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Serial block face-scanning electron microscopy: a method to study retinal degenerative phenotypes

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Abstract

Retinal degenerative conditions can vary in their clinical presentation and often present with subtle phenotypic features before the onset of clinically overt disease. To capture these isolated events that precipitate disease, large representative areas of the retina must be imaged at high resolution. Compared to light microscopic methods, traditional electron microscopy can provide images at sufficient resolution to detect subtle pathologic changes in the retina, but are limited to the area being surveyed. The advent of serial block face-scanning electron microscopy (SBFSEM) provides the resolution needed with the unprecedented advantage of imaging large volumes of retinal tissue. Furthermore, automation of SBF-SEM bypasses errors from manual sectioning and can produce reliable serial sections as thin as 25 nanometers. Moreover, the three-dimensional structures generated can highlight cellular connectivity and interactions in the retina and reveal pathological changes. Using SBF-SEM, we have identified subtle phenotypic features in mouse models of various human retinal dystrophies. This method will allow researchers to identify and monitor the time course of these pathologies. Here, we summarize the SBF-SEM methodology and its application to mouse models of retinal degeneration.

Keywords

Retina; retinal degeneration; scanning electron microscopy; serial block face; vision; photoreceptor(s)

INTRODUCTION

Visual perception occurs through absorption of light by photoreceptor neurons in the retina (Palczewski, 2012). These neuronal layers that support human vision are essential components of a phototransduction cascade that signals the occipital cortex of the brain for visual processing. Rod and cone photoreceptor cells, often collectively called ‘photoreceptors’, are post-mitotic neurons subject to significant stress throughout life. Subtle phenotypic changes in these cells can lead to profound visual pathology, but poor understanding of the precipitating features of disease have limited successful therapies. Moreover, features of clinical disease often present at a stage when pharmacologic therapy can no longer be effective. This is typified by the paucity of effective therapeutics for retinal degenerative conditions such as age-related macular degeneration, Stargardt disease, retinitis pigmentosa, and diabetic retinopathy. However, recent use of mouse models of disease has highlighted certain phenotypic features that hold promise for earlier detection and treatment

of these retinal degenerative conditions (Mustafi et al., 2012, Chen et al., 2013, Du et al., 2013, Zhang et al., 2013).

Although retinal diseases differ with respect to the principle cell type affected, the time frame of degeneration, as well as the underlying genetic drivers, they all require a unifying high-resolution imaging technique to capture subtle phenotypic features that are the predecessors of clinical degeneration. To detect such changes, high-resolution non-invasive images captured over multiple retinal sections would be an important advance. Techniques that rely on light microscopy such as those employing fluorescence labeling have been used to display neural circuitry in transgenic mice (Livet et al., 2007), but cannot resolve neuronal features with diameters below the wavelength of light. Electron microscopy provides the necessary resolution, but traditional transmission electron microscopy (TEM) lacking z-direction penetration, limits the reliability of three-dimensional reconstructions from serial sectioning. Other three-dimensional methods such as cryo-electron tomography (Nickell et al., 2007) are restricted by their size limitations.

Denk and colleagues introduced the technique of serial block face-scanning electron microscopy (SBF-SEM), an innovative method for examining large volumes of neuronal tissue (Denk and Horstmann, 2004). SBF-SEM involves sectioning a resin-embedded tissue sample with an automated microtome inside a scanning electron microscope. Through this automated procedure, many issues of manual sectioning can be resolved and a large volume of tissue can be imaged. Each time the block is sectioned, a high-resolution image is captured through the back scattering of electrons. With the compilation of these images, a high-resolution 3D model of retinal ultrastructure can be formed. These models and images allow a better understanding of both the dynamics of basic retinal physiological processes (Mustafi et al., 2013) and the subtle phenotypic features of various retinal degenerative disorders (Mustafi et al., 2011, Zhang et al., 2013). Here we describe a method for preparing retinal tissue from mice for analysis with SBF-SEM technology. This allows three-dimensional viewing of rod and cone photoreceptors and internal disc elements across the retina, which then can be applied to the structural understanding of retinal degenerative conditions.

