Three-dimensional imaging of podocyte ultrastructure using FE-SEM and FIB-SEM tomography

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Abstract
Podocytes are specialized epithelial cells used for glomerular filtration in the kidney. They can be divided into the cell body, primary process and foot process. Here, we describe two useful methods for the three-dimensional (3D) visualization of these subcellular compartments in rodent podocytes. The first method, field-emission scanning electron microscopy (FE-SEM) with conductive staining, is used to visualize the luminal surface of numerous podocytes simultaneously. The second method, focused-ion beam SEM (FIB-SEM) tomography, allows the user to obtain serial images from different depths of field, or Z-stacks, of the glomerulus. This allows for the 3D reconstruction of podocyte ultrastructure, which can be viewed from all angles, from a single image set. This is not possible with conventional FE-SEM. The different advantages and disadvantages of FE-SEM and FIB-SEM tomography compensate for the weaknesses of the other. The combination renders a powerful approach for the 3D analysis of podocyte ultrastructure. As a result, we were able to identify a new subcellular compartment of podocytes, “ridge-like prominences” (RLPs).

Keywords FE-SEM · Conductive staining · FIB-SEM tomography · 3D reconstruction · Podocyte · Foot process effacement

Introduction
Podocytes are specialized epithelial cells used for glomerular filtration, which are classically divided into three subcellular compartments: the cell body, the primary process and the foot process. Here, we describe two useful methods for the three-dimensional (3D) visualization of these subcellular compartments in rodent podocytes. The first method, field-emission scanning electron microscopy (FE-SEM), is used to visualize the luminal surface of numerous podocytes simultaneously (Kinugasa et al. 2011; Masum et al. 2018; Tagawa et al. 2016). The second method, focused-ion beam SEM (FIB-SEM) tomography, allows the user to obtain serial images from different depths of field, or Z-stacks, of the glomerulus (Arkill et al. 2014; Burghardt et al. 2015). This allows for the 3D reconstruction of podocyte ultrastructure, which can be viewed from all angles, in a single image set. This is not possible with conventional FE-SEM. Our research group analyzed the 3D ultrastructure of podocytes in health, disease and development by utilizing FE-SEM and FIB-SEM tomography (Ichimura et al. 2017; Ichimura et al. 2019; Ichimura et al. 2015; Ichimura and Sakai 2017; Kawasaki et al. 2019). Here, we demonstrate their advantages and limitations in podocyte ultrastructural study.

Materials and methods
Animals
For investigating the 3D ultrastructure of normal podocytes, we used 10-week-old male rats (Charles River Japan,
Perfusion fixation of kidneys

An arterial catheter (PE50, BD Intramedic Polyethylene Tubing, Thermo Fisher Scientific) was retrogradely inserted via the abdominal aorta, below the renal arteries, under anesthesia with pentobarbital. To wash out blood from the vascular system, the inferior vena cava was incised, then, physiological saline was perfused for 2–3 min. The abdominal organs, including kidneys, whiten due to blood removal. Subsequently, 2.5% glutaraldehyde/0.1 M phosphate buffer (PB) was perfused for 3 min, which makes the abdominal organs become hard and turn yellowish. Perfusion of saline and fixative should be performed at 180–200 mmHg using a constant-pressure perfusion apparatus, such as a VPF-1 perfusion apparatus (Nissin EM, Tokyo, Japan). The fixed kidneys were immersed in the same fixative for 2–3 days at 4 °C.

FE-SEM with conductive staining

For the step-by-step protocol of FE-SEM with conductive staining, see Supplementary Method S1.

Conductive staining The cortex of the fixed kidney was cut into small cubes (approximately 4 × 4 × 2 mm). After being washed with 0.1 M PB, the cubes were processed with conductive staining. First, the cubes were immersed in 1% osmium tetroxide (OsO₄) in 0.1 M PB for 30 min at 24 °C; washed with 0.1 M PB for 5 min three times; and then immersed in 1% low molecular weight tannic acid (LMW-TA, Electron Microscopy Sciences, Hatfield, PA, USA) in distilled water (DW), for 2 h at RT. After the cubes were washed with DW for 5 min three times, the same staining was repeated twice. However, the OsO₄ was diluted with DW.

