



# Three-Dimensional Reconstruction of Neurovascular Network in Whole Mount Preparations and Thick-Cut Transverse Sections of Mouse Urinary Bladder

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**Purpose:** Proper functional and structural integrity of nervous and vascular system in urinary bladder plays an important role in normal bladder function and the disruption of these structures is known to be related to lower urinary tract symptoms. Here, we present an immunohistochemical staining method that delineates neurovascular structures in the mouse urinary bladder by using immunohistochemical staining with three-dimensional reconstruction.

**Materials and Methods:** The urinary bladder was harvested from 8-week-old C57BL/6 male mouse. Lamina propria and detrusor muscle layer were dissected for whole mount staining, and thick-cut (60- $\mu$ m) sections were prepared for full-thickness bladder staining. Immunofluorescent staining of bladder tissue was performed with antibodies against CD31 (an endothelial cell marker), smooth muscle  $\alpha$ -actin (a smooth muscle cell marker), NG2 (a pericyte marker), and  $\beta$ III-tubulin (a neuronal marker). We reconstructed three-dimensional images of bladder neurovascular system from stacks of two-dimensional images.

**Results:** Three-dimensional images obtained from thick-cut sections clearly provided good anatomic information about neurovascular structures in the three layers of bladder, such as urothelium, lamina propria, and detrusor muscle layer. Whole mount images of lamina propria and detrusor muscle layer also clearly delineated spatial relationship between nervous and vascular systems. The microvessel density was higher in the lamina propria than in the detrusor muscle layer. Nerve fibers were evenly innervated into the lamina propria and detrusor muscle.

**Conclusions:** This study provides comprehensive insight into three-dimensional neurovascular structures of mouse urinary bladder. Our technique may constitute a standard tool to evaluate pathologic changes in a variety of urinary bladder diseases.

**Keywords:** Blood vessels; Mouse; Nervous system; Three-dimensional imaging; Urinary bladder

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## INTRODUCTION

The urinary bladder is richly supplied by nerves and blood vessels [1-4]. Proper functional and structural integrity of nervous and vascular system in urinary bladder plays an important role in normal bladder function; the storage and emptying of urine [5-7]. Although the underlying mechanisms of bladder dysfunction and associated lower urinary tract symptoms (LUTS) have not yet been understood clearly, the impairments of microvascular structures and peripheral nervous system are regarded to be responsible for the development of these conditions [7]. In human studies, neurovascular injury caused by aging, diabetes, hypertension, or hyperlipidemia are known to be involved in the progression of LUTS [8,9].

Recent studies provide an evidence that crosstalk between neuronal and endothelial cells is crucial to maintain normal function of nervous and vascular systems [10,11]. While neuronal cells promote angiogenesis by secreting angiogenic factors, such as vascular endothelial growth factor, endothelial cells can also regulate development of nervous system by releasing neurotrophic factors [11,12]. It was reported that the expression of endothelin-1 and interleukin-6, which are known as biomarkers for endothelial dysfunction, are upregulated in patients with bladder outlet obstruction [13]. In addition, the derangements in microvascular structures and increase in the number of apoptotic endothelial cells have been well characterized in patients with bladder pain syndrome [14]. Microvascular dysfunction from chronic bladder ischemia and repeated ischemia/reperfusion during a micturition cycle ultimately leads to denervation of the urinary bladder [7,8].

The visualization of changes in tissue structures and cellular distribution is critical to understand the progression of many diseases. Immunohistochemistry has been used widely as a standard tool to evaluate structural changes in a variety of urinary bladder diseases. However, the most of studies characterized the urinary bladder at two-dimensional (2D) level, which provides limited anatomical information. Especially, 2D imaging from thin-cut tissue section is not appropriate to comprehensive analysis of the association between nervous and vascular systems due to limited sample depths. For decades, several methods have been developed to visualize tissue structure at three-dimensional (3D) level [15]; for example, reconstructing images from serially

stained thin-cut tissue sections, and immunostaining of whole mount preparations or thick-cut tissue sections. The disadvantages of serially sectioned samples include the requirement of large number of sections, high-cost of antibodies, and time-consuming. In contrast, limitations of whole mount samples or thick-cut tissue sections are difficulties in its technique and limited penetration of antibodies.

