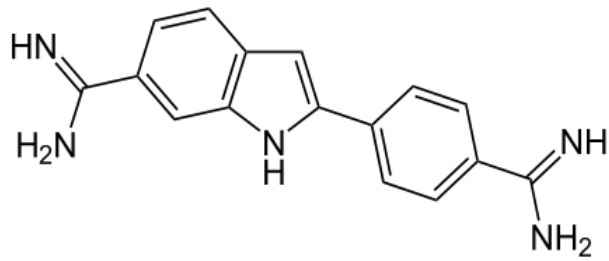

Figure 2.4 Molecular structure of DAPI.

2.2.2 Phalloidin

Phalloidin binds F-actin, preventing its depolymerization and poisoning the cell. Phalloidin binds specifically at the interface between F-actin subunits, locking adjacent subunits together. Phalloidin, a bicyclic heptapeptide, binds to actin filaments much more tightly than to actin monomers, leading to a decrease in the rate constant for the dissociation of actin subunits from filament ends, which essentially stabilizes actin filaments through the prevention of filament depolymerization [10]. Moreover, phalloidin is found to inhibit the ATP hydrolysis activity of F-actin [13]. Thus, phalloidin traps actin monomers in a conformation distinct from G-actin and it stabilizes the structure of F-actin by greatly reducing the rate constant for monomer dissociation, an event associated with the trapping of ADP [13]. Overall, phalloidin is found to react stoichiometrically with actin, strongly promote actin polymerization, and stabilize actin polymers [14].

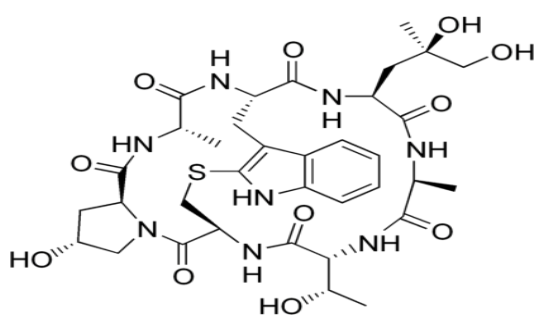

Figure 2.5 Molecular structure of Phalloidin.

Phalloidin is one of a group of toxins from the death cap (*Amanita phalloides*) known as phallotoxins. Phalloidin binds F-actin, specifically at the interface between F-actin subunits. Phalloidin, a bicyclic heptapeptide, binds to actin filaments much more tightly than to actin monomers, leading to a decrease in the rate constant for the dissociation of actin subunits from filament ends, which essentially stabilizes actin filaments through the prevention of filament depolymerization. (Cited from tools.invitrogen.com/contents/sfs/ manuals/

mp00354.pdf)

The properties of phalloidin make it a useful tool for investigating the distribution of F-actin in cells by labeling phalloidin with fluorescent analogs and using them to stain actin filaments for light microscopy. And the amount of fluorescence visualized can be used as a quantitative measure of the amount of filamentous actin there is in cells if saturating quantities of fluorescent phalloidin are used. Therefore, fluorescent phalloidin can be used as an important tool in the study of actin networks at high resolution [10].

In this research, I used phalloidin 488 and 568 for staining f-actin, on the purpose of investigating structure of the tissue. The absorption maximum of phalloidin 488 is at a wavelength of 495 nm and its emission maximum is at 518 nm. The absorption maximum of phalloidin 568 is at a wavelength of 578 nm and its emission maximum is at 600 nm.

2.3 Fluorescence Stereo Microscope

Fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances [11,12].

A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities. The conventional microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample. A fluorescence microscope, on the other hand, uses a much higher intensity light source which excites a fluorescent species in a sample

of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source.

When the reflected light and background fluorescence is filtered in this type of microscopy the targeted parts of a given sample can be imaged. This gives an investigator the ability to visualize desired organelles or unique surface features of a sample of interest. (cited from ttp://serc.carleton.edu/microbelife/ research_methods/microscopy /fluromic.html).

2.3.1 Sample Preparation

Biological fluorescent stains:

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI which bind the minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivatised with a fluorescent reporter. A major example of this class of fluorescent stain is fluorescently labelled-phalloidin which is used to stain actin fibres in mammalian cells [11].

2.3.2 Imaging Method

In most cases the sample of interest is labeled with a fluorescent substance known as a fluorophore and then illuminated through the lens with the higher energy source. The illumination light is absorbed by the fluorophores (now attached to the sample) and causes them to emit a longer lower energy wavelength light. This fluorescent light can be separated from the surrounding radiation with filters designed for that specific wavelength allowing the viewer to see only that which is fluorescing [17].

Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector

by the same objective that is used for the excitation which for greatest sensitivity will have a very high numerical aperture. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and the epifluorescence method therefore gives a high signal to noise ratio. An additional barrier filter between the objective and the detector can filter out the remaining excitation light from fluorescent light. (cited from https://en.wikipedia.org/wiki/Fluorescence_microscope)

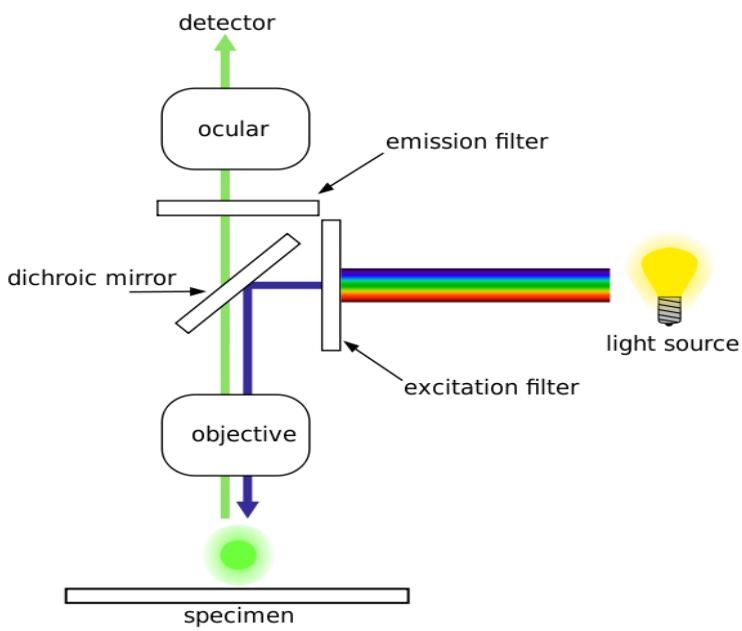

Figure 2.6 The Schematic diagram of the fluorescence stereomicroscope.

2.4 Confocal Laser Scanning Microscopy

Confocal Laser scanning microscopy (CLSM or LSCM) has become an invaluable tool for a wide range of investigations in the biological and medical sciences for collecting the image of serial thin optical sections in living and fixed specimens ranging in thickness up to 100 micrometers.

CLSM is a technique for obtaining high-resolution optical images with depth selectivity [15]. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects [16].

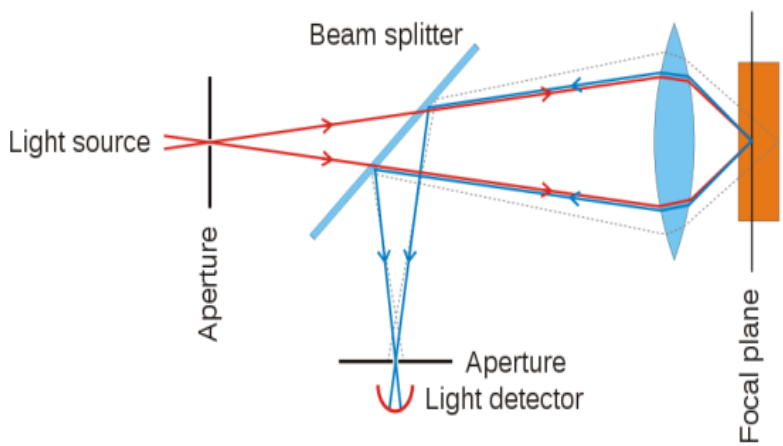

Figure 2.7 Principle of confocal microscopy

2.4.1 Imaging Mode

In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small (ideally diffraction limited) focal volume within or on the surface of a specimen. In biological applications especially, the specimen may be fluorescent. Scattered and reflected laser light as well as any fluorescent light from the illuminated spot is then re-collected by the objective lens. A beam splitter separates off some portion

of the light into the detection apparatus, which in fluorescence confocal microscopy will also have a filter that selectively passes the fluorescent wavelengths while blocking the original excitation wavelength. After passing a pinhole, the light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT) or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer.[15]

The detector aperture obstructs the light that is not coming from the focal point, as shown by the dotted gray line in the image. The out-of-focus light is suppressed: most of the returning light is blocked by the pinhole, which results in sharper images than those from conventional fluorescence microscopy techniques and permits one to obtain images of planes at various depths within the sample (sets of such images are also known as z stacks).[14]

The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected light. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage or objective lens. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes.[15]

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution[15]. Biological samples are often treated with fluorescent dyes to make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules.

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Primo vascular system in the Lymphatic system

Chapter 3. Protocol for the Observation of the Primo Vascular System in the Lymph Vessels of Rabbits

Molecular level understanding of the structure and the functions of the lymphatic system has greatly enhanced the importance of this second circulation system, especially in connection with cancer metastasis and inflammation. Recently, a third circulatory system, the primo vascular system (PVS) was found in various parts of an animal's body, especially as threadlike structures floating in the lymphatic flow in lymph vessels. Although the medical significance of this emerging system will require much work in the future, at the present time, some important suggestions in connection with immune cells, stem cells, and cancer metastasis have already appeared. Experiments to observe the PVS in the lymph vessels near the caudal vena cava of rabbits and rats have been performed by several independent teams, but reproduction requires much skill and technical know-how. In this article, we provide a detailed protocol to detect the PVS inside the lymph vessels of a rabbit. Detection and isolation are the first steps in unraveling the physiological functions of the PVS, which awaits intensive research.

3.1 Introduction

After centuries of playing second fiddle to the blood system, the second circulation, the lymphatic one, came into its own as a key player in cancer metastasis [1].The turning point in lymph research came in the 1990's when the vascular endothelial growth factors (VEGFs) of the lymphatic system were found by Alitalo et al. [2] and by Achen and Stacker [3], but lymphatic research really assumed great importance only after the discovery of the specific marker, LYVE-1, by Jackson et al. in 1999 [4]. Besides the role in cancer metastasis, the lymphatic system could be a major culprit in inflammation due to the protein podoplanin and its signaling molecule CCL21, which is a powerful attractant to immune cells such as dendritic cells and macrophages [5].

In addition to its surprising transformation from long obscurity to prominence

thanks to molecular level findings in the lymphatic system, a less publicized development in the gross anatomy of the lymphatic system, is a potentially epoch-making development in lymphology, is the observation of a threadlike structure floating in the lymph flow. Such structures were first observed in the large caliber lymph vessels near the caudal vena cava of a rabbit with the staining dye Janus Green B [6], and were subsequently observed near the caudal vena cava of a rat with fluorescent magnetic nanoparticles [7]. Since the early observations, the method was further developed to detect the threadlike structures with Alcian blue [8] and with an optical technique without using any dye to stain the threadlike structure [9].

The threadlike structure was a part of a third circulatory system called the primo vascular system (PVS) which is distributed throughout an animal's body, for example, on the surfaces of various internal organs [10], in the heart [11] and in the ventricles of the brain and the central canal of the spinal cord [12]. The PVS was first found by Bong-Han Kim [13] in the 1960's as an anatomical structure of acupuncture meridians, but it has only recently been investigated very extensively [14].

A hint to its medical significance was given by the abundance of immune cells in the PVS [15], which suggested an anti-inflammatory effect of acupuncture [16]. Another important feature was the PVS-genesis around the cancer tissue [17] and its possible role as an additional path, besides the well-known blood vessel and lymphatic vessel, for cancer metastasis [18]. In addition, the abundance of catecholamine-producing cells in the PVS has led to the conjecture of a hormone-conduit function of the PVS [19].

Even though there are descriptions on the methods used in PVS experiments, reproducing the results is not easy because the techniques for detecting the PVS are state of the art. Therefore, a detailed protocol for the experiment is necessary. Here, we provide the protocol for observing the PVS in the lymph vessels around the caudal vena cava of a rabbit. Hopefully, this protocol will help those who want to develop a molecular-level understanding of the PVS by following a path similar to that followed in the development of the lymphatic system, like VEGF's and LYVE-1 [2-4].

3.2 Materials

3.2.1 REAGENTS

Experimental animals: Rabbits (clean-outbred New Zealand White (NZW), female, 10 weeks old, 1.5 kg~1.8 kg) were obtained from SAM TACO Laboratory Animal Company (Osan, Korea) All animal care and studies should abide by international laws and policies according to the Guide for the Care and Use of Laboratory animals.

Zolatil (Virbac Laboratories, Carros, France)

Rompun (Bayer, Korea)

0.9% Saline Solution (Choongwae Pharmaceuticals, Korea)

NaCl (Duksan Pure Chemicals, Korea)

KCl (Junsei Chemical Co. Ltd, Japan)

K_2HPO_4 (Yakuri Pure Chemicals Co. Ltd, Japan)

NaH_2PO_4 (Sigma, USA)

70% Alcohol (Choongwae Pharmaceuticals, Korea)

Alcian Blue 8GX (Sigma, USA)

Alexa Fluor 488 phalloidin (Invitrogen, USA)

ProLong Gold antifade reagent with DAPI: 4', 6-diamidino-2-phenylindole (Invitrogen, USA)

3.2.2 EQUIPMENT

Stereomicroscope (SZX12, Olympus) with a CCD Camera (DP70, Olympus)

Phase Contrast Microscope (BX51, Olympus) with a CCD Camera (Infinity 3, Lumenera)

Vortex-2 Genie (Scientific Industries, USA)

Ultrasonic Cleaner (Mujigae, Korea)

Water Bath (Changshin Scientific Co., Korea)

Pet Specialty Cordless Trimmer (Oster, USA)

1ml Hypodermic Syringe (Kovax- Syringe, Korea)

0, 5-10 ul Finnpipette

Micro Slides (Silane Coating) (size: 76 mm x 26 mm)

100 Deckglaser Cover slips (size 24 mm x 50 mm)

Electric Heating Pad (size: 30 mm x 30 cm)

Disposable Transfer Pipette

PH Meter (Thermo Electron Corporation, USA)

Electrocautery

Glass Funnel

Surgical Instruments and Ophthalmic Surgical Instruments (Tumed, German)

Glass Microfibre Filters 110 mm, Cat No 1820-110 (GE Healthcare Co., UK)

Minisart Syringe Filter, Hydrophilic (Sartoriou Stedim Biotech, Germany)

BD 10 ml Filter Syringe (Becton Dickinson Medicals Ltd, Singapore)

BD Ultra-Fine Insulin Syringe, 31G (Becton, Dickinson and Company, USA)

**The arrangement of the experimental equipment is given in Fig. 3.1

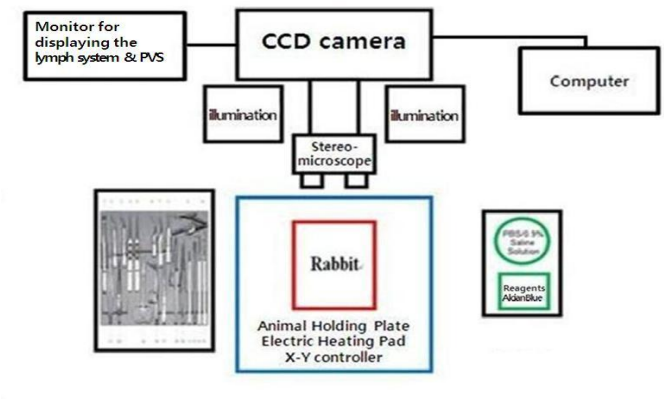

Figure 3.1 Arrangement of the experimental equipment.

3.2.3 REAGENT SETUP

Phosphate buffered saline solution (PBS): Eight (8) g of NaCl, 0.2 g of KCl 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 and 800 ml of distilled water are mixed together to make a solution, and using a pH meter, the pH is set to 7.4. An additional 200 ml of distilled water is added to the previously mixed solution to make 1 L (1000 ml) of 1X PBS solution, which is stored at room temperature.

Alcian blue (AB) staining dye (1.0%): Combine 0.01 g of alcian blue powder with 1 ml of 1X PBS solution to make 1% alcian blue staining dye. After mixing these, apply the vortex machine and put this solution in the heating cabinet (~ 61˚C) for about 30 minutes to dissolve the alcian blue powder completely. Filter this solution with a filter paper in a funnel first, followed by a 2nd filteration using a 0.20-um syringe filter attached in a 10-ml syringe. After the 2nd filteration, load the alcian blue solution in a 1-ml insulin syringe (31 Gauge) while using a warm bath to keep it at a constant temperature of 38~40˚C prior to injection into the lymphatic system.

Phalloidin: Combine 10 ul of 6.6-uM Alexa Fluor 488 phalloidin and 300 ul of PBS. Use the sonicator to mix these well and store the solution at -20℃. Avoid light.

3.3 Procedure

An overview of the entire procedure is given in Fig. 3.2

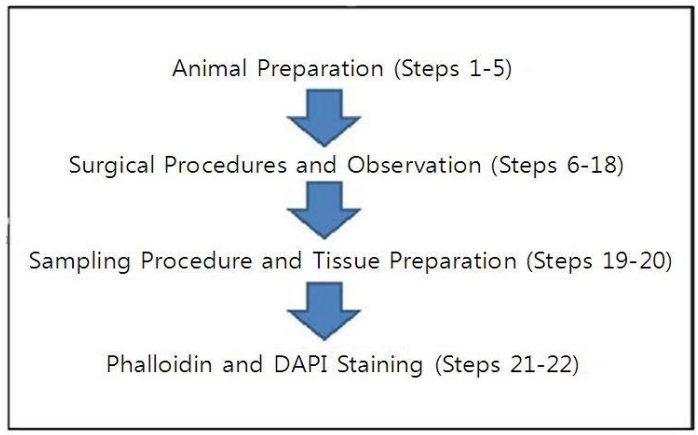

Figure 3.2 Representative overview of the protocol for observation of the primo vascular system in the lymph vessels of a rabbit.