Dehydration, drying and mounting The stained samples were dehydrated with graded series of ethanol (50% for 5 min; 70% for 5 min; 90% for 5 min; 100% for 5 min, five times) and then immersed in tert-butyl alcohol for 5 min, three times. The samples were freeze-dried with an ES-2030 freeze dryer (Hitachi High-Technologies, Tokyo, Japan). The dried specimens were mounted on aluminum stubs with carbon tape (Cat #732, Nissin EM) and paste (Pelco Colloidal Graphite, Ted Pella, Inc. Redding, CA, USA). The mounted specimens were coated with osmium with an OPC80T osmium plasma coater (Filgen, Inc., Nagoya, Japan).

FE-SEM The samples were observed with an S-4800 FE-SEM (Hitachi High-Technologies). Regions of interest were imaged using a backscattered electron detector with an acceleration voltage of 3 kV. The pixel dimensions for a recorded image were 3072 × 2048 pixels. Individual podocytes are colored with different transparent colors using Adobe Photoshop or Illustrator. This procedure was made easier by using a Cintiq 27QHD interactive pen display (Wacom, Tokyo, Japan).

FIB-SEM tomography and 3D reconstruction

For the step-by-step protocol of FIB-SEM tomography and 3D reconstruction, see Supplementary Method S2.

Combinatorial heavy metal block staining The cortex of the fixed kidney was cut into 250-μm-thick slices with a DTK-1000 Microslicer (Dosaka EM, Kyoto, Japan). The slices were processed using the combinatorial heavy metal staining protocol. This protocol was designed to enhance signal for backscatter electron imaging of epoxy resin–embedded mammalian tissues, at low accelerating voltages. The slices were successively immersed in 1% OsO₄, containing 1.5% potassium ferrocyanide (K₄Fe(CN)₆·3H₂O), in 0.1 M cacodylate buffer (CB) for 1 h on ice; 1% LMW-TA (Electron Microscopy Sciences) in 0.1 M CB, for 4 h at 24 °C; 2% aqueous OsO₄ for 30 min at 24 °C; and 1% aqueous uranyl acetate (UO₂(CH₃COO)₂·2H₂O) overnight at 24 °C. Between each staining step, the slices were washed with 0.1 M CB or DW for 5 min, three times.

Dehydration and embedding The stained samples were then dehydrated with a graded series of ethanol (50% for 5 min; 70% for 5 min; 90% for 5 min; 100% for 5 min, five times). The dehydrated samples were successively immersed in propylene oxide for 5 min, twice; 50% epoxy resin in propylene oxide, on a rotational mixer, for 3 h; and then pure epoxy resin, on a rotational mixer, overnight. Subsequently, the samples were embedded in newly made pure epoxy resin and hardened in a heated air incubator for three to 5 days. We used a hardening formula of Oken Epok 812 epoxy resin kit (Oken-shoji, Tokyo, Japan).

Mounting and coating The surface of resin-embedded tissues was exposed using a diamond knife on an Ultracut UCT (Leica Biosystems). The block was mounted onto an HV-8 aluminum stub (Micro Star, Tokyo, Japan). The space between the block and stub was filled with carbon paste (Pelco Colloidal Graphite, Ted Pella, Inc.) and then coated with a thin layer of platinum-palladium, using an MC1000 ion sputter coater (Hitachi High-Technologies) to prevent charging.
FIB-SEM tomography

The exposed surface of embedded tissue was imaged with a Helios NanoLab 660 FIB-SEM (Thermo Fisher Scientific, Waltham, MA, USA), at a high acceleration voltage of 10 kV, to find the area of interest. To prevent beam damage, a platinum layer was deposited on the area of interest, with a 2.5-nA beam current, where gallium ions were accelerated by a voltage of 30 kV. To make the new block-face (imaging-face), a trench was cut using FIB-milling, with a 25-nA beam current, where gallium ions were accelerated by a voltage of 30 kV. Serial FIB-SEM images were acquired with Auto Slice and View imaging software (Thermo Fisher Scientific) on a Helios NanoLab 660 FIB-SEM. New surfaces for serial block-face imaging were generated using FIB-milling, with a 0.77-nA beam current. Serial block-face images (300–800) were obtained every 50 nm in depth, with a backscattered electron detector at an acceleration voltage of 2.0 kV. The pixel size of each FIB-SEM image was 13.5 × 17.1 × 50 nm/pixel (width × height × depth). The pixel dimensions for a recorded image were 3072 × 2048 pixels. Thus, the dimension of the serial images acquired using FIB-SEM was 41.5 × 35.0 × 50 μm (width × height × depth).