Although previous studies demonstrated urinary bladder innervation or suburothelial microvascular structure in human or rodents at 3D level [16-19], anatomic information about neurovascular network in whole layers of urinary bladder are still lacking. In the present study, therefore, we present an immunohistochemical staining method that delineates 3D neurovascular systems of mouse urinary bladder at a high resolution. To visualize neural and vascular network in a mouse urinary bladder, full-thickness frozen section of urinary bladder, and whole mount preparations of lamina propria and detrusor muscle layer were used.

## MATERIALS AND METHODS

### 1. Animals

Eight-week-old C57BL/6 male mice weighing 20 to 25 g were purchased from Orient Bio (Seongnam, Korea). All experiments protocols were approved by the Institutional Animal Care and Use Subcommittee of Inha University (IACUC No. 180727-580).

### 2. Tissue preparation

#### 1) Preparation of transverse full-thickness frozen section of urinary bladder

The mouse urinary bladders were harvested and immersed in 4% paraformaldehyde in phosphate buffer saline (PBS) for 24 hours at 4°C. After washing several times with PBS, samples were embedded in optimal cutting temperature compound medium. The samples were kept inside of the cryostat at -20°C for 20 minutes and thick-cut (60 µm) sections were prepared for full-thickness bladder staining.

#### 2) Preparation of lamina propria and detrusor muscle layer for whole mount staining

The mouse urinary bladders were harvested, and the urothelium and lamina propria were carefully dissected from underlying detrusor muscle with fine micro-

forceps. Urothelial layer was then removed by gentle swabbing with a Q-tip. All procedures were done with the aid of a dissecting microscope (Zeiss, Göttingen, Germany). The remaining whole lamina propria and detrusor muscle layer were flattened and fixed in 4% paraformaldehyde in PBS for 24 hours at 4°C.

### 3. Immunohistochemistry

After washing several times with PBS, samples were then immersed in 3% bovine serum albumin (BSA) for 1 hour at room temperature to minimize nonspecific binding of antibodies. Samples were incubated with antibodies to CD31 (an endothelial cell marker; Millipore, Temecula, CA, USA; 1:50; Cat #MAB1398Z), fluorescein isothiocyanate-conjugated antibody to smooth muscle  $\alpha$ -actin (a smooth muscle cell marker; Sigma-Aldrich, St. Louis, MO, USA; 1:200; Cat #F3777), smooth muscle  $\alpha$ -actin (a smooth muscle cell marker; Abcam, Cambridge, UK; 1:200; Cat #ab5694), NG2 chondroitin sulfate proteoglycan (a pericyte cell marker; Millipore; 1:50; Cat #ab5320), and  $\beta$ III-tubulin (a neuronal marker; Abcam; 1:100; Cat #ab107216) for 48 hours at 4°C. After washing several times with PBS, the samples were incubated with Donkey Anti-Mouse IgG H&L Alexa Fluor® 405 (Abcam; 1:200; Cat #ab175658), rhodamine AffiniPure Goat Anti-Armenian Hamster IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA; 1:50; Cat #127-025-160), rhodamine Affinipure Donkey Anti-Chicken IgY (H+L) (Jackson Immuno Research; 1:50; Cat #703-025-155). Primary and secondary antibodies were diluted in PBS containing 0.1% triton X-100 and 3% BSA (Sigma-Aldrich). Samples were mounted in a solution containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) for nuclei staining.

### 4. Imaging and three-dimensional reconstruction

To visualize microvascular and neural structures in the mouse urinary bladder, images were obtained from full-thickness bladder sections or whole mount preparations of lamina propria and detrusor muscle layer under confocal laser scanning microscopy (FV1000; Olympus, Tokyo, Japan). Confocal images were captured with multi-lasers (405 nm, 48 nm, and 595 nm). For 3D reconstruction, Z-stack imaging was performed from top to the bottom of tissue samples with serial images obtained from 1.0  $\mu$ m-depth. Projection of Z-

stack images and 3D reconstruction were built up by Olympus Fluoview software or ImageJ software (National Institutes of Health IMAGE J 1.34; <http://rsbweb.nih.gov/ij/>).

### 5. Ethics statement

All experiments were conducted in accordance with laboratory animal and institutional animal care guidelines. Protocols used in this study were approved by the Institutional Animal Care and Use Subcommittee of Inha University (IACUC No. 180727-580).