3.3.1 ANIMAL PREPARATION (Timing: 30-45 min)

1. Withhold food and water from the rabbit is required for 48 hours before the experiment for clear observation of the lymphatic system in the abdomen.
2. Anesthetize NZW rabbit (~1.8 kg) with an intramuscular injection of a mixture of zolatile (2.5 ml) and rompun (0.5 ml) by using a 1-ml hypodermic syringe.
3. For the checking the level of anesthetization, apply strong stimulation, such as pressing or twitching, to the rabbit's ear and feet. Wait until rabbit is fully anesthetized.
4. Shave all the hairs in the abdominal area by using the Pet Specialty cordless trimmer.
5. Tie the rabbit's arms and legs with strings to the four corners of the animal holding plate for fixation.

3.3.2 SURGICAL PROCEDURES AND OBSERVATION (Timing: 1-2 hour)

6. Prior to surgery, preheat pH 7.4 phosphate buffered saline (PBS) and AB staining solution loaded in a syringe to 39℃. In order to disinfect the skin on the abdomen, spray 1-2 ml of 70% ethanol over the top of the skin and wipe with aseptic tissue paper.
7. Grip the middle skin of the abdomen with toothed forceps, and incise the outermost skin along the middle line (linea alba line) of the abdomen down to the symphysis pubis and up to the ensisternum with surgical scissors. In this step, the depth of incision should be very shallow, just to see the muscular layer.
8. Cutting with a 1-cm incision on the linea alba line in the straight muscle of the abdomen at the place of the lower 1/3 on the line from the ensisternum (syn: xiphoid process) to the symphysis pubis. Be careful to avoid bleeding during this procedure.
9. When you open, you will have exposure to the large intestines with adipose tissues covered in the greater omentum and to the top of the surface of the urinary bladder. Remove the internal organs to the preferred side (right or left) to reveal the lymphatic vessels in the abdomen. You will see a lymph vessel in adipose tissue as shown in Fig. 3.3. Cover the organs with wet gauze, and continuously spray warm saline solution over the top of the gauze to avoid dryness. Use a disposable transfer pipette to continuously add warm saline drop by drop on top of the lymphatic vessel to avoid dryness in the abdominal area during the observation.

   A. To avoid bleeding, the incision must be on or around the linea alba line because the distribution of blood vessels is the least in this area. However, in case of a bleeding problem, use electrocautery to stop the bleeding. This will burn and seal the blood vessel, which will help to reduce or stop massive bleeding.

   B. To avoid body- temperature drops after opening the abdomen, don’t forget to apply continuous warmth to the rabbit’s body by using a

heating pad.

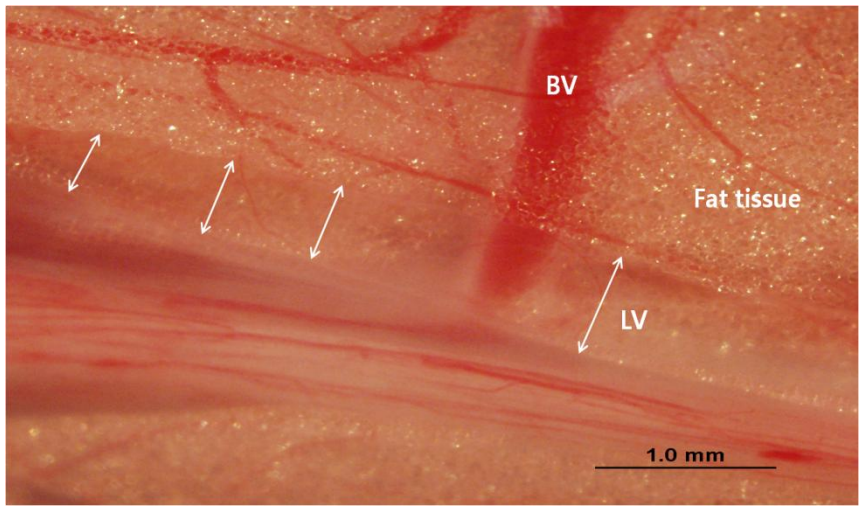

Figure 3.3 Image of a transparent lymph vessel (LV; double arrow) in adipose tissue. BV stands for a blood vessel. Notice that the floating primo vascular system (PVS) in the LV is not observable because of its transparency. Alcian Blue will be injected into this LV. [*J Acupunct Meridian Stud.* 2012 Oct; 5(5):234-40, image taken by Dr. K.H.Bae]

10. If you face difficulties in finding transparent lymphatic vessels or nodes in the surgically- opened abdomen, find the caudal vena cava first and search carefully around the vena cava for a thick lymph vessel. Sometimes, depending on the rabbit's health condition, the vessels are covered with thick adipose tissues and a few layers of membranes. In such a case, try to remove these membranes and tissues carefully to secure a clear view of the lymph vessels. Because many small blood vessels are spread out across the adipose tissues like a net, using a cotton swap to remove these without bleeding is effective.
11. After choosing a lymph vessel beside the caudal vena cava, try without AB staining to detect the PVS candidate floating inside the lymph vessel under a stereo microscope. If you can't find it, decide on an injection point in the chosen lymph vessel and inject a small amount (0.03~0.1 ml) of preloaded 1% AB staining dye into the lymph vessel by using a 31-gauge ultra-fine insulin syringe in 30 seconds. Injecting the dye at a slow

rate until it reaches a satisfactory level of staining is very important; otherwise, it well leak out and stain the surrounding area or damage the vessels. In the case of injecting a larger amount of AB into a lymph vessel you need to search the PVS in the region away from the injection point. Take caution to keep air out of the syringe in the beginning of the injection and insert the needle at an angle of less than 40° relative to the lymph vessel.

12. Right after injecting the AB staining solution into the lymph vessel, you will observe the flow of a blue-colored AB solution upward along the lymph fluid movement inside the lymph vessel. Depending on the vessel you chose, the AB solution will stain many small branches of the lymph vessels that can be found around the injection point. Warm saline is continuously dropped on the lymphatic vessel to avoid dryness.
13. In order to promote the natural circulation of the lymph fluid inside the vessel to wash the staining dye, place the internal organs you moved to the side back into their proper positions to provide the body temperature, close the outermost skin with forceps, and let sit for 15~ 20 minutes.
14. Open the abdomen again and move again all the internal organs to the side and repeat step 9.
15. Observe the injected lymph vessels carefully under a stereomicroscope (STZ10, Olympus) with a CCD camera (DP70, Olympus), detect the PVS stained by AB, and take images. Usually you will see freely-floating, thin, blue PVS lines inside the washed lymph vessel and its various smaller branches. Because lymph vessels are interconnected with each other as a branch, very close observation of the PVS lines at a junction of the lymph vessels is needed (Fig. 3.4). In addition to that, most of the time, you will see stained lymphatic valves along the vessels, and sometimes, you will see very thin primo-vessel lines passing through the valves.

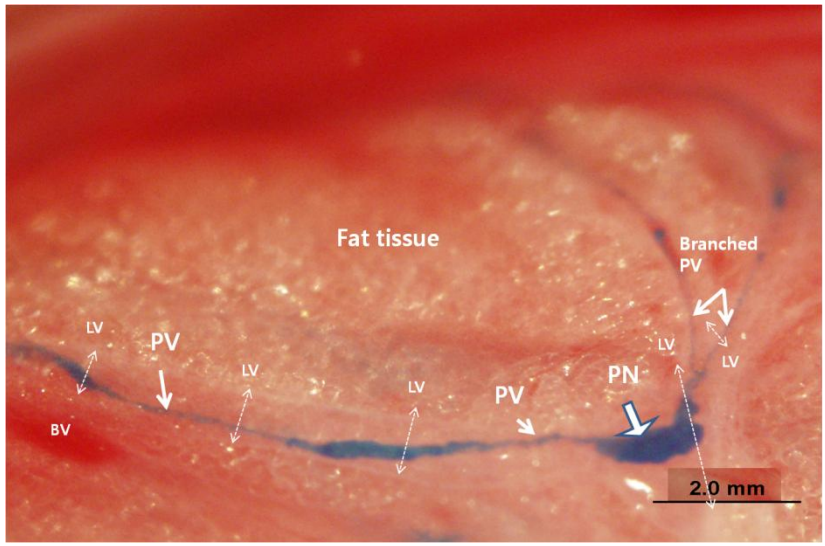

Figure 3.4 Representative stereomicroscopic image of Alcian blue (AB)-stained threadlike primo vessel (PV, thick arrow) and primo node (PN, bold arrow) within the lymph vessel (LV). The AB injected into the lymph vessel was washed out by the natural flow of lymph fluid, and only the stained PV and PN emerged. The hyaluronan-rich primo vessel (thick arrow) is more prominently stained with AB than the surrounding lymphatic vessel (LV, double arrow). Notice that where the LVs branched, the PVs also branched. [*J Acupunct Meridian Stud.* 2012 Oct;5(5):234-40, image taken by Dr. K.H.Bae]

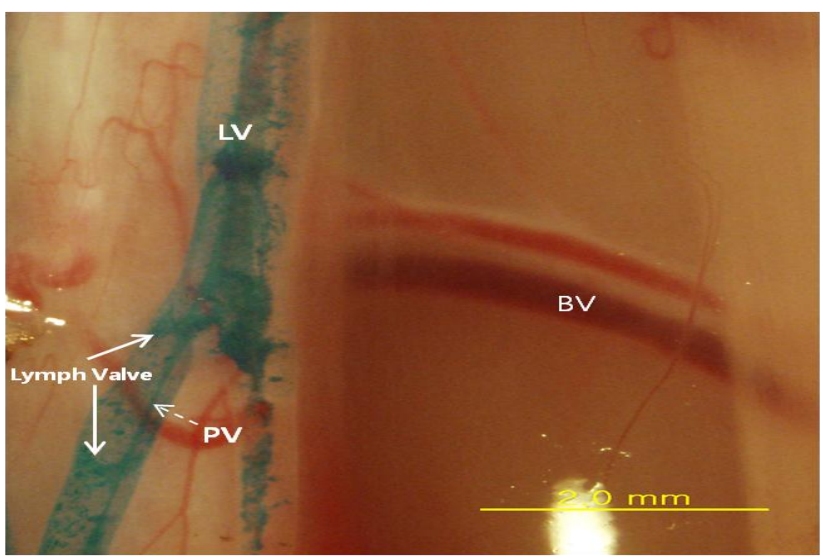

Figure 3.5 This image shows stained lymphatic valves (thick arrows) and a primo vessel (PV, dotted arrow) that passes the valves. BV= blood vessel; LV= Lymph vessel.

16. Sampling: When you detect the PVS lines inside the lymph vessels, you may cut the lymph vessel that contains the PVS with micorscissors and put the specimen on a glass slide (Fig. 3.6). If possible, cautiously incise the lymph vessel along the vessel's wall by using microscissors. Pick up the PVS line with microforceps carefully, and pull it out towards your body gently (Fig. 3.7). This specimen needs to be placed over a glass slide for the next step.

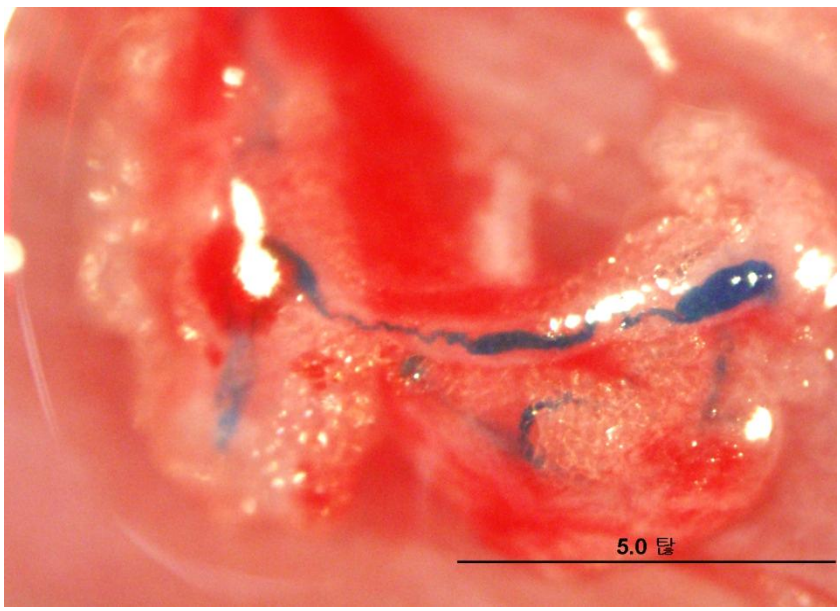

Figure 3.6 Stereomicroscope image showing an isolated specimen of the primo vessel inside the lymphatic vessel. In this picture, the floating primo vessel is shown together with the surrounding lymphatic vessel.
[*J Acupunct Meridian Stud.* 2012 Oct;5(5):234-40, image taken by Dr. K.H.Bae]

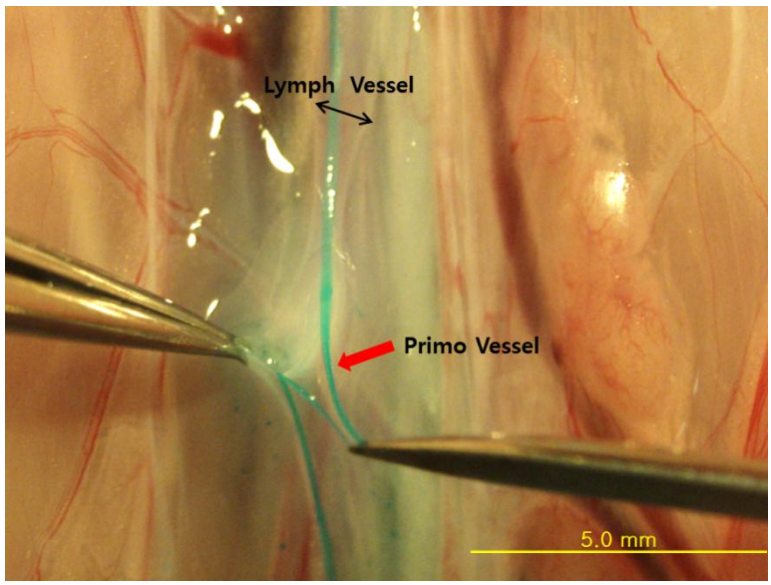

Figure 3.7 A PV (Primo vessel) specimen drawn out from a lymph vessel.
[*J Acupunct Meridian Stud.* 2012 Oct;5(5):234-40, image taken by Dr. K.H.Bae]

17. If much debris is attached in the PVS line, place this sample in a petri dish with a few drops of PBS in it, put the petri dish in an ultrasonic cleaner, and turn it on for 15 minutes to remove the debris from the specimen by liquid agitation.
18. The isolated PVS specimens from the lymph vessels are to be fixed with either 4% PFA solution or 10% NBF solution for a day or two for further analysis and are to be stored at 4℃ in a refrigerator.

3.3.3 PREPARATION OF ISOLATED PVS TISSUE (Timing: 15 min)

19. Wash the fixed samples with 1X PBS solution two or three times. Always pay attention to the branch of the PV and the PN, and as much as possible handle every branch carefully.
20. Put either the fresh or the fixed PV sample (from step 19) on a glass slide with 1-2 drops of PBS 1X solution. In order to characterize the nuclei in the PVS, stain the specimens with Alexa Fluor 488 Phalloidin (Invitrogen, USA) and 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen, USA). Wash the samples in PBS, place them on slides, and under a microscope,

carefully and slightly flatten the tissues for future examination.

3.3.4 PHALLOIDIN AND DAPI STAINING (Timing: 45 min)

21. After washing the fresh samples three times with PBS, stained them with phalloidin for 30 min, followed by a three - times PBS wash. Then, stain them with Prolong Gold Antifade reagent with DAPI for 5 min. When you apply DAPI staining dye to the sample, mix thoroughly, but take care not to create too many bubbles. If the final wash is in a buffer with a pH close to 7, such as PBS, equilibration is not necessary. Drain excess solution from the slide. Apply two separate drops of Gel Mount – DAPI on the sections, and then carefully lower the cover slip on the sections. Let stand in the dark for a few minutes, seal the cover slip with a transparent manicure, and then observe the DAPI stain with UV excitation. Carefully ensure that the sample is not exposed to light during the phalloidin and DAPI staining procedure.

22. Analyze the finished specimens with a fluorescence phase contrast microscope (Olympus BX51, Olympus) to observe the rod-shaped nuclei (Fig. 3.8).

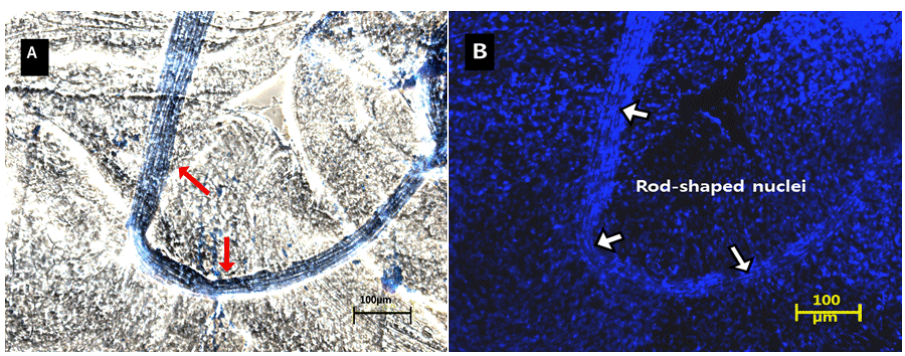

Figure 3.8 Phase-contrast microscope image of an Alcian blue (AB) stained threadlike primo vessel. (A) Under a phase-contrast microscope, the threadlike primo vessel (thin line) is shown along the red arrows within the very thin lymph vessel. (B) Rod-shaped nuclei (thick white arrows) are shown in the primo vessel stained with DAPI. [*J Acupunct Meridian Stud.* 2012 Oct; 5(5):234-40, image taken by Dr. K.H.Bae]

3.3.5 Time distribution during the entire procedure:

Steps 01-05:	**35 min–40 min**
Steps 06-18:	**1-2 hours**
Steps 19-20:	**15 min**
Steps 21-22:	**45 min**

3.4 Anticipated results

The large lymph vessels along the caudal vena cava of a rabbit are the target to search for a PVS floating in the lymph flow. The lymph vessels in the abdominal cavity form a fine network where many thin branches are interconnected. These lymph branches are usually imbedded in adipose tissues (Fig. 9). If we inject AB into a large-caliber lymph vessel, AB reveals the PVS, which is branched and forms a network following the lymph network.