3D reconstruction

The alignment of serial FIB-SEM images, segmentation of target podocytes and 3D reconstruction were performed using AMIRA 6.1 reconstruction software (Thermo Fisher Scientific). We also used a Cintiq 27QHD interactive pen display for the segmentation procedure (Wacom). The 3D images could be presented as a movie or 3D-printed model.

Results and discussion

FE-SEM

The FE-SEM procedure, for 3D ultrastructural analysis of podocytes, is divided into eight major steps (Fig. 1): (1) perfusion fixation at a high hydrostatic pressure; (2) conductive staining; (3) dehydration; (4) freeze-drying; (5) mounting on a stub; (6) coating with heavy metal; (7) image acquisition; (8) coloring (optional) (for the step-by-step protocol for FE-SEM, see Supplementary Method S1). All steps should be adequately performed to prevent artificial structural alterations. In particular, the first and second steps are crucial in realizing a clear presentation of the podocyte surface ultrastructure.

It is essential that glomerular capillaries are fixed in the distended state (using step 1), to prevent their collapse and, thus, distortion of the podocyte architecture.

Fig. 1. Outline protocol for FE-SEM. Procedure for FE-SEM is divided into eight steps (1–8). (a) Freeze-dried samples on an aluminum stub. (b) FE-SEM image of normal rat podocytes. (b′) Individual podocytes in the FE-SEM image, which have been colored by Adobe Illustrator image software. The step-by-step protocols for FE-SEM are described in Supplementary Method S1.

The staining procedure is often conducted only using 1% OsO4. This is a simple method but the podocytes can end up appearing distorted and somewhat shrunken (Fig. 2b). Instead,
we consider the more invested conductive staining method, which maintains the size of the cells and the integrity of the glomerular capillaries without artificial distortions during subsequent steps (Fig. 2a).

The coloring of individual podocytes, in the FE-SEM images, is useful for understanding the architecture and mutual relationship of the podocytes (Figs. 1b' and 3). Such coloring can be easily performed by commercially available image software, such as Adobe Photoshop or Illustrator.

FE-SEM, with conductive staining, is an easy and efficient method for visualizing the luminal surface of numerous podocytes in different glomeruli, simultaneously. It is also useful for analyzing the outline of the 3D podocyte architecture without structural distortions. However, there are several
The basal surface and the regions between glomerular capillaries are quite difficult to observe by FE-SEM. Thus, it cannot be used to image the entire surface of individual podocytes.

**FIB-SEM tomography**

The procedure for FIB-SEM tomography, for the 3D structural analysis of podocytes, is divided into 11 major steps (Fig. 4): (1) perfusion fixation at a high hydrostatic pressure; (2) combinatorial heavy metal staining; (3) dehydration; (4) embedding in resin; (5) surface exposure; (6) mounting on a stub; (7) coating with heavy metal; (8) generating a new block-face; (9) serial image acquisition; (10) segmentation of podocytes; (11) 3D reconstruction (for the step-by-step protocol for FIB-SEM tomography, see Supplementary Methods S2). All steps should be performed to prevent artificial structural alterations. In particular, steps (1), (2) and (9) are necessary to achieve a clear presentation of the podocyte ultrastructure and 3D reconstruction.

Combinatorial heavy metal staining is used to increase the contrast in FIB-SEM images. Several protocols for this staining method have been reported by different research groups (summarized in Kubota et al. 2018). Our protocol is relatively simple. Uranyl acetate (UO₂(CH₃COO)₂) is generally used for this staining method; however, it is an internationally controlled material and requires extreme handling caution. Instead, samarium triacetate (Sm(CH₃COO)₃) or gadolinium triacetate (Gd(CH₃COO)₃) could be used (Nakakoshi et al. 2011; Odriozola et al. 2017).

The manual segmentation of podocytes from serial FIB-SEM images is made much simpler by using an interactive pen display (Fig. 5), which is also useful for the coloring of podocytes in FE-SEM images.