## RESULTS

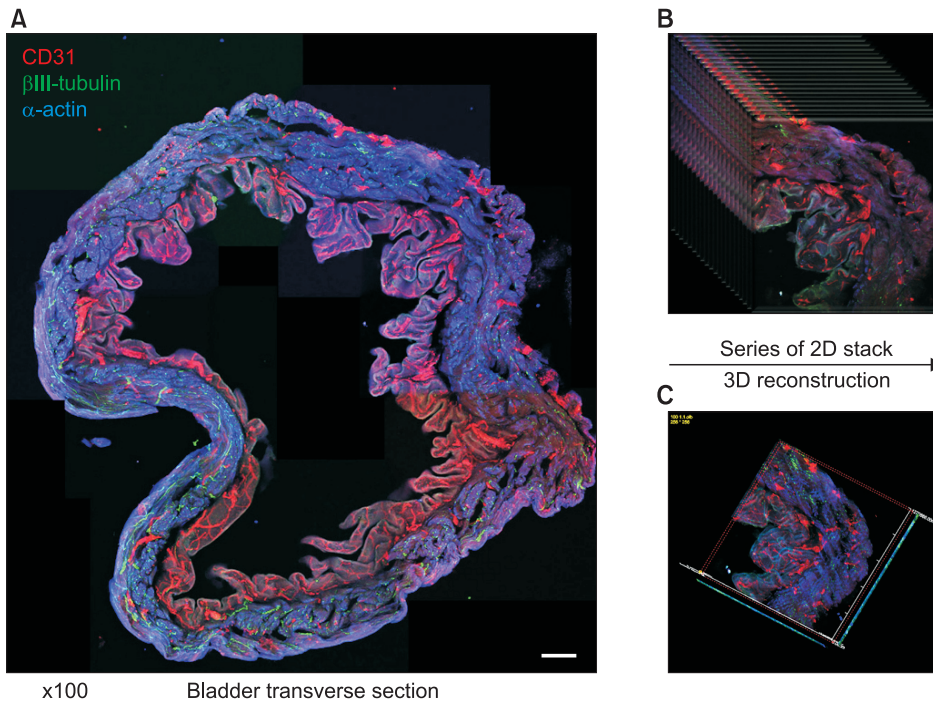
### 1. Three-dimensional reconstruction of neurovascular structures in mouse urinary bladder

High-resolution 3D images of neurovascular system in mouse urinary bladder were presented in Fig. 1 and Fig. 2. The image for full-thickness transverse section of urinary bladder was reconstructed with stacks of eight 2D images, which allow visualization of entire neurovascular system (Fig. 1A). The urothelium and lamina propria form bladder mucosa and have numerous folds that disappear when the bladder is filled (Fig. 1A). The reconstructed 3D images clearly demonstrate the distribution of CD31-positive endothelial cells,  $\alpha$ -actin-positive smooth muscle cells, and  $\beta$ III-tubulin-positive nerve fibers, and their relationship in urothelium, lamina propria, and detrusor muscle layer (projection depth: 25  $\mu$ m) (Fig. 1B, 1C).