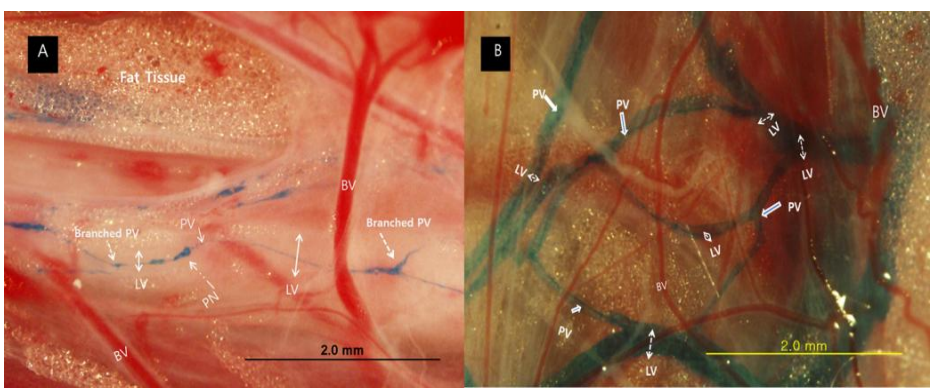

Figure 3.9 (A) Alcian blue dye was injected into the lymph vessel and it flowed through the network of lymph vessel (LV), leaving the stained primo vessel visible. Arrows indicate the stained primo vessel (PV) floating inside the lymph vessel.
(B) The lymph vessels (LV) were connected to one another like a fine web-like network in adipose tissue, as were the PVs inside the lymph vessels. [*J Acupunct Meridian Stud.* 2012 Oct;5(5):234-40, [A] image taken by Dr. K.H..Bae]

The PVS can be identified straightforwardly by observing the distributions of nuclei in the longitudinal sections to distinguish the PVS from blood or lymph vessels. The nuclei of the endothelial cells of the primo vessels are arranged in a fashion of parallel broken lines, and they are rod-shaped, about 10-20 micrometers in length. This feature can be easily checked with DAPI staining. Another characteristic of the primo vessel is the bundle structure which is composed of several subvessels. This bundle structure is a key feature of the primo vessel that uniquely distinguishes it from blood or lymph vessels.

In order to completely identify the PVS in the lymph vessel, the usual histological, immunohistological, and electron microscopic studies should follow. We omitted these processes in this article because they were addressed in an earlier work and in well-established standardized works [6-9]. The aim of the current work is to introduce the detailed process for detecting and identifying the PVS in lymph vessels.

We used AB staining dye to trace the primo vessel system afloat within the lymph flow in the abdominal area of a rabbit. Due to AB's water-soluble nature, AB disappears according to the natural washing process with lymph fluid inside vessels, leaving the stained PVS observable. The method with AB has limitations when we try to remove the primo vessel and the primo nodes which are placed in very thin lymph vessels. The motivation for the current work is to provide information for further research to disclose the complete network of primo vessels and to develop the tools required to overcome the difficulties in performing the delicate work in the present protocol.

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Chapter 4. Method for Observation of the Primo Vascular System in the Thoracic Duct of a Rat

Even though the primo vascular system (PVS) has been observed in large-caliber lymph vessels by several independent teams, the presence of the PVS in the thoracic duct has been reported by only one team, probably because reproducing the experiment is technically difficult. This brief report presents a new, relatively straight forward method, which is a simple modification of the previous method of dye injection into the lumbar node, to observe the PVS in a thoracic duct of a rat by injecting Alcian blue into the renal node. When this new method was applied to a rat, the branching of the thoracic duct and the primo vessel in that duct was clearly displayed. Thus, this new method is expected to extend the network of the PVS from abdominal lymph ducts to thoracic ones.

4.1 Introduction

Observation of the primo vascular system (PVS) in the thoracic lymph ducts of rodents was reported earlier [1], but reproducing that experiment was difficult; thus, no further work by other independent groups has been performed. In this brief report, we present a different method to observe the PVS in the thoracic duct of a rat.

Our method is to inject Alcian blue staining dye into the renal lymph node near the kidney. In fact, dye injection techniques have been used to find the PVS in the lymph vessel between the lumbar and the mesenteric nodes in rabbits [2-5] and rats [6, 7]. In these previous experiments, the dye was injected into the lumbar nodes, and the stained primo vessel was traced only up to the diaphragm because the researchers were trying to observe the PVS under in-vivo conditions. In the current work, which is still in progress, in order to expand the range of PVS observations, we opened the thoracic cavity thirty minutes after injecting the dye into the renal lymph node, and we were able to observe the PVS in the thoracic duct in situ, but not in vivo.

Recently, the primo nodes in the lymph vessels of rats were found to be

enriched with immune cells, such as macrophages, mast cells, and neutrophils .Thus, the PVS might play an important role in the immune mechanism [8]. Therefore, extending the network of the PVS as much as possible to obtain larger numbers of specimens would seem to be an appropriate endeavor. The current brief report is intended to serve that purpose.

4.2 Materials and Methods

4.2.1 Animals

For the laboratory animals, male Sprague-Dawley (SD) rats (n= 7, 9 weeks old) were purchased from DooYeol Biotech (Seoul, Korea). Rats were kept in constant temperature and humidity conditions (23℃, relative humidity: 60%) with a 12/12 light/dark cycle, and were provided water and commercial rat chow ad libitum. The procedures involving the animals and their care were in full compliance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996.)

4.2.2 Surgery and Alcian blue injection

We anesthetized the rats by using an intramuscular injection of a mixed solution of 1.5 g/kg of urethane (or 50 mg/kg of zoletile) and 1 mL of Rompun. With surgical scissors we incised the outermost skin along the linea alba of the abdomen from the navel down to the symphysis pubis and again up to the ensisternum. Then, we cut the straight muscle of the abdomen to expose the internal organs and moved the organs to the side for observation of the target renal lymph node.

The Alcian blue solution was prepared from 0.1 g of Alcian blue (Sigma, St. Louis, MO, USA) dissolved in 10 ml of phosphate-buffered saline (PBS, pH 7.4) and was filtered by using a 0.22-μm syringe filter (Millipore, Bedford, MA, USA) with a 10-mL syringe (BD, Franklin Lakes, NJ, USA). After the rat's abdomen had been incised along the linea alba, the Alcian blue solution was preheated to 37℃ in

a water bath and was prepared for injection into a renal lymph node. After the staining dye had been injected into the left renal node, in order to promote the natural circulation of the lymph fluid inside the vessel for the purpose of washing the staining dye, the internal organs that had been moved to the side were replaced in their original positions, and the abdominal skin was closed with forceps. This step was necessary to raise the body temperature. The rats were sacrificed with an intracardiac injection of 0.7-mL urethane at about 30 minutes after the Alcian blue staining dye injection. The thorax of the rat was opened along the right side of the sternum, and the heart and the lungs were removed to observe the thoracic duct. For clear observation, careful incision and removal were necessary to minimize bleeding.

For in-situ observation of the primo vessel inside the thoracic duct, we used a stereomicroscope (SZX12, Olympus, Tokyo, Japan). We put the rat in 10% neutral buffered formalin solution for one day and then washed it for two hours with tab water. We took the PVS specimen extracted from the thoracic duct, put it on a microscope slide, and examined it with a microscope after staining.

4.2.3 Staining and Microscopy

We applied 4', 6'-diamidino-2-phenylindole (DAPI) and phalloidin reagents for staining of nuclei and f-actins in the cells, respectively. After a 1-hour DAPI (Invitrogen, Prolongⓡ Gold Antifade Reagent with DAPI, St. Louis, MO, USA) staining, we washed the sample three times with PBS solution. The phalloidin (Invitrogen, Rhodamine Phalloidin, St. Louis, MO, USA) staining was done in the same way as the DAPI staining.

The prepared sample was investigated under a phase contrast microscope (Olympus Model #BX51, Tokyo, Japan) in order to observe the distributions of the nuclei and the f-actin of the primo vessels that had been stained with DAPI and phalloidin, respectively. Confocal laser scanning microscopy (CLSM; Nikon, C1 plus, Tokyo, Japan) was used to examine optical sections of the threadlike primo vessel.

4.3. Anticipated results

The thoracic duct is a continuation of the largest-caliber abdominal lymph vessel along the caudal vena cava (Fig. 4.1(A)). The Alcian blue was injected into the renal node, flowed into the thoracic duct, and stained the PVS floating in the thoracic duct as indicated in Figure 4.1(B). A magnified view of the thoracic duct (dotted line) and the primo vessel (blue curve) is given in Figure 4.1(C). The primo vessel was a continuous thread from the thread in the abdominal lymph vessel below the diaphragm. There were several branching and rejoining of the thoracic lymph duct and one of them is shown in Figure 4.1(D). The primo vessel floating inside the thoracic lymph duct also branched and rejoined (blue curves). The thoracic lymph duct before the branching point was stripped, and a blue primo vessel was exposed.

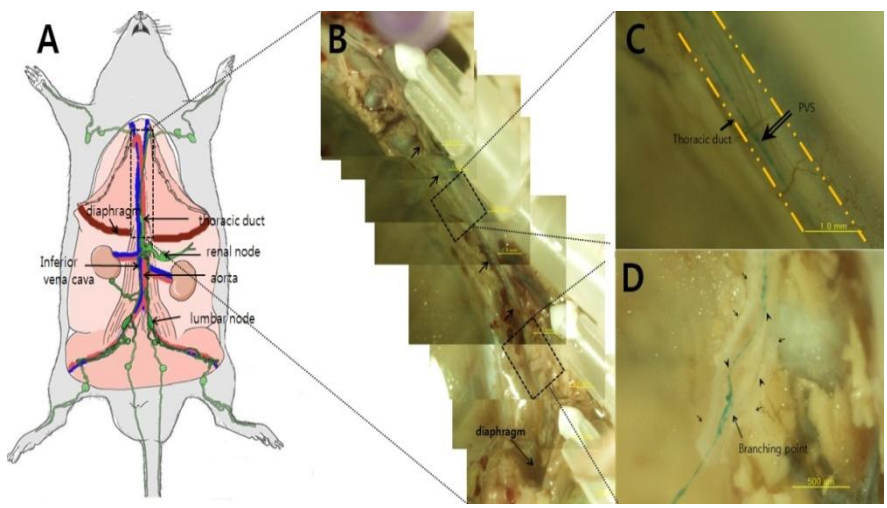

Figure 4.1 Anatomical location of the primo vascular system (PVS) in the thoracic duct of a rat. (A) Schematic anatomical view of the rat. Large-caliber lymph vessels are depicted with green curves, and large arteries and veins with red and blue curves, respectively. (B) Stereomicroscopic image of the thoracic duct indicated with arrows. (C) Magnified view showing the primo vessel (PVS, open arrow) in the thoracic duct (arrow and two dotted lines). (D) Another magnified view showing branched primo vessels (arrowheads) in the thoracic duct (arrows). (E) Stereomicroscopic image of the left renal node indicated with arrows. [*Evidence-Based Complementary and Alternative Medicine*, volume 2013 (2013)]

The branched primo vessel was extracted from the duct and put on a slide, as shown in Figure 4.2 (A). A magnified view of the branch showed only partial Alcian blue staining in Fig. 4.2 (B), and the reason for that partial staining is not yet understood. The branching of the thoracic duct and the primo vessel in it was a common phenomenon, as shown in Figure 4.3, where branching and rejoining is seen to occur twice. The image in panel 4.2 (B) is a magnified view of the image in panel 4.2 (A), which was taken in situ with a stereomicroscope.

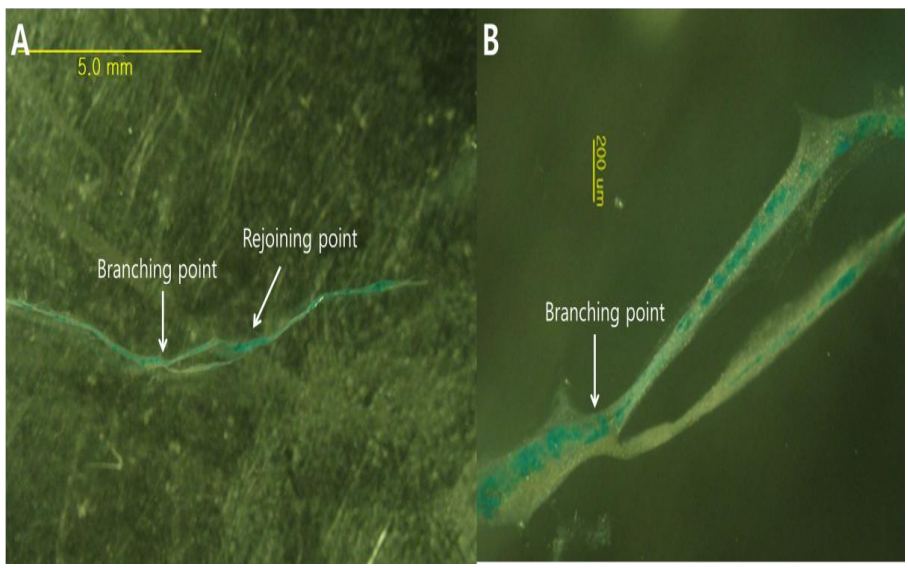

Figure 4.2 Stereomicroscopic images of the branched primo vessels (PVs) of Fig. 4.1(D), which were extracted from the thoracic duct and put on a slide. Panel 4.2 (B) is a magnified view of 4.2 (A).
[*Evidence-Based Complementary and Alternative Medicine* ,volume 2013 (2013)]

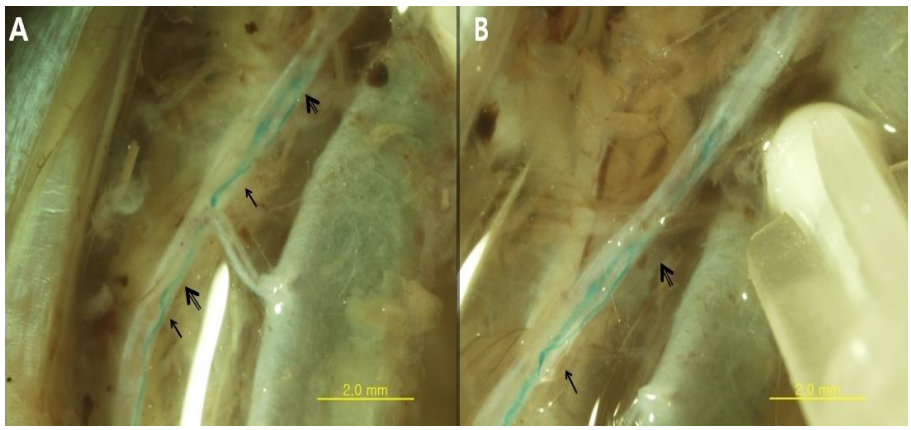

Figure 4.3 Stereomicroscopic in-situ image of a branched and rejoined primo vascular system (blue stained lines) in the thoracic duct of another subject rat. (A) Two regions of branching (arrows)-rejoining (open arrows) are observed, and the (B) is the magnified image of (A).
[*Evidence-Based Complementary and Alternative Medicine* ,volume 2013 (2013)]

In order to confirm that the stained threadlike structure was a primo vessel, we applied the previously-established simple criteria of DAPI staining of nuclei and phalloidin staining of f-actins [9]. As shown in the DAPI image, the alignment of rod-shaped nuclei in parallel with the primo vessel was in good agreement with the criteria in Fig. 4.4(B). Notice that the rod-shaped nuclei were present only inside the region defined by two broken lines. Outside the region, round-shaped nuclei, which are aggregated lymphocytes, were observed. The phase contrast image more clearly showed the aggregated round-shaped lymphocytes scattered around the primo vessel whose boundaries were indicated by the two broken lines shown in Fig. 4.4(A). Also, the distribution of the f-actins in the cytoplasm was in agreement with that for a typical primo vessel and was distinctively different from that for a lymph or a blood vessel (Fig. 4.4(C)); that is, the phalloidin signals were aligned along the vessel. The confocal laser scanning microscope image more clearly showed the rod-shaped nuclei with blue color in Fig. 4.4(D).

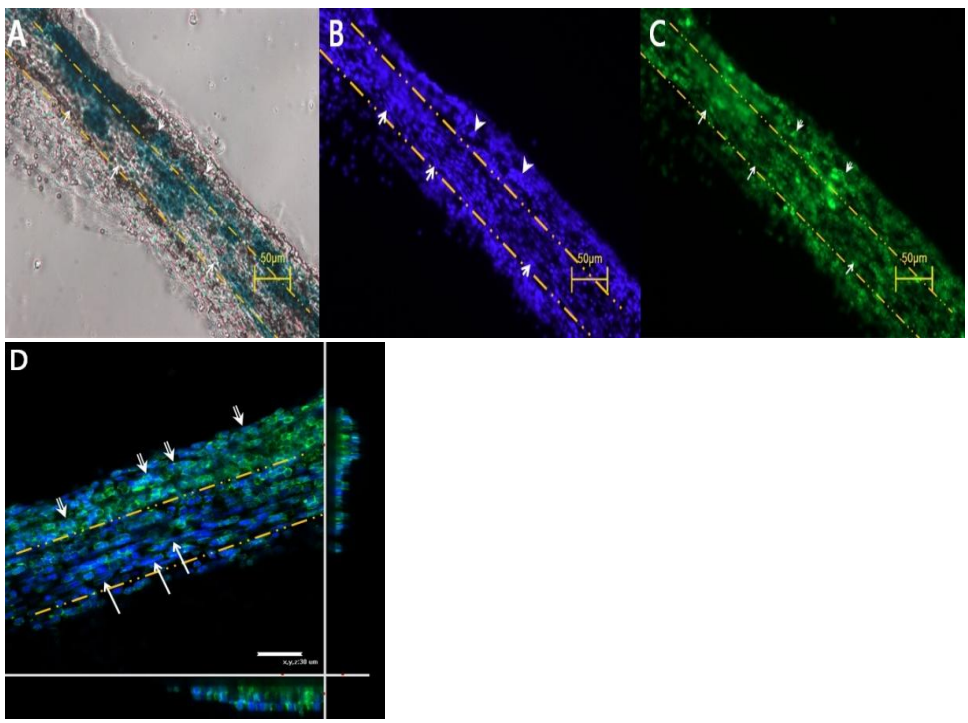

Figure 4.4 Morphological features of a primo vessel (inside the two dotted lines) in a thoracic duct. (A) Phase contrast microscopic image of the primo vessel. (B) Rod-shaped nuclei longitudinally arranged along the primo vessel (inside the yellow-lined region) stained with DAPI. (C) F-actin signals in the cell plasma of the primo vessel stained with phalloidin. (D) Confocal laser scanning microscopic (CLSM) image of the f-actin signals (green) and the nuclei (blue) of the primo vessel. The under and the side panels show cross-sectional views. Rod-shaped nuclei (arrows) are longitudinally arranged along the primo vessel, and lymphocytes (double arrows) are aggregated around the primo vessel.
[*Evidence-Based Complementary and Alternative Medicine* Volume 2013 (2013)]

The lengths of the nuclei were 8.3-14 μm, as expected from Bong-Han Kim's work [10]. Another important morphological datum is the diameter of the primo vessel, and it was, on average, 62 ± 28 μm. The morphological size data for the primo vessels from the thoracic ducts of the subject rats are given in Table 4.1.