The 3D reconstruction data can be represented by a 3D-printed plastic model (Fig. 2d′) and as a movie (Supplementary Movies S1, S2 and S3). Such models and movies are useful for comprehensively demonstrating the 3D ultrastructure of podocytes, especially in histology and pathology education for medical students.

The 3D-reconstructed images of single podocytes, generated from serial FIB-SEM images, allow the visualization of...
FIB/SEM tomography

1. Perfusion fixation
2. Combinatorial metal staining
3. Dehydration
4. Embedding in resin
5. Exposure of tissue
6. Mounting on a stub
7. Coating with Pt-Pd

8. Generating a new surface

9. Serial image acquisition

10. Segmentation of podocytes
11. 3D reconstruction
Fig. 4. Outline protocol for FIB-SEM tomography. Procedure of FIB-SEM is divided into eleven major steps (1–11). (a) Resin-embedded block mounted on an aluminum stub and coated with platinum-palladium (Pt-Pd). (b) Schematic drawing of the positional relationships of the sample (yellow), the FIB column (pink) and the SEM column (green). The cross-sectional surface, called the “block-face” (asterisk), is generated by FIB milling. The block-face is perpendicular to the surface of the sample. The SEM column is situated obliquely to scan the block-face. (b′) Low-magnification image of the block-face. (c) The contrast-inverted serial FIB-SEM images are similar to a conventional transmission electron microscope (TEM) image of an ultrathin section. The generation and imaging of the block-face is automatically performed to acquire serial FIB-SEM images. (d) Reconstructed images of a single podocyte produced from the serial FIB-SEM images, using reconstruction software. (d′) 3D-printed podocyte. The detail and step-by-step protocols for FIB-SEM are described in Supplementary Method S2.

Fig. 5. Interactive pen display used for manual segmentation in serial FIB-SEM images. (a) Interactive pen displays used in our laboratory (Cintiq 27QHD, Wacom, Tokyo, Japan). (b) Segmentation of a targeted podocyte in serial FIB-SEM images. The area in red indicates the segmented podocyte.
Although FIB-SEM tomography is a powerful tool for precisely analyzing the 3D ultrastructure of podocytes, this method has several technical disadvantages. The imaged region is completely lost due to the required FIB milling. Moreover, the observational area is restricted in FIB-SEM tomography, rendering it impossible to visualize the entire glomerular surface. Array tomography is a potential solution to this problem. It has been used to obtain serial images from a series of ultrathin
sections mounted on silicon wafer and glass plate substrates (Koga et al. 2018; Koike et al. 2017). These ultrathin sections have been shown to be physically stable and repeatable. In future investigations, we aim to establish a “serial ultrathin sections library” of renal biopsy specimens and to carry out analysis using array tomography.

In our laboratory, we manually segmented podocytes on 200–500 serial FIB/SEM images using Amira software. This manual segmentation procedure is the main rate-determining step in our 3D structural analysis of podocytes, although it becomes easy to perform using the interactive pen display, as described above. For instance, it took approximately 25 and 50 h to segment podocytes for producing the reconstruction images shown in Figs. (6c, 7a), respectively. Various image analysis software containing an automated segmentation tool have been reported and some of them are commercially and freely available (Moen et al. 2019). However, there has been no successful case that podocytes could be automatically segmented at a high level of quality—the special complexity of podocyte structure makes it quite difficult to automatically segment them. To make the 3D ultrastructural analysis of podocytes more efficient, a novel tool needs to be developed using artificial intelligence that is specified for automated segmentation of podocytes.

**Conclusion** The different advantages and disadvantages of FE-SEM and FIB-SEM tomography allow each technique to compensate for the weaknesses of the other. The combination of these produces a powerful approach for the 3D analysis of podocyte ultrastructure. As a result, we were able to identify a new subcellular compartment of podocytes, RLPs.

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Compliance with ethical standards

Conflict of interest  The authors declare that they have no conflict of interest.

Informed consent  Not applicable.

Ethical approval  Animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Juntendo University School of Medicine (approval no. 300226) and were carried out in accordance with the Guidelines for Animal Experimentation of Juntendo University School of Medicine. This article does not contain any studies with human participants performed by any of the authors.

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