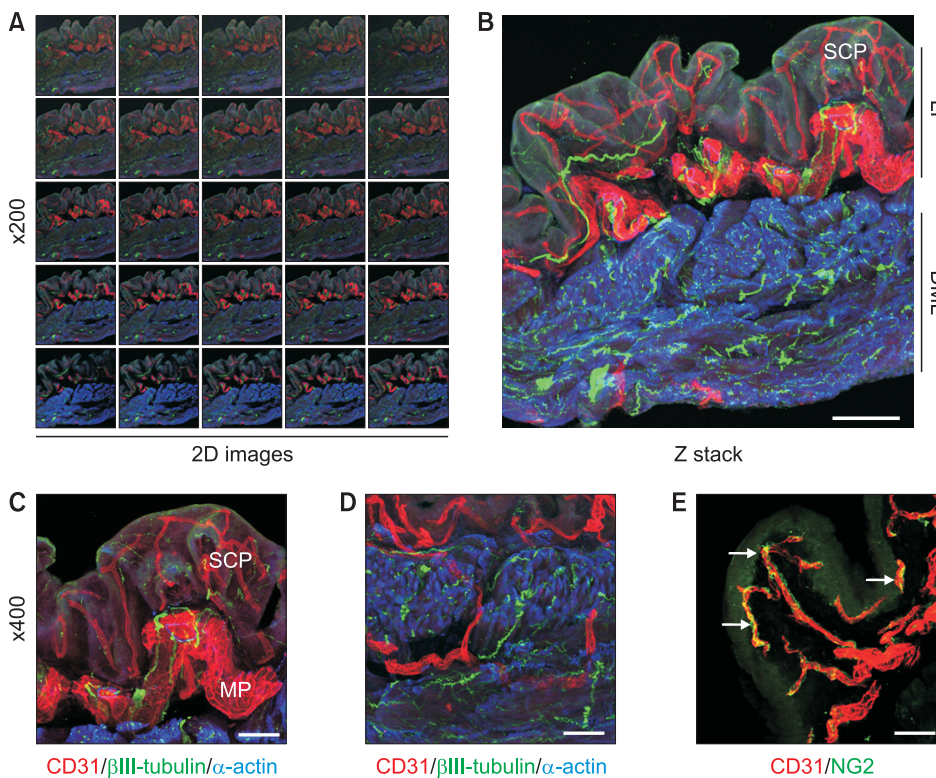
Z projection was performed from a series of 2D images, which permits the 2D dataset to be visualized as a 3D image (Fig. 2A, 2B). The three dimensional images clearly demonstrate lamina propria and detrusor muscle layer. CD31-positive suburothelial capillary plexus and mucosal plexus were well recognized in the lamina propria (Fig. 2B, 2C). The microvessel density was higher in the lamina propria than in the detrusor muscle layer (Fig. 2B). The reconstructed 3D images clearly delineate typical wavy appearance of nerve fibers that evenly innervate to lamina propria and detrusor muscle (Fig. 2B-2D). CD31-positive endothelial cells were covered with NG2-positive pericytes, especially in the suburothelial capillary plexus underneath urothelium (Fig. 2E).

In contrast, immunofluorescent double staining of bladder tissue with antibodies to CD31 and smooth mus-





**Fig. 1.** The principle of three-dimensional (3D) reconstruction of neurovascular structures in mouse urinary bladder. High-resolution 3D image was reconstructed from stacks of confocal two-dimensional (2D) images, which permit to render the volume and visualize microvascular structure and nerve innervation. (A) Transverse section of whole urinary bladder. The image was merged from stacks of eight 2D images by ImageJ software. (B, C) High-magnification images of transverse section of urinary bladder. (B) Twenty-five confocal images were obtained from the thick-cut transverse section of urinary bladder. The step size was kept at 1.0  $\mu\text{m}$  constantly. (C) 3D reconstruction of urinary bladder from stacks of twenty-five 2D images. CD31 (red): an endothelial cell marker; smooth muscle  $\alpha$ -actin (blue): a smooth muscle cell marker;  $\beta$ III-tubulin (green): a neuronal marker.



**Fig. 2.** Three-dimensional reconstruction of neurovascular structures in mouse urinary bladder. (A) Twenty-five confocal images were taken from different focal planes (60- $\mu\text{m}$  tissue section). (B) The entire sample volume were rendered and visualized by generating Z-stack image. DML: detrusor muscle layer, LP: lamina propria. High-magnification Z-stack image of bladder neurovascular system: urothelium and lamina propria (C), and detrusor muscle layer (D). CD31 (red): an endothelial cell marker; smooth muscle  $\alpha$ -actin (blue): a smooth muscle cell marker;  $\beta$ III-tubulin (green): a neuronal marker. (E) High-magnification Z stack image of vascular system in lamina propria. Please see abundant microvascular structures in mucosal plexus (MP) and suburothelial capillary plexus (SCP). CD31-positive endothelial cells were covered by NG2-positive pericytes, especially in the suburothelial capillary plexus underneath urothelium (arrows). CD31 (red): an endothelial cell marker; NG2 (green): pericyte marker.

cle  $\alpha$ -actin and subsequent 3D reconstruction (projection depth: 25  $\mu\text{m}$ ) revealed that  $\alpha$ -actin-positive smooth muscle cells are mainly expressed in perpendicular blood vessels of suburothelial capillary plexus, and in blood vessels of mucosal plexus and detrusor muscle (Fig. 3).

## 2. Neurovascular structures of lamina propria in a whole mount preparation

High-resolution 3D reconstruction of neurovascular system in lamina propria of urinary bladder was performed by using whole mount immunostaining (Fig.