Subject	Diameter of Lymph Vessel (mm)	Diameter of Primo Vessel(μm)
1	1.0	58.8
2	0.4	88.2
3	0.9	85.0
4	0.3	73.5
5	1.0	72.1
6	0.5	36.4
7	0.4	18.3
Average± S.D.	0.6±0.3	61.8±28.3

Table 4.1 Morphological size data for the primo vessels from the thoracic ducts of seven male, nine weeks old rats.

4.4 Discussion

In this brief report, we presented a repeatable method for observing the PVS in the thoracic duct of a rat. Even though the thoracic duct is the largest-caliber lymph vessel, the primo vessel found in this duct is not necessarily much thicker than those found in less-large-caliber lymph vessels. The average diameter of the primo vessels in our case was 61.8 ± 28.3 μm while the average value of the primo vessels found in the lymph vessels in abdominal cavities was 52 ± 30 μm (rat) [6]. This uniform size was also in agreement with the sizes of the primo vessels found in blood vessels, and on the surfaces of internal organs [9]. In our case, the diameter was somewhat larger because of the aggregation of lymphocytes around the primo vessel, as seen in Figure 4.4. A future task is to remove these aggregated lymphocytes to obtain pure specimens.

This work is the first report on a primo vessel seen in the whole thoracic duct in the longitudinal direction, although a cross sectional image was presented earlier

[1]. In addition, in this work, the branching and the rejoining of the thoracic duct and its associated primo vessel, as mentioned in Bong-Han Kim's work [10], were first demonstrated.

The primo vessel specimen taken from the thoracic duct showed the characteristic hall-marks of a primo vessel: namely, the DAPI images of the shapes and the distribution of nuclei and the phalloidin images of f-actins. We did not address further histological analysis in this brief report partially because the results for the basic H&E-stained specimen has already been reported [1] and partially because an immunohistochemical examination of its extended part in the abdominal lymph vessels was thoroughly done in another work [8]. Another reason was that our main purpose was to develop a repeatable method that could be reproduced by other independent groups. We were able to demonstrate that the method of injecting Alcian blue into a lumbar node could be modified to inject it into a renal node to detect a primo vessel in the thoracic duct.

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Chapter 5. Observation of Primo Vascular System Floating in Lymph Ducts of Rats

An epoch-making development in gross anatomy of lymph system has emerged: the primo vascular system (PVS) which is a threadlike structure floating in lymph vessels like a thoracic duct. The PVS formed a complex network distributed throughout an animal body which was first proposed by Bong-Han Kim as the conduit for the acupuncture Qi. The lymph-PVS is a sub-system of the PVS of which observation is one of the most convincing demonstrations of the PVS. It was difficult to notice its existence even with a microscope due to its transparency, but now it can be made visible with the aid of suitable staining dyes. The lymph-PVS was observed in rabbits, rats, and mice by several independent teams. Since the involved techniques are rather complicated we provide a detailed protocol for surgery, injection of the staining dye, and detection, extraction and identification of the PVS in a rat.

5.1. Introduction

The discovery of the specific marker LYVE-1 [1] was a culmination in a series of investigations on the lymphatic endothelial cells triggered by finding of the vascular endothelial growth factors [2, 3]. The lymphatic system played for a long time only obscure roles as the second circulation to the blood system. But its medical importance became prominent as a key player in cancer metastasis [4] and a major culprit in inflammation due to the protein, podoplanin and its signaling molecule CCL21 [5].

In this revolutionary period another astounding development in lymph research appeared. It was the discovery of a floating threadlike structure in lymph fluid whose existence was not noticed due to its optical transparency. In fact it is a sub-system of the primo vascular system (PVS) which was found by Bong-Han Kim in the early 1960's [6] as the third circulatory system, an anatomical structure corresponding to the acupuncture meridians, the classical Qi flowing path, but had been ignored for a long time until the rediscovery by the Seoul National University team [7] in 2002. Since then there have been increasing number of reports on the

PVS which is comprised of the threadlike structure called primo vessel (PV), its thickened corpuscle-like parts named primo node (PN), and fluid flowing in it. The PVS has been observed in various organs: In blood stream [8, 9], in hearts [10], in brain [11], and on the surfaces of internal organs like stomach, intestines, bladder, and liver [12, 13]. The animals studied were mostly mice, rats and rabbits, and rarely pigs, dogs, and cows. The PVS was also found in adipose tissues [14] which are sources of pluripotent stem cells [15]

The presence of PVS was impressively demonstrated as a floating threadlike structure in the lymphatic flow with the aid of staining dyes. It was observed in the large caliber lymph ducts near the caudal vena cava of a rabbit with the staining dye Janus green B [16], and of a rat with the fluorescent nanoparticles [17], and with Alcian blue [18]. The PVS in a thoracic duct of a mouse was observed without use of a staining dye [19], other similar works on the PVS in lymphatic flow were subsequently reported [20-23].

One of the important investigations on the medical significance of the PVS was about the abundance of immune cells in PNs like mast cells (20%), eosinophils (16%), neutrophils (5%) and histiocytes (53%) with a few lymphocytes (1%). There were also round immature cells, which were possibly stem cells (3%). These stem-like cells showed some positive signatures for hematopoietic stem cells [23], and embryonic-like stem cells [24]. This is in agreement with the immune function augmentation by acupuncture [25]. Another medical significance was possible implication on cancer metastasis through the PVS that were developed on the surface of tumors [26-28]. The flowing fluids carried hyaluronic acids [6] and hormones like adrenaline and noradrenaline [29]. The flow speed was measured to be 0.3 ± 0.1 mm/sec [30].

There have been descriptions on the methods used in PVS experiments [16-23], but it is not easy, without detailed protocols, to obtain the PVS specimen for molecular-level investigations to find vascular endothelial growth factors and specific markers for the PVS, for instance. Here, we provide a detailed protocol for surgery, injection, observation, isolation, identification, and histological analysis of the PVS in lymph ducts of a rat so that independent researchers can reproduce the experiments.

5.2 Materials

5.2.1 REAGENTS

Experimental animals: Rats (Sprague–Dawley, male, 9-weeks old, 280 ~300 g) were purchased from DooYeol Laboratory Animal Company (Seoul, Korea). The animals were housed in a constant temperature-controlled environment (23 ℃) with 60% relative humidity. All animals were exposed to a 12 hour light-dark cycle and had *ad- libitum* access to food and water. Procedures involving animals and their care conformed to institutional guidelines (approval number: WJIACUC 20130212-1-07), which were in full compliance with current laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996).

Anesthesia: Zolatil (Virbac Laboratories, Carros, France), Xylazine (Bayer, Korea).

General use: Phosphate- buffered saline solution (PBS). 0.9% saline solution (Choongwae Pharmaceuticals, Korea).

Histology: Tissue-Tek OCT freezing compound (Sakura Finetek, Japan), base molds (15×15× 5 mm, Fisher Scientific, USA), gel/mount medium (Biomeda Corp, USA, no. M01).
AB staining: Alcian blue (AB) 8GX (Sigma, USA), PBS pH 7.2 (1X) (Life Technology Corp, USA). DAPI staining: 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen Molecular Probes, cat. no. D1306; 1:10,000).
Phalloidin staining: Alexa Fluor 488 Phalloidin (Invitrogen, USA).
DiI stainig : 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Sigma, USA).
Hematoxylin and Eosin (H&E): Hematoxylin (Sigma, USA), ethanol (Ducksan Chemicals Co. Ltd, Korea), eosin (Sigma, USA), xylene (Ducksan Chemicals Co, Ltd, Korea).
Gordon & Sweet’s silver staining: $NH_4Fe(SO_4)_2$ (Sigma, USA), formaldehyde

solution (Duksan Chemicals Co. Ltd, Korea), $AuCl_4Na$ (Invitrogen, USA), $KMnO_4$ (Fluka Chemical Corp,Switzerland), $AgNO_3$ (Kojima Chemicals Co, Ltd, Japan), NaOH (Kanto Chemical Co, Inc, Japan), sodium thiosulphate (Sigma, USA, Cas 7772-98-7), H_2SO_4 (Daejung Chemicals & metals Co, Ltd, Korea), neutral red (CI 50040) (Sigma-Aldrich Co, LLC, USA**).**

Immunohistochemistry: EMP-3: Acetone (Ducksan Chemicals Co. Ltd, Korea), CAS block (Invitrogen, USA, cat. no. 008120). Antibodies: (EMP-3), Primary: Rabbit anti-EMP3 (Santa Cruz Biotechnology, USA , cat. no. sc-135143), Secondary: Goat anti-mouse AlexFluor® 568 (Invitrogen Molecular Probes, USA, cat. no. A11034).
vWF: Bovine serum albumin (BSA) (Biotechnology, Canada), triton x-100 (Shino Pure Chemicals Co, Ltd, Japan). Antibodies: (vWF), Primary: mouse anti-vWF (Santa Cruz Biotechnology, USA, cat. no. sc-365712), Secondary: goat anti-rabbit AlexFluor® 488 (Invitrogen Molecular Probes, USA, cat. no. A11037).

5.2.2 EQUIPMENT

Microscopes and light source: Stereomicroscope (SZX12, Olympus) with a CCD camera (DP70, Olympus, Japan), phase contrast microscope (BX51, Olympus, Japan) with a CCD camera (Infinity 3, Lumenera), confocal laser scanning microscope (CQ plus, Nikon, Japan).
Light source and optical fiber illuminator (Halogen lamp, KLS-100H-LS-150P, Kwangwoo Co, Ltd, Korea).

Surgical instruments: Surgical instruments and ophthalmic surgical instruments (Tumed, German). Electric surgical unit (Surgitor, Korea), electrocautery (Umeco, Korea). Disposable Gentax latex glove (Geneall Biotechnology, Korea), Pet Specialty cordless trimmer (Oster, USA), masking tape (Scitech Korea Inc, Korea), gauze (Scitech Korea Inc, Korea), surgical drapes (Scitech Korea Inc. Korea), electric heating pad (size: 30 mm x 30 cm; Woojin Tech, Korea).

Syringes and filters: Hypodermic syringe (Kovax- Syringe, Korea), BD ultra-fine insulin syringe, 31G (Becton, Dickinson and Company, USA), BD 5- ml filter syringe (Becton Dickinson Medicals Ltd, Singapore), BD 10- ml filter syringe (Becton Dickinson Medicals Ltd, Singapore). Glass microfibre filters 110 mm (GE Healthcare Co., UK, cat. no. 1820-110), minisart syringe filter, hydrophilic (Sartoriou Stedim Biotech, Germany).

Staining and histology instruments: pH meter (Thermo Electron Corporation, USA), Glass funnel (Dongsung Science, Korea), round bottom test tube, 5 ml (BD Falcon, USA), coplin jar (Fischer Scientific, USA), pap pen (Invitrogen, USA), Vortex-2 Genie (Scientific Industries, USA). 5 –to-10- µl finnpipette (Lab Systems, Korea), disposable transfer pipette (Lappia, Korea). Micro slides (silane coating; size: 76 mm x 26 mm; Mutopure Chemicals Co, Ltd, Japan), 100 deckglaser cover slips (size : 24 mm x 50 mm; Knittel Glass, Germany), Leica CM1800 cryostat (Leica, Germany).

5.3 REAGENT SETUP

5.3.1. Animals

Spague Dawly(SD) rats (260~300 g, 9 weeks old) were used. Males are preferred as they develop less abdominal fat, making the surgery easier.

5.3.2. PBS

Eight (8) g of NaCl, 0.2 g of KCl 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 and 800 ml of distilled water are mixed together to make a solution, and using a pH meter, the pH is set to 7.4. An additional 200 ml of distilled water is added to the previously mixed solution to make 1 L (1000 ml) of 1x PBS solution, which is stored at room temperature.

5.3.3. Alcian blue staining dye (1.0 %)

Combine 0.01 g of alcian blue powder with 1 ml of 1x PBS solution to make 1% Alcian blue staining dye. After mixing these, apply the vortex machine and put this solution in the heating cabinet (~ 61˚C) for about 30 minutes to dissolve the Alcian blue powder completely. Filter this solution with a filter paper in a funnel first, followed by a 2nd filtration using a 0.20-um syringe filter attached in a 10-ml syringe. After the 2nd filtration, load the Alcian blue solution in a 1-ml insulin syringe (31 Gauge) while using a warm bath to keep it at a constant temperature of 38~40˚C prior to injection into the lymphatic system.

5.3.4. DAPI

To make a 5 mg/mL DAPI stock solution (14.3 mM for the dihydrochloride or 10.9 mM for the dilactate), dissolve the contents of one vial (10 mg) in 2 mL of deionized water (dH2O) or dimethylformamide (DMF). The less water-soluble DAPI dihydro-chloride may take some time to completely dissolve in water and sonication may be necessary. 1:15,000 dilution in water of a 5 mg/ml stock is a good dilution for DNA staining.

CAUTION! DAPI is a known mutagen and should be handled with care. The dye must be disposed of safely and in accordance with applicable local regulations.

Note: Neither of these DAPI derivatives is very soluble in phosphate-buffered saline (PBS). For long-term storage the stock solution can be aliquoted and stored at ≤–20°C. For short term storage the solution can be kept at 2–6°C, protected from light. When handled properly, DAPI solutions are stable for at least six months.

5.3.5. Phalloidin

Combine 10 ul of 6.6-uM Alexa Fluor 488 phalloidin and 300 ul of PBS. Use the sonicator to mix these well and store the solution at -20°C. Avoid light.

5.3.6. DiI (membrane staining solution)

1) Prepare DMSO or EtOH stock solutions: The stock solutions should be prepared in DMSO or EtOH at 1-5 mM.

CRITICAL! The unused portion of the stock solution should be stored at -20 oC. Avoid repeated freeze cycles.

2) Prepare working solutions: Dilute the stock solutions (from Step 1)) into a suitable buffer such as serum-free culture medium, HBSS or PBS to make 1 to 5 M working solutions.

CRITICAL! The final concentration of the working solution should be empirically determined for different cell types and/or experimental conditions. It is recommended to test at the concentrations that are at least over a tenfold range.

5.3.7. Harris' H&E

Acid Alcohol Solution (1%):

Mix Well 1ml of Hydrochloric acid and 50ml of 70% ethanol.

Ammonia Water Solution (0.2%):

Mix well 2ml of Ammonium hydroxide (Concentrated) and 1000ml distilled water.

Eosin-Phloxine B Solution:

Prepare the stock solutions first, and then create the working solution as needed.

Eosin Stock Solution:

Mix to dissolve 1 g Eosin Y and 100 ml distilled water.

Phloxine Stock Solution:

Mix to dissolve 1 g Phloxine B and 100ml distilled water.

Eosin-Phloxine B Working Solution:

Mix well 100 ml Eosin Stock solution, 10 ml Phloxine stock solution, 780 ml 95% ethanol and 4 ml Glacial acetic acid

Hematoxylin Solution (Harris):

Mix well 100 g of Potassium or ammonium(alum) and 1000ml distilled water.

Heat to dissolve. Add 50 ml of 10% alcoholic hematoxylin solution and heat to boil for 1 minute. Remove from heat and slowly add 2.5 g of mercuric oxide (red).

Steps:

1. Heat to the solution and until it becomes dark purple color.
2. Cool the solution in cold water bath and add 20 ml of glacial acetic acid (concentrated).
3. Filter.

5.3.8. Golden & Sweet's silver staining

Acidified Potassium Permanganate XE "Potassium permanganate:acidified.

0.5% Potassium Permanganate ---------- 9.5ml

3% Sulphuric Acid ---------- 0.048ml

Make fresh, discard after use.

CAUTION! Corrosive, avoid contact and inhalation

Silver solution:

To 5 ml of 10% aqueous silver nitrate, add strong ammonia drop by drop until theprecipitate which first forms is just dissolved. Add 5 ml of 3% sodium hydroxide then add strong ammonia drop by drop until the resulting precipitate is almost completely dissolved. Make up to 50 ml with distilled water and pour into a clean coplin jar.

CRITICAL STEP!

If too much ammonia is added there is a great loss of sensitivity.

CAUTION! Corrosive, possible carcinogen.

2% aqueous oxalic acid:

Oxalic acid (Analytical) ---------- 0.2 g

Distilled water ---------- 10 ml

Mix well, label with initial and date. Solution is stable for 1 year.

CAUTION! Corrosive, avoid contact and inhalation.

4% aqueous iron alum:

Iron alum (Ferric Ammonium Sulphate) ---0.4 g

Distilled water ---------- 10 ml

Mix well, label with date and initial. Solution is stable for 6 months.

10% Formalin:

Formaldehyde (Ajax, 37 – 40g/L) ---------- 1 ml

Distilled water ---------- 9 ml

Make fresh, discard after use.

CAUTION! Carcinogen.

0.2% gold chloride:

Gold chloride (Sodium tetrachloroaurate, BDH) ---- 0.2 g

Distilled water ---------- 100 ml

Rinse the entire contents of bottle of gold chloride (sodium chloroaurate) into an acid clean glass bottle. Store in the refrigerator. Stable for 1 year.

CAUTION! Avoid contact and inhalation**.**

2% Sodium thiosulphate:

Sodium thiosulphate ---------- 0.2 g

Distilled water ---------- 10 ml

Store in the refrigerator, stable for 3 months.

Neutral Red Stain – acidified:

Neutral red (CI 50040) ---------- 1g

Distilled water ---------- 100 ml

glacetic acid ---------- 1 ml

Dissolve the dye in the distilled water. Add the acid. Mix well. Filter into the reagent bottle. Keep well. Stable for 1 year.

CAUTION! Irritant, avoid contact and inhalation

5.3.9 EMP-3

Primary antibody: Dilute the primary antibody in freshly prepared CAS blocking solution to reach 1:200 ratio. Mix well 1 ul primary antibody to 200ul in blocking solution. A 0.5-ml tube requires 200 µl of diluted antibody. Diluted primary antibodies can be reused up to three times. Some antibodies may last even longer. Store antibodies between uses at 4 ℃ for no more than 1 month.