4A). The complexity of vascular structures and nerve fibers, and their relationship were clearly delineated by Z projection (Fig. 4A) and 3D reconstruction (Fig. 4B-4E). The 3D images demonstrate abundant distribution of vascular structures and nerve fibers in the lamina propria. The relatively large blood vessels were covered

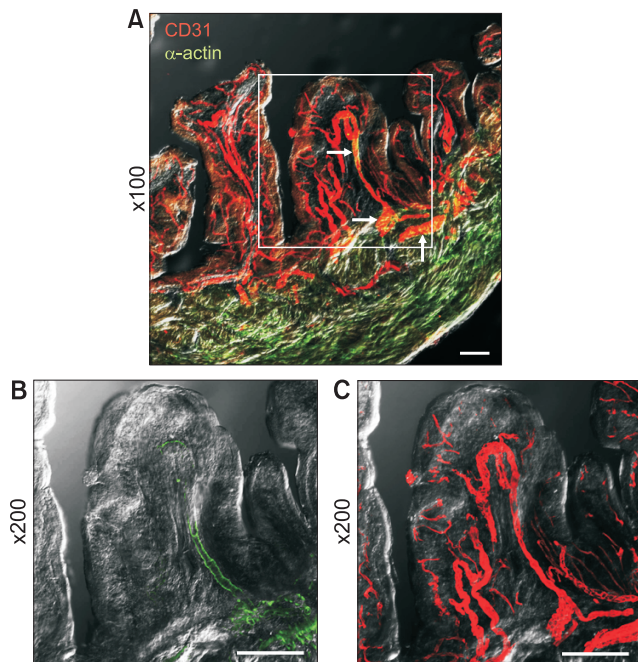
with  $\alpha$ -actin-positive smooth muscle cells. Moreover, nerve fibers also run very close to the large-caliber blood vessels (Fig. 4A).

### 3. Neurovascular structures of detrusor muscle layer in a whole mount preparation

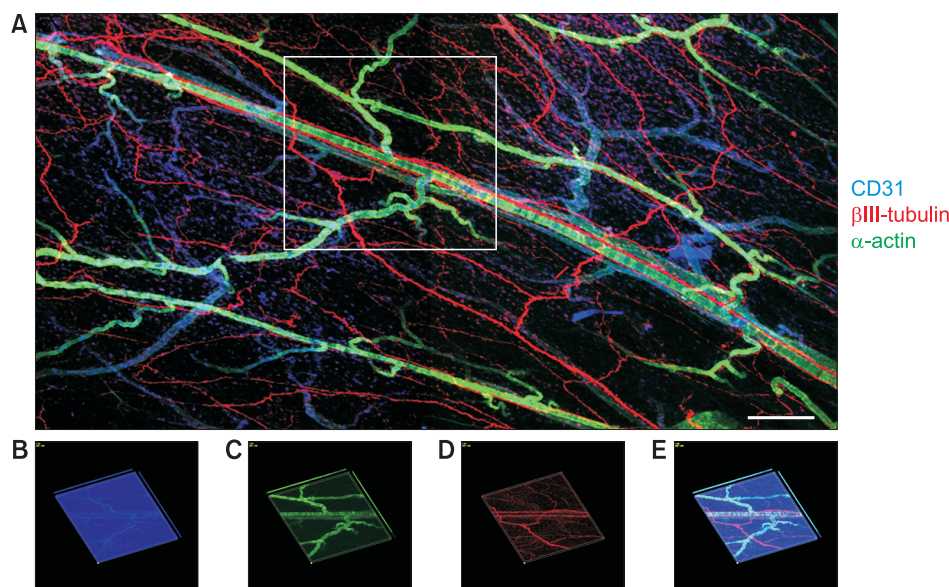
We also successfully demonstrated neurovascular structures in detrusor muscle layer by using whole mount immunostaining. Similar to the findings in the lamina propria, the large-diameter blood vessels are covered with  $\alpha$ -actin-positive smooth muscle cells and run parallel to the nerve fibers (Fig. 5).