CRITICAL! As little as 200 µl of diluted antibody can be used in a 0.5-ml tube when quantities of antibodies are very limited.

Secondary antibody Dilute the secondary antibody in freshly prepared 1x PBS solution down to 1:500. Mix well 0.4ul secondary antibody to 500ul in 1XPBS solution. A 0.5-ml tube requires 200 µl of diluted antibody. Prepare fresh and discard secondary antibodies after use.

CRITICAL! As little as 200 µl of diluted antibody can be used in a 0.5-ml tube when quantities of antibodies are very limited.

5.3.10 vWF.

Primary antibody: Dilute the primary antibody in freshly prepared 1% BSA blocking solution to reach 1:50 ratio. Mix well 4 ul primary antibody to 200ul in blocking solution. Diluted primary antibodies can be reused up to two times. Store antibodies between uses at 4 ℃ for no more than 1 month.

Secondary antibody: Dilute the secondary antibody in freshly prepared 1x PBS solution down to 1:100. Mix well 2ul secondary antibody to 200ul in 1XPBS solution. A 0.5-ml tube requires 200 µl of diluted antibody. Prepare fresh and discard secondary antibodies after use.

5.3.11 IHC blocking solution

Prepare 1% (wt/vol) BSA, 0.5% (vol/vol) Triton X-100 in PBS. Prepare fresh each time of use.

Troubleshooting:

1. Use acid clean glassware, or rinse 5x with distilled water.
2. When making working silver solution, if over 30 drops of ammonium hydroxide are used to turn the solution, then the ammonium hydroxide is too old. Start over with fresh ammonium hydroxide.
3. When adding the 3 % sodium hydroxide solution to the silver solution it should turn black, if not make fresh sodium hydroxide.
4. Because of the alkalinity of the solution, it may cause some tissues to fall off the slides, celloidinize.
5. Change distilled water after every slide.

5.4. Equipment Setup

Dissecting instruments: Two large scissors, small micro scissor, two large forceps, two micro dissecting tweezers, small forceps, one pair of fine straight forceps, one pair of curved forceps, one micro dissecting straight forcep, one pair of angular micro dissecting forceps and one 31G insulin syringe.

CRITICAL!

1) **All** instruments and other equipment used to perform surgery **must be sterilized** prior to use.

2) In addition to sterile microdissecting instruments, two sets of sterile instruments should be used. Separate sets are used for the skin and for the peritoneal wall and for dissection and extraction of the primo system in the lymph vessels to reduce the chances of contamination as you proceed through the tissue layers.

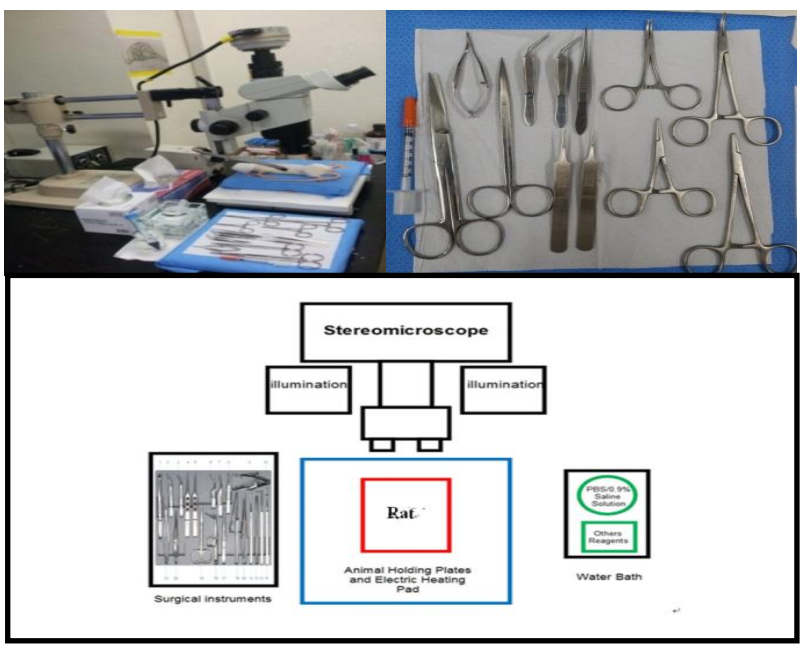

Figure 5.1 Equipment setup.

5.5. Procedures

- Steps 01 - 04 Preparation of animal prior to *in vivo* operation.
- Steps 07 - 16 In vivo operation for detecting the PVS network.
- Steps 17 - 19 PVS Tissue harvest and fixation.
- Steps 20 - 27 Analysis and confirmation of the PVS

Figure 5.2 An overview of the entire procedure.

5.5.1 ANIMAL PREPARATION (Time: 35-45 min)

1. Rats were fasted for 48 hours to reduce fats in order to minimize bleeding during the surgery and to obtain a clear view of lymphatic system in thick fat tissues which contain many blood capillaries and cover the abdominal organs. Avoidance of bleeding during the surgery and precise injection of the staining dye are critical factors for which lean rats are needed.
2. Anesthetize the rat with an intramuscular injection of a mixture of zolatil (0.3 ml) and xylazine (0.1 ml) by using a 1-ml hypodermic syringe. Anesthesia takes approximately 15 minutes. For the checking the level of anesthetization, apply strong stimulation, such as pressing or twitching, to the rat's ear and feet. Wait until rat is fully anesthetized.
3. Shave all the hairs in the abdominal area by using the Pet Specialty cordless trimmer. Fix the rat with its head away from the operator by taping its feet to the operating table. Cover the rat's eyes with gauze to prevent the exposure from light (Fig 5.3).

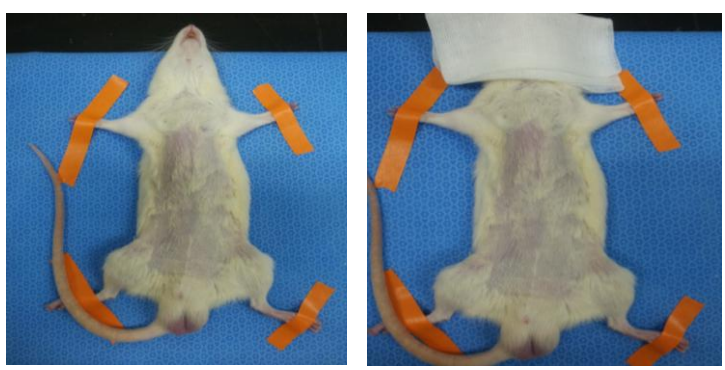

Figure 5.3 A process of animal preparation.

4. Position the fiber optic illuminators for optimum illumination. Stereomicroscope and monitoring system are to be adjusted for good observation.

5.5.2 SURGICAL PROCEDURES AND OBSERVATION (Time: 3 ~5 hour)

Surgical Procedure Set-up Notes:

- Prior to the surgery sterilize surgical supplies and instruments.
- Supplies used for the sterile surgical procedure for each rat include one drape to be layed out as a sterile surface for the placement of instruments, another drape with a pre-cut hole about 1.5 cm x 3 cm in the center to be placed over the rat. The pre-cut hole on the drape will align with the incision made on the rat.
- Instruments needed for the surgery include one standard pair of scissors to be used to cut suture material, two forceps and a fine needle holder for handling and gripping of the suture, and a scalpel with a blade.
- Preheat pH 7.4 PBS and AB staining solution loaded in a syringe to 39 ℃.

5.5.2.1 Laparectomy:

CAUTION!

Careful dissecting techniques are needed not to damage blood vessels and other fine structures..

5. Position the anesthetized rat on its back on a heating pad to maintain body temperature. Aseptic techniques are used during the surgical procedure. Wear sterile gloves and do not allow instruments and supplies to contact non-sterile surfaces other than the rat tissues. In order 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 line) of the abdomen down to the symphysis pubis and up to the ensisternum with surgical scissors. In this step, the depth of incision should be very shallow, just to see the muscular layer.

7. Hold the abdominal muscle upward and make a small incision along the linea alba in the straight muscle of the abdomen at the place of the lower 1/3 on the line from the ensisternum (syn: xiphoid process) to the symphysis pubis by using a small scissors. Insert the scissors into the incision slit and cut abdominal muscle along the linea alba to the xiphoid process. Be careful to avoid bleeding during this cutting. The incision must be on or around the linea alba because the distribution of blood vessels is the least in this area. Do not insert the scissors too deeply into the peritoneal cavity. Keep the tip of your scissors pointed upwards in order not to damage the underlying structures.

5.5.2.2 Locating lumbar nodes:

8. Gently move the intestines to the animal's right side in order to expose the area of the caudal vena cava and cover them on gauze soaked in saline. Continuously spray warm saline solution over the top of the gauze to avoid dryness.
9. Use the stereo microscope for the identification of the lumbar lymph nodes. Expose a node by careful removal of adipose tissues with curved forceps. Then, grasp the node and tease away the surrounding connective tissues with a cotton bud for AB injection into the node. Use a disposable transfer pipette to continuously add warm saline solution drop by drop on top of the lymphatic vessel to avoid dryness during the observation.

CRITICAL STEP!

a) If you face difficulties in finding transparent lymphatic vessels or lumbar lymph nodes in the surgically-opened abdomen, find the caudal vena cava first and search carefully around this region for lumbar lymph nodes. Sometimes, depending on the rats, the vessels are covered with thick adipose tissues and few layers of membranes. In such a case, remove these membranes and adipose tissues carefully to secure a clear view of the lymph vessels and nodes. Because many small blood vessels spread out across the adipose tissues like a net, using a cotton swap is

recommendable for their removal.

b) Use curved forceps and cotton bud in order to expose the chain of lymph nodes located along the inferior vena cava. Usually a lumbar lymph node on each side is composed of two or more small nodes. For injection convenience and accuracy choose the largest node.

c) Damaging large blood vessels must be avoided. If necessary, maintain hemostasis by pressing them with a cotton bud. However, in case of massive bleeding, use electro-cautery to stop bleeding.

5.5.2.3 Injection of AB and visualization of the PVS in lymph ducts

10. Inject a small amount (0.1~0.2 ml on each side) of preloaded 1 % AB solution, preheated to 37 ℃ in a warm bath, into the lumbar node both sides by using a 31- gauge ultra-fine insulin syringe. Injecting the dye at a slow rate is essential because otherwise, it will leak out and stain the surrounding area. After injection the original transparent lymph ducts in Fig. 5.4 (a) become blue colored due to the AB flow upward along the lymph fluid in Fig. 5.4 (b). The AB will stain many small branches of the lymph ducts as well. Drop warm saline occasionally on the lymphatic ducts to avoid dryness.

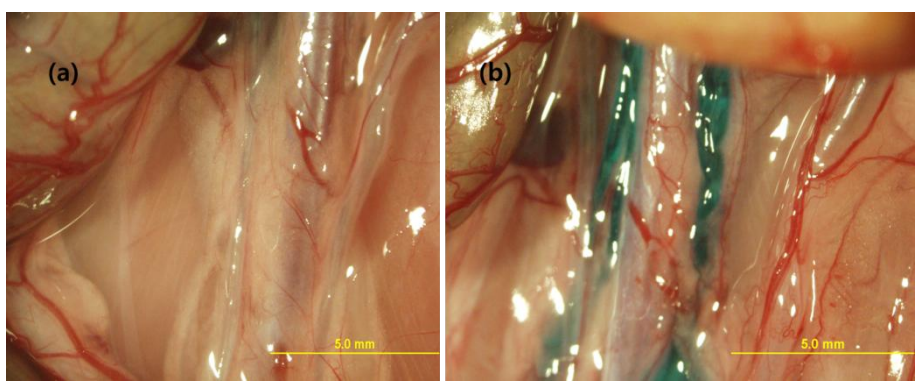

Figure 5.4 Pictures showing the distribution of lymphatic vessels in peritoneal cavity. (a) an image of before AB staining dye injection at lumbar nodes (b) an image of right after AB staining dye injection at lumbar nodes.

CAUTION!

1) Keep air out of the syringe in the beginning of the injection and insert the needle at an angle of less than 40 ° relative to the lymph nodes. Wait several minutes after inserting the needle into the node until the node becomes stabilized.
2) If blood vessels in the lumbar node are damaged during injection of AB bleeding occurs and the blood coagulates with the PVS. It is a cause of the thickening of primo vessels with adhered blood cells.

11. In order to promote the circulation of the lymph fluid for washing the staining dye thoroughly, place the internal organs you moved to the side back to their original positions, and close the outermost skin with forceps to provide the body temperature. The effective way to keep the body temperature is to cover the rat's body with tissue paper 3~4 times. Let it sit for 60~120 minutes for sufficient washing. Put the anesthetized rat on its back on a heating pad. Applying direct heat to the rat by using infra red light is better to avoid. Due to the natural flow the AB in the lymph ducts are mostly washed and the lymph ducts become clear again, Fig. 5.5 (c). However, the PVS is stained and emerges as a floating blue threadlike structure in the transparent flow of lymph ducts after washing. This is the essence of visualizing technique of the current protocol.

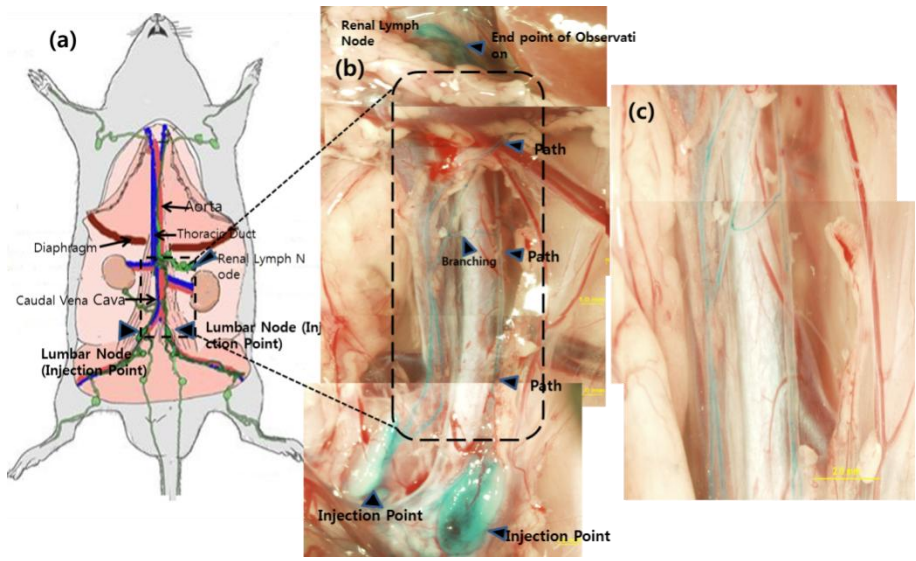

Figure 5.5 A diagram showing whole procedure. (a) a picture showing where the AB injection points (b) a whole picture of after AB solution injected. (c) a magnified view of square box of Fig. 5.4 (b) showing the PVs and how these vessels are branched.

5.5.2.4 Observation of PVS in abdominal and thoracic lymph ducts

12. Open the abdomen and move again the internal organs to the side and repeat step 11.
13. **PVS in abdomen**: For better detection of the PVS system within lymph vessels, remove the fat tissue to secure a clear view of the lymph vessels. Take a close look with microscope at the PVS and trace it up to the cisterna chyli near the diaphragm. Because lymph vessels are interconnected (Fig 5.6) with each other, very close observation of the PVs at a junction of the lymph vessels is needed. You will often see a primo vessel passing through lymph valves shown in Fig. 5. 7 (a, b).

CRITICAL STEP!

a) The difficult in tracing the PVS system between renal lymph node area and cisterna chyli arises due to tangled blood vessels. There are also many complicated structure of membranes, fats and blood vessels. A careful and fine handling is necessary for finding a network of PVS without bleeding.

b) Even though large lymphatic ducts are situated at designated locations, many small lymphatic vessels are distributed irregularly. Considering this point search as many of them as possible in order not to miss the PVS in them.

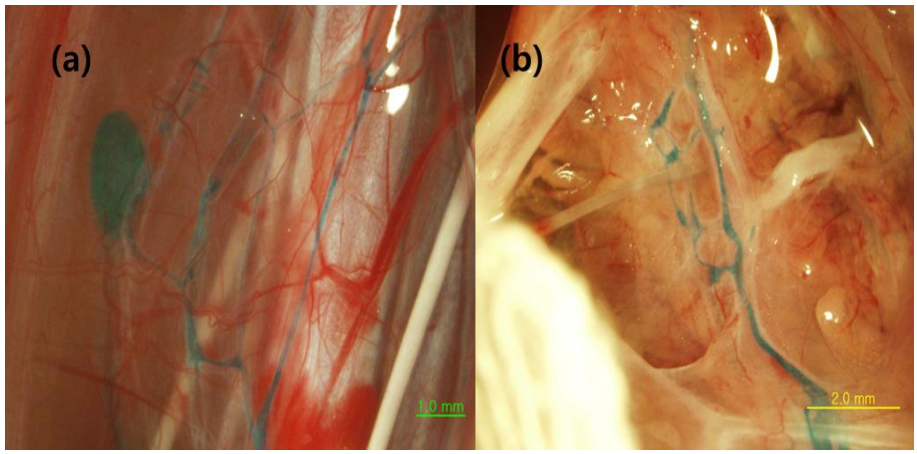

Figure 5.6 A network of lymph ducts and primo vessels within lymph ducts.

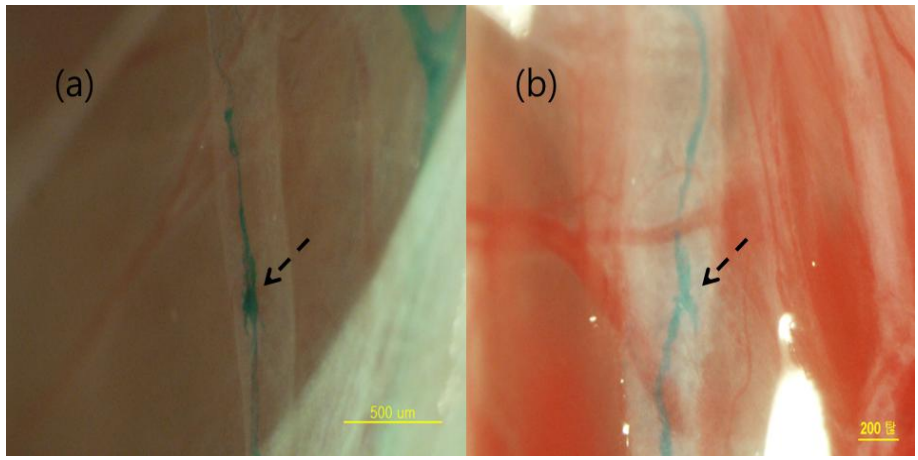

Figure 5.7 Images of PV passing through a lymph valve.

14. Euthanize the rat by intra cardiac injection of 0.7 ml (3.2 g/ kg) urethane for further detection of PVS network in the thoracic cavity. Check the rat's vital signs to confirm the euthanasia.
15. Open the diaphragm and observe carefully the lymphatic system

connection between thorax and abdomen. For the purpose of thoracic duct exposure, cut the rib cage without damaging the organs underneath and pin the ribs outwardly opened. The superior vena cava and thoracic duct can be located by lifting the heart and carefully separating the lobes of the lung. The thoracic duct is a thin transparent tube just beside and the right hand side of the superior vena cava.

16. **PVS in thoracic duct**: Observe the thoracic duct carefully, search for the threadlike structure stained by AB. You will see freely-floating, thin, blue PVs inside the thoracic duct.

5.6 PVS Tissue harvest and fixation (Time: 1 hour)

17. **Sampling:** the PVS from the lymph vessel: When you detect the PVS inside the abdominal or thoracic lymph ducts, you may cut the lymph vessel that contains the PVS with micro-scissors and put the specimen on a glass slide (Fig. 5.8(a)). Incise the lymph vessel along by using micro-scissors and pick up the PVS with micro-forceps carefully, and pull it out towards your body gently. This specimen needs to be placed over a glass slide for the next step in Fig. 5. 8(b).

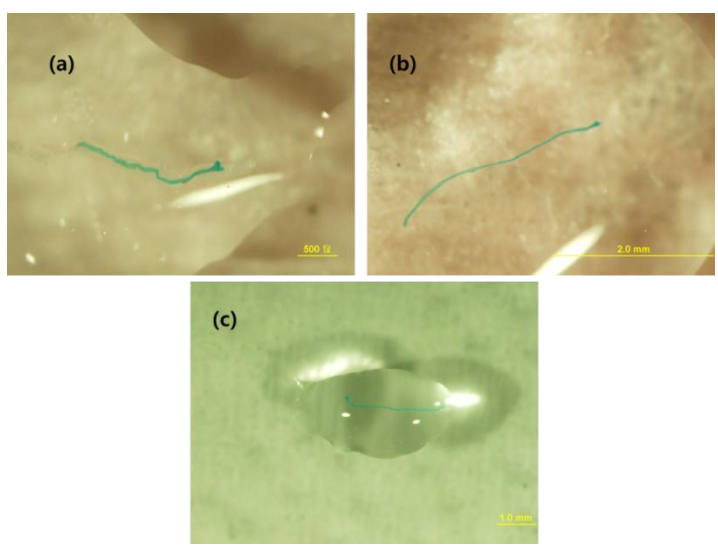

Figure 5.8 (a) A specimen of a lymph vessel with a PVS on a slide.
(b) Separated PVS: stereo Image, (c) Phase image.

CRITICAL STEP!

a) Use sharp end forceps to tear at the bottom of a targeted lymph vessel containing the PVS by pulling carefully from both sides. Don't pull with too much stretching of vessels.

b) On site check list for identifying PVS from artifacts such as coagulation of AB with lymph fluids in a string like form: 1) It is a floating threadlike structure not attached to the lymph walls. 2) Elasticity. The PVS thread is elastic but the AB coagulation is not so and therefore easily broken.3)Thickness. The diameter of a primo vessel is around 20 -30 μm. 4)Presence of primo nodes. The primo nodes are thicker parts of the primo vessel and their number, thicknesses and lengths are varying. 5. It passes through lymph valves. 6. It branches at the branching of the lymph vessels.

18. If the thickness of a primo vessel is larger than 50 μm it is likely that the primo vessel has adhered blood or lymphocytes.
19. The isolated PVS specimens from the lymph vessels are to be fixed with either 4% PFA solution or 10% NBF solution for a day or two for further analysis and are to be stored at 4℃ in a refrigerator.

CRITICAL STEP!

Formalin fixation will inactivate infectious agents. However, it is recommended that one follows universal precautions when working with formalin-fixed tissues.

5.7. Analysis and confirmation of the PVS

5.7.1 Morphological analysis with DAPI and phalloidin staining (Time: 40 min)

20. Wash the fixed samples with 1x PBS solution two or three times. Always pay attention to and keep the branches of the PV well. Put either the fresh or the fixed PV sample on a glass slide with 1-2 drops of PBS 1x solution. In order to characterize the f-actins and the nuclei in the PVS, stain the

specimens with phalloidin and DAPI. Wash the samples in PBS, place them on slides, and under a microscope, carefully and slightly flatten the tissues for examination.

21. After washing the primo vessels extracted from the lymph vessel three times with PBS, for the staining of nuclei and f-actins in the cytoplasm of endothelial cells of a PV, DAPI and phalloidin are used respectively. Stain the specimen with 6.6 μM phalloidin for 20 min, followed by a three - times PBS wash. Then, stain them again with 300 nM Prolong Gold Antifade reagent with DAPI for 20 min. When you apply DAPI, mix thoroughly, but take care not to create too many bubbles. Drain excess solution from the slide. Apply two separate drops of Gel Mount – DAPI on the sections, and then carefully lower the cover slip on the sections. Let the slide stand in the dark for a few minutes, seal the cover slip with a transparent manicure, and then observe the DAPI stain with fluorescent microscope.

CAUTION!

Avoid light on the sample during the Phalloidin and DAPI staining procedure.

22. Analyze the specimens with a fluorescence phase contrast microscope (Olympus BX51, Olympus) and a confocal laser scanning microscope (CLSM; C1 plus, Nikon, Japan) to observe the rod-shaped nuclei and f-actins.

CRITICAL!

Morphological characteristics of the PV: thickness (20 -30 μm), bundle structure of several sub-vessels, rod-shaped endothelial nuclei (length 15-20 μm) aligned along the vessel like broken parallel curves, sometimes presence of DNA containing granules inside sub-vessels. F-actins are also aligned along the PV. Non-pure thick samples have coagulated lymphocytes surrounding the PV.

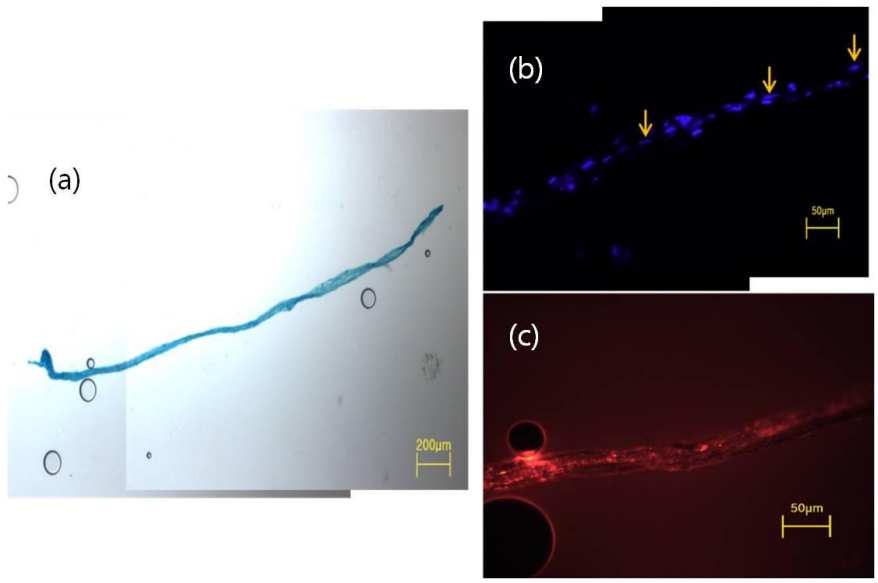

Figure 5.9 Morphological characteristic analysis of PVS (a) Phase contrast image of a PV sample. (b) Dapi staining image of specimen of (a) (Yellow arrows represent rod -shaped nuclei), (c) Phalloidin staining image of sample (a).

5.7.2 Histology and Immunostaining (Time: 2~ 3 d)

Since the procedures in this stage are conventional ones we put them in SI.

23. **Preparation of tissue specimens**: The lymph duct including primo vessel inside was prepared for the cross sectional examination. Place the tissue sections in base molds and cover with OCT compound and wrap frozen OCT-tissue blocks in aluminum paper. Apply snap-frozen in liquid nitrogen and store at at -80℃ until use for sectioning

24. **Sectioning**

    1) For tissue stored at -80℃: remove from freezer and equilibrate at -20℃ for approximately 15 minutes before attempting to section. This may prevent cracking of the block when sectioning.
    2) Remove the tissue from -20℃ and leave it at room temperature for 10 minutes.
    3) Section tissue at a range of 5 μm using a Leica CM 1800 cryostat, and place on glass microscope slides.

4) Allow sections to air dry on bench for a few minutes before fixing (this step will allow sections adhering to slides).

25. **Fixation**

5) After sections have dried on the slide, fix in optimal fixative as directed below. Either one is okay for an experiment and fixed sections can be store at -80℃ in desiccant bags.

a) **10% Neutral buffered formalin:** 7 minutes at room temperature. Proceed with staining procedure immediately.

b) **Cold acetone:** 7 minutes at -20℃. Air dry. Proceed with staining procedure immediately.

CRITICAL! Fixation method depends on the antibody used, each investigator should test the fixation method appropriate for their antibody of choice.

CRITICAL! Formalin fixation will inactivate infectious agents. However, it is recommended that one follows universal precautions when working with formalin-fixed tissues.

6) Sample Preparation

a) Air-dry for one hour at room temperature.

b) Dip the slide in distilled water for 5 minutes in a coplin jar to melt down the frozen OCT.

26. **Histology**:

H&E; DiI; Gordon & Sweet's Silver Staining. (Figure 5.12)

***Hematoxylin and Eosin Staining of Tissue and Cell Sections (H & E):**

Hydrate the tissue section

i. Put the slide into the 100% ethanol after tissue section for 10 seconds.

ii. Rinse the slides with distilled water to remove ethanol for 10 seconds.

Stain nucleus with Hematoxylin

iii. Put the slide into a container filled with Hematoxyline solution for 5 minutes.

CRITICAL STEP!

a) Filter before each use to remove oxidized particles.
b) Hematoxylin must be protected from the light and filtered before use to eliminate oxidized golden sediments.
c) The time of staining could be different depending on Hematoxylin. That's because Hematoxylin is diluted by repeated uses. It takes more time to stain the tissue if old Hematoxylin is used. However, there is no significant effect on the results of staining. New Hematoxylin: about 5 minutes, old Hematoxylin (about 7 days old): over 15 minutes.

iv. Dip the slide into a Coplin jar containing in distilled water for 5 times.

v. Dip the slide into a Coplin jar containing in 1% HCl in 70% EtOH for 30 seconds (acid alcohol dipping).

vi. Rinse in distilled water, 5 dip.

vii. Bluing in 0.2% ammonia water for 30 seconds to 1 minute.

viii. Wash in distilled water for 1 minute.

Stain cytoplasm with Eosin and dehydrate

ix. Rinse in 95% alcohol, 10 dips.

x. Counterstain in eosin-phloxine B solution (or eosin Y solution) for 30 seconds to 3 minutes.

xi. Dehydrate through 70% alcohol by slide dipping for 5 times.

xii. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 3 minutes each.

xiii. Clear in 2 changes of xylene for extracting alcohol, 5 minutes each

CRITICAL!

If using plastic slides or staining in plastic culture dishes, do not use xylene or xylene-based mounting media, because they dissolve plastics.

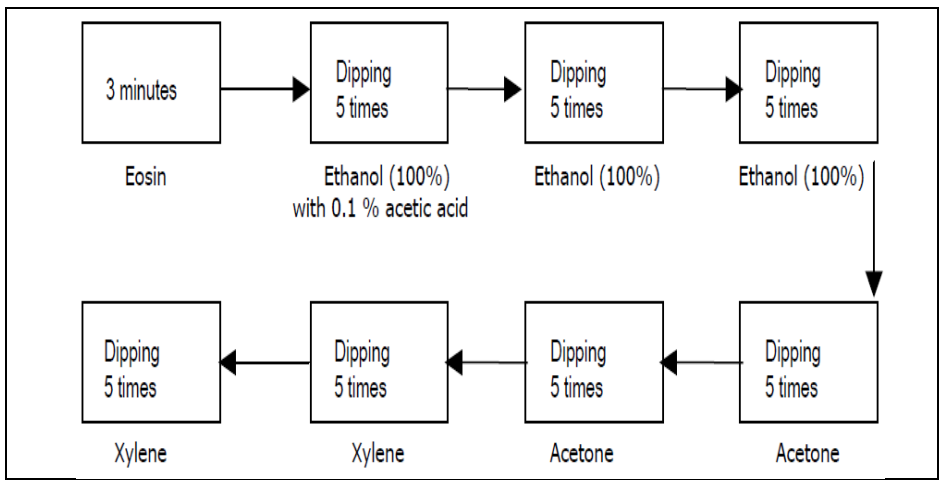

Figure 5.10 A procedure of H &E staining.

Mounting and Observation

xiv. Add one or two drops of mounting medium and cover with a coverslip.

xv. The stained specimen is investigated under a phase contrast microscope (BX 51, Olympus, Japan)

***Golden & Sweet's Silver Staining for Reticulin:**

i. Prepare the slide for staining.

ii. Oxidise in acidified potassium permanganate for 3 minutes

iii. Rinse in distilled water.

iv. Decolourise with 2% oxalic acid for 1 min

v. Rinse in distilled water.

vi. Mordant in 4% iron alum for 10 minutes

vii. Rinse in distilled water, 3 changes.

viii. Impregnate in ammoniacal silver solution for 11 seconds, shake excess solution off slides.

ix. Rinse quickly in distilled water, 2 changes.

x. Immediately reduce with 10% aqueous formalin for 2 minutes

xi. Wash in running tap water for 2 minutes

xii. Tone in 0.2% gold chloride for 2 minutes.

xiii. Rinse in distilled water.

xiv. Fix with 2% aqueous sodium thiosulphate (hypo) for 2 minutes

xv. Wash in water for 2 minutes

xvi. Counterstain with neutral red for 2 minutes

xvii. Dehydrate, clear, mount and coverslip.

PAUSE POINT!

Dehydrate sections:

a. Dipping the slide three times in 70% ethanol.

b. Incubate sections in 95% ethanol two times for 2 minutes each.

c. Repeat in 100% ethanol, incubating sections two times for 2 minutes each.

27. Immunohistochemistry**:** EMP-3; vWF (Figure 5.12)

Immunohistochemistry (IHC) for Frozen tissues:

EMP-3:

Hydrate the tissue section:

i. Put the slides into a rack for IHC

ii. Dip the slides into the acetone in the -20˚C for 5 minutes

CAUTION! Acetone is toxic. Work in a fume cupboard, and wearing gloves and a protection mask is recommended.

iii. Air-dry slides at RT for 5 minutes

iv. Wash sections in PBS buffer twice at RT for 5 minutes

CRITICAL!

Prepare a container which is large enough to afford 2 racks. Put the rack at one side of the container and, then, make water flow from the other side. Don't make the tissue side face the water.

v. Repeat the step iii-v once more

vi. Encircle the tissues with a PAP pen to prevent them slipping out of the slide. Using a PAP Pen, mount hydrophobic barrier rings onto glass slides by circling tissues to limit reagent use for staining. By doing so approximately 400 µl of liquid (blocking or staining solutions) per slide is required.

vii. Using CAS block solution, blocking the sample 2 hours at room temperature.

CRITICAL! After blocking, store the slides in humid chamber with a lid on it to inhibit the slide to be dried.

Primary Antibody:

viii. Adjust AB (AB=Rabbit anti-EMP-3 (H-130: SC-135143,Santa cruz), with dilution solution (1:200 in CAS blocking solution).

ix. Put the slide on the humid chamber, drop AB solution enough to cover the tissue.

x. Keep the samples in the humid box for overnight at 4℃.

xi. Drain the blocking solution out of the slide (not washing).

CRITICAL! When you spread solution on the slide, be careful to cover tissues completely.

xii. Dip the rack in PBS buffer at RT for 5 minutes.

Secondary Antibody:

xiii. Put the slides in the humid chamber again

xiv. Drop 2-3 drops of Goat anti-rabbit AlexFluor® 488 (A11034, Invitrogen) of secondary antibody to the slide and store it for 2 hours at RT. (**Darkroom**)

CRITICAL! Must do step (xv) in darkroom.

Colorimetric Detection:

xv. Wash the slide with PBS buffer when you believe the tissues are stained well enough for examination.

Counter Stain :

xvi. Stain the slides with DAPI nucleic acid stain (1:10000 diluted in 1X PBS) for 15 minutes at RT. (**Darkroom**)

CRITICAL! Must do step (xvi) in darkroom.

xvii. Wash three times with PBS buffer 3 times in a coplin jar applying gentle shaking.

Mounting:

xviii. Drop 1-2 drops of mountant onto the slide.

xix. Put a cover glass onto the slide. Remove excess mounting medium with a piece of filter paper.

CRITICAL! Be careful not to create vapor between the cover glass and the slide.

***Von Willebrand Factor , (vWF)**

i. Prepare the sample slide for **vWF** staining.

ii. Spread 0.5% triton X-100 solution over the fixed tissue section and leave it for 5 minutes.

iii. Wash with PBS solution

iv. Repeat the step (ii)-(iii)

v. Block each section with 1% BSA blocking solution for 2 hours at room temperature.

CRITICAL! After blocking, store the slides in humid chamber with a lid on it to inhibit the slide to be dried.

Primary Antibody:

vi. Adjust AB (AB=Mouse anti-vWF (SC-365712,Santa cruz), with dilution solution (1:50 in1% BSA blocking solution).

vii. Put the slide on the humid chamber, drop AB solution enough to cover the tissue.

viii. Incubate the samples in the humid box for overnight at 4℃.

xi. Drain the blocking solution out of the slide (not washing).

CRITICAL! When you spread solution on the slide, be careful to cover tissues completely.

x. Wash the sections with PBS solution

CRITICAL! When you spread solution on the slide, be careful to cover tissues completely.

Secondary Antibody:

xi. Put the slides in the humid chamber again

xii. Drop 2-3 drops of Goat anti-mouse AlexFluor® 568 (A10037, Invitrogen) of secondary antibody (1:100 diluted in 1X PBS solution) to the slide and store it for 2 hours at RT. (**Darkroom**)

Colorimetric Detection:

xiii. Wash the slide with PBS buffer when you believe the tissues are stained well enough for examination.