## DISCUSSION

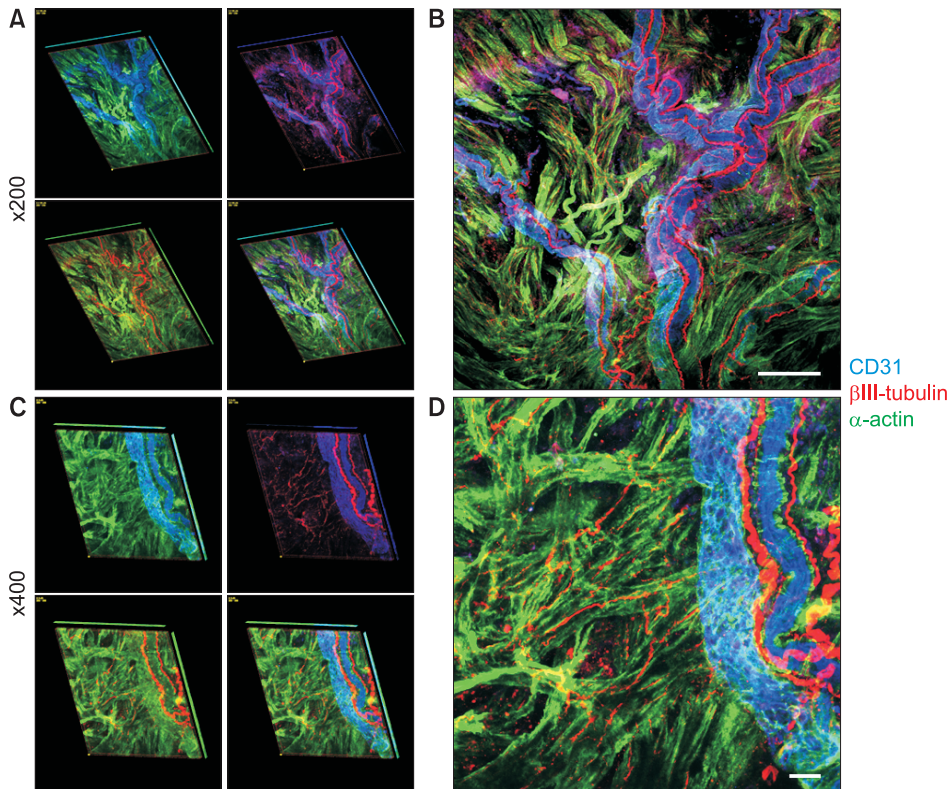
The wall of urinary bladder consists of transitional epithelial lining supported by lamina propria and muscularis [3]. Although conventional 2D imaging from thin section of urinary bladder is often useful to visualize some cell types, the spatial distribution of certain anatomic structures, such as nervous and vascular systems, cannot be readily appreciated in two-dimensions. We successfully visualized neurovascular structures in whole mount preparations of lamina propria and detrusor muscle as well as full-thickness thick-cut bladder sections by increasing incubation time with primary antibodies up to 48 hours. It was reported that incubation time in primary antibodies should be extended up to 7 days for complete staining in rectal whole mounts [19].



**Fig. 3.** Three-dimensional (3D) reconstruction of vascular structures in mouse urinary bladder. 3D reconstruction from stacks of 25 two-dimensional images at magnifications of  $\times 100$  (A) and  $\times 200$  (B, C).  $\alpha$ -actin-positive smooth muscle cells are mainly expressed in main arteries of suburothelial plexus, mucosal plexus, and detrusor muscle layer (arrows). CD31 (red): an endothelial cell marker; smooth muscle  $\alpha$ -actin (green): a smooth muscle cell marker.



**Fig. 4.** Neurovascular structures in a whole mount preparation of the lamina propria. (A) The complexity of vascular structures and nervous innervation, and their relationship delineated by three-dimensional (3D) reconstruction of whole mount staining. (B-E) Reconstruction of a 3D image from stacks of two-dimensional images in selected area (white square). CD31 (blue): an endothelial cell marker; smooth muscle  $\alpha$ -actin (green): a smooth muscle cell marker;  $\beta$ III-tubulin (red): a neuronal marker.



**Fig. 5.** Neurovascular structures in a whole mount preparation of the detrusor muscle layer. (A-D) The distribution of intramuscular nerve fibers and vascular structures delineated by three-dimensional (3D) reconstruction of whole mount staining. 3D reconstruction from stacks of two-dimensional images at magnifications of  $\times 200$  (B) and  $\times 400$  (D). CD31 (blue): an endothelial cell marker; smooth muscle  $\alpha$ -actin (green): a smooth muscle cell marker;  $\beta$ III-tubulin (red): a neuronal marker.

In the present study, nerve fibers run very close to the large vessels, but not in small vasculatures in whole mount preparations of both lamina propria and detrusor muscle layer. The dense perivascular plexuses of  $\beta$ III-tubulin-positive nerve fibers around blood vessels are clearly documented with whole mount preparations. The detrusor muscle layer mainly consists of smooth muscle cells, nerves, and blood vessels. Different from lamina propria, it is very difficult to visualize neurovascular structure of detrusor layer, because of the dense distribution of smooth muscle and its non-transparent nature. Few studies have provided detailed anatomy of nervous and vascular system in detrusor muscle layer. In this study, we have been succeeded to reconstruct 3D neurovascular network by use of whole mount immunofluorescent staining with antibodies against to endothelial cells, smooth muscle cells, and neuronal cells. The intramuscular innervation and blood supply as well as their relationship were clearly recognized at high resolution, which permits to expand our understanding of neurovascular anatomy in detrusor muscle layer. To the best of our knowledge, this is the first study demonstrating neurovascular structures in a whole mount preparation of detrusor muscle layer. Similar to the results from previous results [20], the

major targets of nerve fibers were urothelium and blood vessels.

We performed immunohistochemical staining with antibodies to CD31, smooth muscle  $\alpha$ -actin, and NG2 to visualize blood vessels in the urinary bladder. The mucosal plexus runs parallel to the surface of mucosal folds and gives off straight and perpendicular blood vessels interconnecting with the suburothelial capillary plexus. The suburothelial capillary plexus shows rich in vascular density and uneven distribution of capillaries. On the contrary, the capillary network was poorly developed in the detrusor muscle layer. The rich vascular supply in lamina propria may be attributable to its three times higher metabolic rate than that of the detrusor muscle [21].

NG2-positive pericytes are mainly distributed in the suburothelial capillary plexus underneath the urothelium, whereas  $\alpha$ -actin-positive smooth muscle cells are predominantly expressed in relatively large-diameter blood vessels. This finding is similar to the results from previous results demonstrating distinct distribution of pericytes and smooth muscle cells in the mucosa of mouse urinary bladder [18]. Pericytes are a population of contractile cells surrounding endothelial cells of microvessels, such as arterioles, capillaries, and venules.



The functional roles of pericytes include the regulation of vascular contractility, permeability, and development [22]. We recently demonstrated the dropout of pericytes and subsequent increase in vascular permeability in the corpus cavernosum of diabetic mice [23,24]. Therefore, further studies are required to determine the differential distribution of pericytes and their functional role in a variety of bladder disorders.

It was also demonstrated the difference in cell morphology between venules and arterioles;  $\alpha$ -actin-positive smooth muscle cells are distributed circumferentially in arterioles, whereas venules has stellate-shaped smooth muscle cells [18]. Our technique also clearly demonstrated distinct morphologic features between venules and arterioles at a high-magnification image (data not shown).

Immunohistochemically stained whole mounts and thick-cut sections of mouse urinary bladder have provided more valuable information for defining the innervation and the distribution of blood vessels than in sections of conventional thickness. By using this technique, we could delineate the entire urinary bladder innervation and vascular supply. Especially, whole mount preparations of lamina propria and detrusor muscle layer have nicely demonstrated the running course of the nerve fibers as well as their association with vascular structures. With advent of genetically modified mice, information on the fine details of neurovascular structures of the mouse urinary bladder will give us an additional value. Further studies are needed to determine the differential distribution of specific nerve fibers, such as sympathetic, parasympathetic, and somatic nerves.

## CONCLUSIONS

The visualization of neurovascular structures is crucial to expand our understanding on the pathophysiology of urinary bladder disorders in a variety of conditions. We have developed an effective, rapid, and highly reproducible imaging technique to visualize neurovascular systems in mouse urinary bladder. Our 3D imaging technique that utilizes immunofluorescent staining of full-thickness frozen section of urinary bladder or whole mount preparations of lamina propria and detrusor muscle layers clearly demonstrates association between nervous and vascular systems. Our technique may constitute a standard tool to evaluate

pathologic changes in a variety of urinary bladder diseases.

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## Conflict of Interest

The authors have nothing to disclose.

## Author Contribution

Conceptualization: NMN, CSP, JKS, JKR. Data curation: NMN, KMS, MJC, KG, AL. Formal analysis: MHK, DYC, JO, GNY. Software: MJC, MHK. Funding acquisition: JKR. Writing—original draft: NMN, KMS, JKR. Writing—review & editing: JKS, JKR

## Data Sharing Statement

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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