Counter Stain :

xiv. Stain the slides with DAPI nucleic acid stain (1:10000 diluted in 1X PBS) for 15 minutes at RT. (**Darkroom**)

xv. Wash three times with PBS buffer 3 times in a coplin jar applying gentle shaking.

Mounting:

xvi. Drop 1-2 drops of mountant onto the slide

xvii. Put a cover glass onto the slide. Remove excess mounting medium with a piece of filter paper.

CRITICAL! Avoid bubbles formation while mounting.

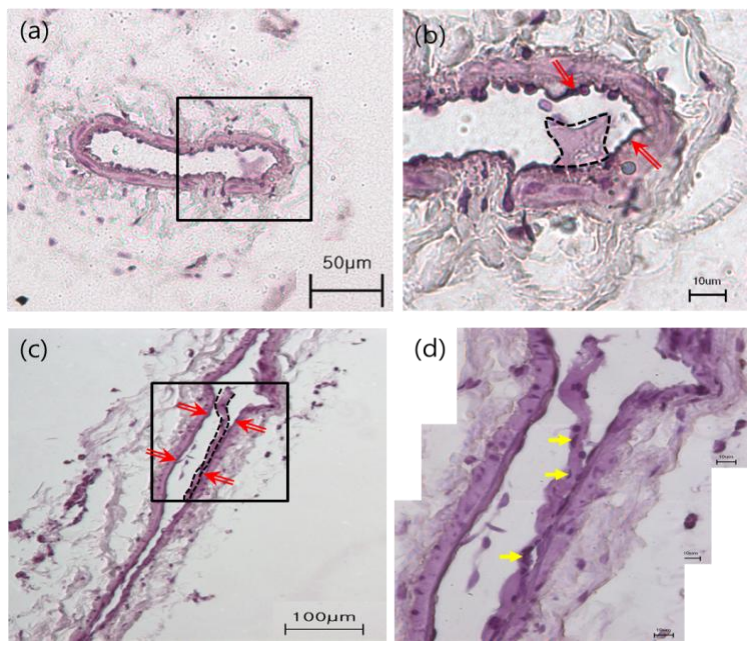

Figure 5.11A H&E Images of PVS (Cross section & Longitudinal section).

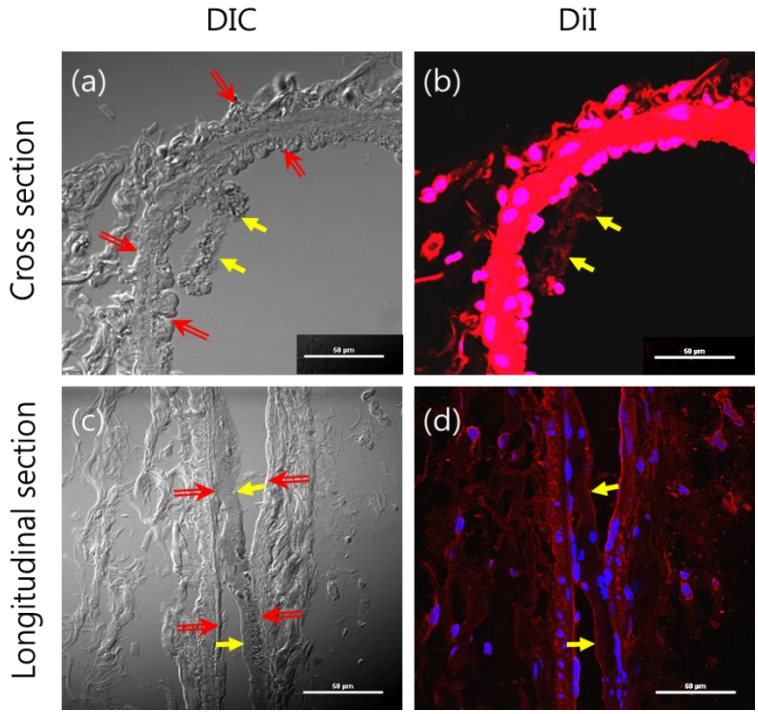

Figure 5.11B DIC (a,c) and DiI (b.d) image of PVS (Cross section & Longitudinal section)

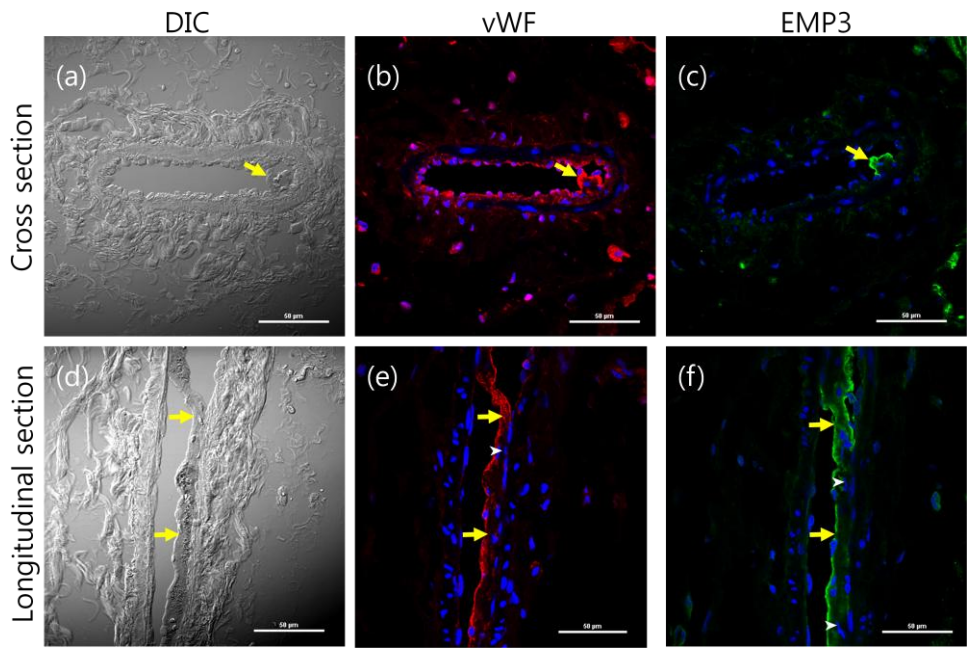

Figure 5.12 The immunohistochemistry images of DIC**,** vWF, and EMP-3 of a PV.

CRITICAL!

1) Histological characteristics of the PV provide the proof for the claim that the PVS inside a lymph duct is a genuine new tissue rather than an artifact. H&E shows the nuclei and cytoplasm in this tissue. DiI shows the surrounding membrane which is a separate independent structure from the lymph ducts. Coagulation or aggregated lymphocytes or fibrins do not have a wrapping membrane. Silver staining shows the presence of reticular fibers while the inner wall of the lymph vessel does not have such fibers. EMP-3 positive expression shows that the PV has epithelia cells at the exterior side, and vWF positive expression shows the presence of endothelial cells which is consistent with the rod-shaped nuclei in the PV.

2) Further evidence of the wrapping membrane with electron microscope was reported earlier, and further confirmed with an atomic force microscopy data (not shown). The CD34 and LYVE-1 negative expression of the PV in earlier works clearly showed that the PVS is different from blood or lymph vessels.

5.8 Anticipated results

5.8.1 Anatomy

The network of lymph ducts near the caudal vena cava and the thoracic duct of a rat are the target to search for a PVS. The PVS forms a network of blue thin threadlike structures stained with AB in the lymph ducts of various thicknesses. The primo vessels pass through lymph valves, and form branches where lymph ducts branch.

5.8.2 Identification

A floating blue threadlike structure should be tested for its elasticity in order not to be confused with an artifact temporarily formed by staining dye aggregations which are easily crushed to pieces by shaking lymph duct. A primo vessel is elastic

that can be felt when it is extracted from the lymph duct *ex vivo* on a slide. The thickness is about 20 -30 μm. If it is thicker than 50 μm, it is likely that it is enshrouded with lymphocytes and fibrin. This coagulation is due to the bleeding in the lymph node during injection. A primo vessel is a bundle of several sub-vessels and its phase contrast microscope image shows few parallel lines along the vessel. This bundle structure is a key feature of a primo vessel that uniquely distinguishes it from blood or lymph vessels. The thickness of a primo vessel is fairly uniform except at the primo nodes. The presence of primo nodes along a primo vessel is irregular and their sizes and shapes are also varying. They can be recognized as cucumber shaped thick parts of a primo vessel

The PVS can be identified positively by observing the distributions of nuclei in the longitudinal sections to distinguish the PVS from blood or lymph vessels. The nuclei of the endothelial cells of a primo vessel are arranged in the fashion of parallel broken lines, and they are rod-shaped, about 10-20 μm in length. This feature can be easily checked with DAPI staining with CLSM images. These rod-shaped nuclei are thought to be the endothelial cells lining the sub-vessels. The endothelial nature of these cells was only proved by the vWF criteria and not yet confirmed by other characteristics. Development of anti-bodies like LYVE-1 will be a critical progress toward a complete tracing of the whole network of PVS.

5.8.3 Histological analysis

In order to completely identify the PVS in the lymph duct, the histological and immunohistological analysis should follow. The DiI staining shows the outer membrane of the primo vessel and node, the H&E shows the distribution of cells and nuclei, and silver staining shows there are reticular fibers.

The immunostaining with EMP-3 revealed that the outer parts of the PVS are composed of epithelial cells while vWF staining shows that the inner cells are endothelial nature.

5.9 Further research

Establishing a protocol for the PVS sampling is the first step toward full enunciation of this new circulatory system. First of all, finding the vascular

endothelial growth factors, and specific markers corresponding to LYVE-1 will be a milestone for molecular level research on PVS and fully tracing the PVS network to reveal its connection with other classes of PVS like those on the surfaces of internal organs [13] and in a heart [10] and blood vessels [8,9].

The abundance of immune cells like histiocytes and mast cells in PN was already studied [23]. The PN might be a niche for the hematopoietic stem cells [23]. This will naturally lead to biological functions of the PVS. For instance, the primo vessel associated with the xenografted tumor may provide a safe haven for a select population of cancer stem cells [28], and the primo vessel as an additional metastasis path for cancer cells [27].

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Chapter 6. Atomic Force Microscopy Study of Rat Primo Vessels of Rats.

The microstructures of the primo vascular systems (PVS) of rats were investigated using atomic force microscopy (AFM). The PVS with an average diameter of 20 ~30 μm were extracted from inside the abdominal lymph vessel of the rat. Atomic force microscope images of fixed specimens revealed that PVS had relatively smooth surface with irregular random bumps on the surface. This was distinguishably different from the outer surface of lymph vessels. The advantage of the AFM compared to electron microscopy (EM) is that EM requires complicated sample preparation processes and therefore may not show the natural state of the specimen due to deformation in the processes while AFM does not require such processes. The current work was the first attempt to study the surface ultrafine-structure of the PVS by using AFM. The smooth surface of the natural PVS was confirmed in this study which was expected from optical microscopy.

6.1 Introduction

Acupuncture is increasingly accepted and used widely worldwide as an effective alternative medicine to that of modern medicine. It is one of fastest growing areas of traditional medicine. Even though evidence of its therapeutic mechanism has been reported, the anatomical structural support for its function still remained unclear. Once the anatomical structure corresponding to the acupuncture meridian is established the science of acupuncture should have a solid foundation [1].

Even though many observations have reported the physical reality of acupuncture point and meridians, until now, however, no anatomical or histological structures corresponding to the acupoint have been found [2].

The first discoverer, Bong-Han Kim claimed in this regard in the early 1960's and reported the anatomically distinctive tissue at the acupoints and meridians. He named new findings after him. The primo vascular system (the former Bong-Han System) is consisted of primo vessels (PVs), primo node (PN), primo fluid (PF) flowing in PV and microcell (another name for Sanal). He traced the PVs and

found that they formed a network throughout the body. Moreover, he found threadlike structures float inside blood and lymph vessels and on the surfaces of internal organs [3]. But questions and serious objections rose by scientists to against the existence of the primo vessels and they thought PVS was the possibility of confusion with lymphatic vessels [4, 5].

After this question has been raised, it has been addressed by the demonstration of anatomical differences between these structures and by the observation of the PVS inside large lymph vessels [6]. The AFM is a powerful instrument in the nanometer-scale science and technology. It is a very high-resolution type of scanning probe microscopy, with resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. It is one of the foremost tools for imaging, measuring, and manipulating matter. AFM is suitable to measure the mechanical properties of living material such as tissue or cells. Not just that, the direct on-axis optical microscope which uses a motorized focus stage provides a much better vision than the optical microscopes provided on conventional large sample AFMs. In another word, it is a very effective tool to image and manipulate structures on a variety of surfaces.

AFM has several advantages over the scanning electron microscope (SEM). Unlike the electron microscope which provides a two-dimensional projection or a two-dimensional image of a sample, the AFM provides a three-dimensional surface profile. Samples viewed by AFM do not require any special treatments (such as metal/carbon coatings) that would irreversibly change or damage the sample, and does not typically suffer from charging artifacts in the final image. While an electron microscope (EM) needs an expensive vacuum environment for proper operation, most AFM modes can work perfectly well in ambient air or even a liquid environment. This makes it possible to study biological macromolecules and even living organisms. In principle, AFM can provide higher resolution than SEM. It has been shown to give true atomic resolution in ultra-high vacuum (UHV) and, more recently, in liquid environments. High resolution AFM is comparable in resolution to scanning tunneling microscopy and transmission electron microscopy (TEM). AFM can also be combined with a variety of optical microscopy techniques, further expanding its applicability [7].

In previous article published by M.S Kim in 2010 revealed small holes on the lateral surface of the primo vessels of a rabbit by imaging with phase contrast x-ray microscopy. Phase-contrast x-ray microscopy is good for examining the micro structural properties of biological systems, as well as condensed matters. Unlike other techniques, phase contrast x-ray microscopy can describe the inside and outside of specimens in a wide range without destroying the specimen [8]. And also taking a relatively short period of time for sample preparation and measurements is another advantage to use this microscope. With all these advantages, proved that the revealed holes on the primo vessels are quite different from the other channels of the cell membranes, including nerve systems and blood and lymphatic capillary vessels. Similar to x-ray microscopy, AFM allowed us to see three-dimensional image of the PVS' surface structure, therefore, we hopefully could have same result or maybe obtaining better data than before on the PVS of rats.

In a complementary sense to phase x-ray microscopy of rabbit PVs, we have been examined the characterized specific anatomical, histological, and ultrastructural features of PVs and PNs using electron microscopy (EM). A detailed microscopic analysis with light and EM were showed the one of the main feature in PVs which were a bundle of several small tubular structure or primo vessels, whose walls were formed by a thin single layer of endothelial cells possessing characteristic rod-shaped nuclei. Using EM, it is clearly exhibited the distinct structural characteristics at the ultrastructural level even though PVs were shared some properties with the endothelium of blood and lymphatic capillaries [9].

For an accurate analysis, it is very important to pick the right tools for the right sample. PVs and PNs are very small biological tissues in size which is less than 40 μm in diameter; therefore, it was very hard to obtain the reliable data due to easy loss of samples during the sample preparation and formation of artifacts during EM imaging. However, AFM imaging, there is no need for pre-processing stage of samples so it reduces the chance of sample damaging, and at same time, makes it possible to image pure PVS. Also due to much high-resolution data than EM, it makes us to observe more detailed image than others. In this paper, we report on a preliminary morphological study of the vessels which all have its uniqueness and own characteristic by AFM imaging to discriminate the PVs from lymph vessel.

6.2 Materials and Methods

6.2.1 Preparation of Rats and Surgical Procedure

Five Spague-Dawley males of ~270g were obtained from Doo Yeol Laboratory Animal Company for use in this study. The rats were housed in a constant-temperature controlled environment (23 ℃) with 60% relative humidity under a 12 h light/dark cycle. All of the rats had ad libitum access to food and water. The procedures involving the animals and their care were in full compliance with current international laws and policies (Guide for the case and Use of Laboratory Animals, National Academy Press, 1996). The rats were anesthetized with urethane (1.5g kg^{-1}) administered intraperitoneally, and all surgical procedures were performed under general anesthesia. Under deep anesthesia the abdominal sides of the rats were incised, and the lumbar nodes near the caudal vena cava were located and exposed by removing the fat. Alcian blue staining dye (0.02 ml for each node) was slowly injected into the two nodes (over 4 min) by using a 31-gauge needle. PVS were collected from the inside lymphatic vessels, immediately fixed with 10% NBF solution for sample preparation for AFM imaging.

Lymphatic vessels

Collect a large lymph vessel from peritoneal cavity sized about 2cm and fix with 10% neutral-buffered formalin (NBF) solution for a day. In order to take an image of outer lining of lymph vessel, cut the lymph vessel carefully and avoid contaminating on the outer surface of vessel.

Microscope

The observations of the threadlike structures were done with a stereomicroscope (SZX12, Olympus).
Atomic Force Microscope images were obtained with a NX-Bio model.
Scanning ion conductance microscope was performed with a XE-Bio Model.

Atomic Force Microscopy

Small segments (~1mm x 7mm in size) of the specimens were fixed in a 10% neutral-buffered formalin solution for a day at 4 ℃. The fixed materials were washed with distilled water three times and were placed in a cover glass (18mm x 18mm). In order to fix the sample within a cover glass, glued both sample ends with a transparent color manicure. Next, a prepared sample cover glass were attaching to the inside surface of a petridish cover. The samples were immersed in 4% formaldehyde solution for 40 minutes and were rinsed samples carefully out with distilled water. Then they were put into flat box overnight at room temperature for dehydration. Samples were micrographed in a NX-Bio atomic force microscope (Park systems, Suwon, Korea).

6.3 Results

Comparison of outer surface of lymph vessel and primo vessel

In this article, we present for the first time scanning atomic force microscope (AFM) images of the outer surface of PVs and LVs of rats.

6.3.1 Outer Surface of Lymphatic Vessel (LV)

As shown in Fig. 6(a), is clearly visible image of Alcian blue stained primo vessel inside a LV. Its thickness about 120 ~150 μm and length about 2 ~3mm were observed. In comparison to PVs ,we were able to uncover the following detailed morphological charateristics of the outer surface lymph vessels: we found that the abundant fibrils served as an extracellular matrix in the outer surface of LV (AFM). The diameter of fibrils were measured and they were about 150~200 nm in diameter which showed in Fig. 6(d). The fibrils of the this group are thicker and got crosslinked to each other where many fibers were overlapped. The individual fibers were not distinguished clearly, making it difficult to characterize at this time. Further researches about identifying types of fibrils by its fine structure and diameter has to be followed for identifying the types and accurate analysis in discriminating the real PVs from other tissues or artifact.

6.3.2 Primo vessel surface

The primo vessels observed on the inside of lymphatic vessels are thin, semitransparent, and freely movable strands. The anatomical work has been shown that the PV is different from known tissue such as lymph vessels in the previous works. The outer diameter of the PV in Fig. 6(e), was estimated to be 15~20μm in this sample. The AFM images were quite different between two samples. Fig. 6(h & f), clearly shows that the primo vessel contains relatively smooth surface structures than a LV and small bumps as indicated by white arrow. These structures were located on the primo vessel surface. Moreover, as Fig.6f showed that there weren't fibrils structure attached on the surface of PVs.

Bumps on the primo vessels could still be observed clearly, and the size of this was 250nm x 250 nm. Not similar structures like this were found on the LVs.

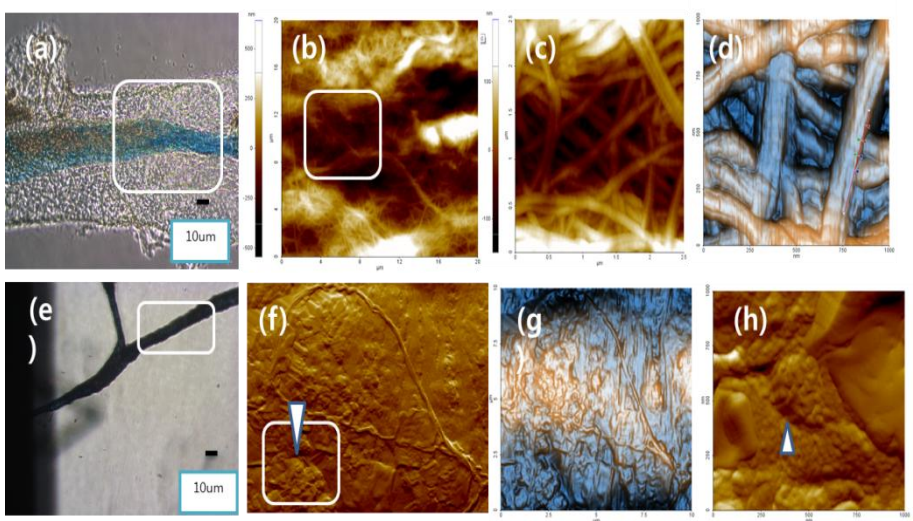

Fig 6.1 Lymph vessel (6a) Phase contrast image of AB stained primo vessel within lymphatic vessel. (6b) AFM image from outer surface of lymph vessel. (6C) Magnified view of fibrils cross-linked to each other on the LV's surface. (6d) Enhanced color mode conversion of Fig. (6c).
Primo vessel (6e) Phase contrast image of AB stained pure primo vessel. (6f) AFM image of fig (6e)'s square box which contains smooth surface of PV with a bump. (6g) Enhanced color mode conversion of Fig. (6f) for comparison. (6h) magnified AFM image of bumps appeared on Fig. (6f).

6.4 Discussion

For the purpose of comparison, we studied the ultrafine structures of the PVs and LVs of a rat by using a poweful imaging tool, namely, AFM which was done for the first time. In this paper, we provide images that clearly distinguish the different outer surface compostion of PV and LV. A detailed microscopic analysis with an AFM was performed to characterize specific anatomical features of them. Simply the PVs consisted of a relatively smooth surface without extra fibers attached. In contrat, the LVs were covered with many fibrils of which size were about 150~200 nm in diameter. These were crosslinked to each other with overlapping. AFM images clearly exhibited the morphological differences between these samples, which was not seen before with optical microscopes.

In previous works were focused mainly on examination the micro structural properties of PVs. As the middle step of whole PVS research objects, we were used the phase contrast x-ray microscopy to describe the inside and outside of PV specimens in a wide range without destroying the specimen [10-12].

Among the advantages of using x-ray microscope, the key is the taking a relatively short period of time for sample preparation and measurements. Based on the x-ray microscope advantages' stated above, we had a chance to observe the holes located on a lateral surface of primo vessels and assumed that these holes act as selective channels for solution flow. However, in order to prove this hypothesis, it needs to obtain the same data for further examinations.

Moreover, in an effort to uncover the detailed study of PVS on its structure at the cellular level, high-resolution imaging techniques were needed of using electron microscopy. A previous paper (BC. Lee et al., 2007) presented the bundle structure of PVs which consisted of many subducts with a typical thickness of about 10 μm. It clearly showed the subducts and their surface structures. This bundle structure is one of the characteristic features that distinguish PVs from lymph vessels [9]. AFM was used to reveal more precise and detailed

morphological features. Furthermore we proved that the PVs were distinctively different from lymph vessels by comparison of both samples.

However, this article is not the first time in using introducing the AFM imaging of PVS. In 2009, the first trial of AFM imaging of primo microcells (former name of sanals) were performed .The ultrastructure of the primo microcells was investigated at that time and unique threadlike structure of lyophilized primo microcells observed using SEM and AFM.. It showed the primo microcell's first step of proliferation which was described by Bong-Han Kim for the first time [13].

In spite of many advantages of AFM imaging of the PVS it still has shortcomings. One of the major downsides is the single scan image size, which is of the order of 150 x 150 micrometers, compared with millimeters for a scanning electron microscope. Another disadvantage is the relatively slow scan time, which can lead to thermal drift on the sample. Traditionally, an AFM cannot scan images as fast as a SEM, requiring several minutes for a typical scan, while a SEM is capable of scanning at near real-time, although at relatively low quality. The relatively slow rate of scanning during AFM imaging often leads to thermal drift in the image making the AFM less suited for measuring accurate distances between topographical features on the image.

In order to overcome these disadvantages and researchers rely on a newly developed microscope. The 'scanning ion conductance microscope (SICM)' was introduced to obtain more precise images of samples for biological living tissues in live conditions. SICM provides also a label-free technique, and its spatial and height resolution are significantly better than the diffraction limit of a confocal fluorescence microscope. It was specially designed for the sub-micrometer resolution scanning of soft non-conductive materials. In addition, the some modes of SICM have the advantage of minimizing (or free from) the loading force, which is unavoidable in AFM. This was the key for solving the fact of minimizing the sample surface damaging. In the near future, using the advantage of SICM, we plan to image and identify circulating cell types including primo microcells.

The microscopic studies presented in this article are an essential step toward establishing novel circulatory system thus they will serve as a stepping stone towards the solution to the long-standing question of the PVS. In addition to the morphological elucidation, the observation of various types of cells, like mast cells, macrophages, and eosinophils in the PNs may be very important to show its immunological role, which in turn, implies the physiological effects of PVS.

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국문 초록

본 연구에서는 쥐의 림프 내 프리모시스템 (PVS) 가시화를 위하여 알시안블루 염료를 이용한 프로토콜을 확립 하였으며, 림프 내 프리모관과 림프관 내벽의 표면 특성을 원자힘 현미경(AFM, Atomic Force Microscope)을 이용하여 프리모관 표면에 있는 조직들과 림프관 내벽의 표면의 차이점이 무엇인지를 조사 분석하였다. 이 결과들을 바탕으로 프리모관의 x-ray microscopy 영상과 비교하였다.

토끼와 쥐 복대정맥 옆의 요추림프절에 알시안블루를 주입하여 림프관 안쪽에 떠있는 PVS 관찰 기술을 정립하였다. 이 프로토콜에 따라 림프관내 프리모관을 채취하여 형태적 특징을 DAPI 와 Phalloidin 염색 후 공초점현미경으로 확인하였다. 기존 연구에서 토끼와 쥐의 복부에 위치한 림프관에서만 관찰되었던 프리모시스템을, 본 연구에서는 흉관에서도 관찰하는 기법으로 확장하였다.

프리모시스템에 대한 H&E 및 면역염색으로 조직학적 분석을 하였다. DiI의 염색을 통해서 프리모관의 외막을 확인할 수 있었으며, H&E는 프리모관을 구성하는 세포의 세포질과 핵의 분포를 관찰할 수 있었다. EMP-3 면역 염색은 PVS의 외막이 상피세포로, vWF는 내부 세포가 내피세포로 이루어져있음을 확인하였다. 프리모관은 EMP-3와 vWF에는 염색이 되지만 림프관의 중요한 마커로 사용되는 CD31이나 LYVE-1에는 염색이 되지 않는 특징을 가지고 있다. 이를 통해 림프관내 프리모관이 림프관과 구별되는 조직학적 특징을 가지고 있음을 확인하였다.

원자힘현미경을 이용하여 림프관의 내벽과 프리모관의 표면형상 분석을 시행하여 이들의 차이점을 규명하였다. 림프관의 내피세포는 탄성섬유로 이루어진 필라멘트가 interstitial collagen에 의해 부착되어있고, 그에 반해 PV의 표면은 상대적으로 매끈하고, 표면에 위치한 외공(hole)들

을 관찰 할 수 있었다. PV의 외공은 PV의 표면과 내부의 연결 통로로서 selective channel 역할을 할 것으로 짐작된다.

결론적으로 림프관 내 프리모시스템의 해부학적 관찰 및 조직학적 분석 프로토콜을 개발했다. 원자힘현미경을 써서 프리모관의 표면특성을 조사하여 림프관 내벽과의 차이점을 규명했으며, x-ray microscopy 결과와 일치하는 프리모관의 외공구조를 자세히 밝혔다.

주요어: **프리모시스템, 림프, 알시안블루, 프로토콜, 원자힘현미경, 외공, 표면특성, X-ray microscope**

학번: 2011-24240

감사의 글

이 논문의 마지막 장을 정리하는 감사의 글을 적으려 하니 참으로 많은 생각들이 스쳐서 지나갑니다. 대학 생활을 하는 동안 연구의 길은 생각 하지 못한 채, 다른 길을 걷고 있던 저에게 운명처럼 프리모 연구의 기회가 주어졌고 항상 관심 밖이었던 생각과는 달리 한번 해보자라는 흥미와 용기가 생겨 올 해로 2년이 넘는 연구실 생활이 시작하게 되었습니다. 신의 이끌림이었다고 표현을 하는 것이 정확한 표현인지 모르겠지만 그렇게 밖에는 표현을 하지 못할 것 같습니다. 오랜 외국생활 덕분에 새로운 분야에 도전하는 일은 제게 그리 두려운 일이 아니었습니다. 하지만 막상 연구를 시작하고 보니 생각과는 달리 막막함과 두려움이 가득하였던 거 같습니다. 그 과정들이 저에겐 쉽지 않은 시간이었기에 더 새록새록 곁에서 도움주신 분들에 대한 감사한 마음이 드는 것이 아닌가 하는 생각이 듭니다.

그 분들 중 제일 처음으로 감사하다는 말씀을 전하고 싶으신 분이 소광섭 교수님이십니다. 공부를 더 해보고 싶습니다 라는 저의 짧은 이메일 후에 여러 혼돈 속을 헤매이고 있던 저에게 새로운 가능성을 제시해 주셨고 이끌어 주셔서 지금의 저를 이 자리에 있게 해주신 분이십니다. 막막함에 한숨과 눈물짓고 있을 때면 조용히 따뜻하게 다독거려 주시고 이끌어 주시고 또 프리모연구의 중요성을 항상 일깨워 주셔서 나태해지려는 저에게 다시 한번 도전이라고 외칠 수 있는 힘을 주셨습니다. 교수님의 연구에 대한 끝없는 열정을 곁에서 보고 배울 수 있던 이 기회가 주어진 것에 그저 감사하다는 말로는 부족하겠지만 제 진심을 담아 교수님, 감사합니다 라고 꼭 말씀 드리고 싶습니다. 앞으로도 매 순간순간 교수님의 가르침들을 잊지 않고 기억하는 제자가 되도록 노력하겠습니다.

그리고 제 지도 교수님이신 송윤규 교수님께도 감사의 말씀 전하고 싶습니다. 늦깎이 석사학생이어서 모르는 것이 많아 때론 엉뚱한 질문을 드리기도 하였지만 언제나 웃음 지으신 얼굴로 편안하게 대해 주시고 지도 해주셨습니다. 제가 속한 연구실이 달라 많은 시간을 교수님 밑에서 지도를 받지 못한 점이 지금도 많이 아쉽습니다. 또한 제 부족한 논문을 심사하시느라고 힘써주신 이학종 교수님께도 정말 감사 드립니다. 교수님들께서 저의 부족한 부분을 아시면서도 심사를 통과시켜 주시고 열심히 했다고 말씀해 주신 것은 앞으로 더 발전 하는 모습을 기대하심이라고 생각하고 더욱더 정진하는 과학자가 되도록

노력하겠습니다.

또 생각나는 분들이 있네요. 국립암센터에서 열심히 연구하고 계시는 따뜻한 큰 언니 같은 황선희 선생님, 옆방 연구실에서 C동으로 이사가신 스마일 박상윤 교수님, 마지막 석사주제로 고민 하고 있을 때 시원스레 이거 하자 하고 제시해주신 Park systems에 조상준 박사님, 실험 주제에 대한 고민을 함께 나누고 고생한 정구은 선생님께도 이 자리를 빌어 감사의 말씀 전하고 싶습니다.

실험실 생활을 대학원 생활보다 먼저 시작하여 벌써 2년 반이라는 시간이 지나갔습니다. 그 시간을 우리 연구실 가족들과 함께 해서인지 한 사람 한 사람이 더 소중하게 떠오릅니다. 많이 바쁘셔서 자주 뵙진 못했지만 항상 저를 보며 샤롱아 하고 불러 주시고 힘들 때 웃음을 짓게 해주신 이민선 교수님, 약침학회 시절부터 항상 조용히 옆자리를 지켜 주시며 어려울 때마다 '지윤씨' 하고 하나하나 짚어주시며 알려 주시던 김정대 박사님, 미국에서 생활을 하셔서 누구보다 저의 고민을 많이 알아주시고 유쾌함으로 토닥거려주신 차용철 박사님, 엄마처럼 챙겨주시고 스마일 퀸이라고 불러주시던 백미옥 선생님, 옆집 오빠처럼 편안하게 이것저것 짚어주시고 도움 주셨던 문상호 선생님께 먼저 감사의 말씀을 전하고 싶습니다. 이 분들 모두 프리모실험에 대한 열정들이 대단하셔서 제가 항상 뵈며 옆에서 힘을 얻을 수 있었습니다.

우리 실험실 맏언니인 배경희 박사님, 처음 연구실 생활에 모르는 것이 많은 저를 데리고 다니시면서 이것저것 알려 주시고, 조용히 제가 해나가는 모습들을 지켜보아 주셨습니다. 언니의 따뜻함과 조언들이 저에겐 얼마나 도움이 되었는지 모릅니다. 이 시간을 빌어 감사했습니다 라는 말을 하고 싶습니다.

그리고 연구실에서 치마 입는 여자는 니가 처음이다 라며 첫 날부터 웃음폭탄을 던져준 프리모 삼남매 중 맏이 임재관 박사님! 매 순간 고비마다 절 붙잡아주고 혼내기도 하고 다독거리기도 하고 부딪치기도 한 분이 임박사님인 거 같습니다. 쥐를 보며 '엄마야' 하고 도망가던 저를 당근과 채찍으로 하나하나 알려주고 이끌어 줘서 지금의 제가 이 글을 쓰고 있는 것이 아닐까 하는 생각이 듭니다. 재관오빠! 필요한 순간순간 많이 챙겨주고 마음 써줘서 많이 고마웠어요. 이젠 같은 연구실이 아니어서 자주 보진 못하지만 항상 오빠가 하고자 하는 연구들이 좋은 성과가 나길 바랍니다.

우리 프리모 삼남매 중 막내 민호! 나이는 저보다 어리지만 많이 어른스럽고 야무진 성격을 가지고 있어서 이 누나에게 실험과 진로에 대한 많은 조언과 도움말을 해준 막내였습니다. 연구에 대한 열정도 누구보다 남달라 항상 노력하는 민호를 보며 스스로를 되돌아 보기도 했습니다. 누나가 아프다고 하면 한 번의 찡그림도 없이 침도 놔주고, 챙겨주던 따뜻한 모습 잊지 못할 것 같습니다. 우리 민호, 꼭 멋진 연구자가 되기를 바래. 민호야 파이팅!

또 순박한 청년이란 별명을 가진 승환이, 누구보다 지금 제 옆에서 '누나' 하며 챙겨주고 걱정해주는 따뜻한 마음을 지닌 동생입니다. 연구실 정리대장, 만능 순돌이 아빠처럼 뚝딱뚝딱 말만하면 고쳐주고 만들어주는 만능맨, 제가 하는 일마다 적극 나서주고 함께 해주어서 얼마나 고마운지 모르겠습니다. 지금처럼 연구에 쏟은 집중력과 열정을 살려서 꼭 하고자 하는 일 이루기를 바랍니다.

항상 같이 실험을 하며 늦은 시간에도 꿋꿋히 곁을 지켜주고 응원해준 자상한 동생 상연이, 같은 주제로 연구를 하며 고생도 많이 했는데, 잘 챙겨주고 같이 연구에 대한 고민들을 나눌 수 있어서 좋았습니다. 멋진 동생 상연아, 꿈을 향해 전진!

그리고 실험실의 막내 주환이, 앞으로 나노프리모센터의 새로운 꿈나무가 되기를 바라며 '밥먹으로 가요 누나' 하며 늘 챙겨주던 모습 잊지 못할 것 같습니다. 실험실 분위기 메이커 주환이, 파이팅!

지금은 함께 하지 못하는 연구실 동료들인 따뜻한 명진언니, 톡톡튀는 성하, 마음 따뜻한 정숙이, 화통한 성격을 가진 금옥, 상지대 지부장 영일, 대학원 선배이자 룸메이트 동생이었던 귀요미 은진에게도 고맙다는 말을 전하고 싶습니다. 몸은 멀리 있어도 응원해주고 걱정해주는 우리 연구실 식구들이 있어 얼마나 든든한지 모르겠습니다.

마지막으로 늘 곁에서 믿고 응원해주고 배려해 준 저의 가족들에게 너무 감사하다는 말씀을 전하고 싶습니다. 그리고 사랑하는 우리 가족이 버팀목이 되어 주었기에 제가 하고 싶은 일을 할 수 있었습니다.

많은 분들의 도움으로 제가 석사학위를 받게 된 것 같습니다. 여러분의 응원과 격려를 마음속에 간직하며 그 사랑을 발판으로 더 전진하는 모습 보이는 정지윤이 되도록 노력하겠습니다. 감사합니다.