BASIC PROTOCOL 1: PREPARATION OF RETINAL TISSUE FOR SERIAL BLOCK FACE IMAGING

Here we describe the steps needed to isolate eye tissue from mice and procedures to prepare the tissue for downstream SBF-SEM experiments covered under Basic Protocol 2. Optimal procedures are presented for tissue fixation, staining, and washing that generate the best signals when subjected to electron imaging. These will vary by user and could require subtle optimization after examination of images from the final block-embedded sample.

NOTE: All protocols and procedures involving animals must be approved by an Institutional Animal Care and Use Committee (IACUC).

Materials

Mice, in this case C57BL/6J animals (Jackson Laboratory)

Basic dissection kit (forceps, scissors, scalpel, micro-dissection scissors) Surgical dissecting microscope 18½ gauge needle

Phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4)

Paraformaldehyde solution (4% in PBS)

Shaker set to 37°C

Osmium tetroxide (OsO₄) solution (2% in PBS)

Potassium ferrocyanide (3% in PBS)

Uranyl acetate solution (0.25% in PBS)

Ethanol

Propylene oxide

Poly/Bed 812 Embedding Kit (Poly/Bed, DDSA, NMA), with DMP-30 (Ted Pella, Inc)

Tissue culture rotator (Ted Pella)

Desiccator with vacuum

96 well plate

Incubator set to 73°C

Block mold

Tissue Preparation

1. Mice should be euthanized by cervical dislocation.
2. The tissue around the eye socket should be carefully dissected to expose the eye. It is important not to place pressure on the eye with forceps as this can disrupt the integrity of the retina-retinal pigmented epithelium (RPE) connection (Figure 1A, B).
3. The eye should be placed in 4% paraformaldehyde in PBS and placed under a surgical microscope at room temperature.
4. An incision should be made with an 18½-gauge needle and, using surgical scissors, the cornea should then be dissected away and the lens and vitreous carefully removed (Figure 1C).

Eye cup fixation and heavy metal staining

- 5 Place the dissected eye cups in 4% paraformaldehyde in PBS at 37°C for 4 hours with gentle agitation on a shaker to infuse the tissue with fixative. Eye cups can be placed in wells of a 96 well plate to facilitate future wash steps.
- 6 Wash the eye cups with PBS three times, which entails pipetting the solution in and out of the well. All incubation steps should be done on a shaker with gentle shaking to perfuse the tissue.
- 7 Incubate eye cups in a 1:1 solution of 2% OsO₄ and 3% potassium ferrocyanide for 1 hour.
- 8 Remove the solution from each well and incubate the eye cups in a new mixture of 2% OsO₄ : 3% potassium ferrocyanide for 1 hour.
- 9 Wash eye cups with filtered water three times, with 5 minutes between each wash.
- 10 Incubate eye cups in 0.25% uranyl acetate overnight at 4°C. Wrap the well plate in foil to protect it from light.

Eye cup dehydration

- 11 Carry out the dehydration steps of the eye cups in small vials with the caps open to air with the vials gently rotating in a tissue culture rotator at room temperature.
- 12 Start the dehydration process of the eye cups by initially placing them in increasing concentrations of ethanol for 10 minutes each. Concentrations will be 30%, 50%, 75%, 85%, 95% and 100% ethanol in water.
- 13 Continue dehydrating the eye cups in increasing concentrations of propylene oxide for 15 minutes each. Concentrations will be 50%, 75%, and 100% propylene oxide in ethanol.

Eye Cup Resin Block Polymerization

- 14 Place the eye cups in 30% Epon resin (can be made from Poly/Bed 812 Embedding Kit with DMP-30) in propylene oxide for 2 hours.
- 15 Place the eye cups in 50% Epon resin in propylene oxide overnight
- 16 The next morning, transfer the eye cups to 75% Epon resin in propylene oxide for 4 hours.
- 17 Finally, transfer the eye cups to 100% Epon resin for 2 hours under a vacuum to remove all air bubbles.
- 18 Place the eye cups in molds with 100% Epon resin and incubate at 73°C for 4 days to allow resin crosslinking to occur.

BASIC PROTOCOL 2: PREPARATION OF RETINAL TISSUE FOR SERIAL BLOCK FACE IMAGING

Here we describe the steps needed to prepare the block embedded retinal tissue for SBF-SEM. These steps for the block set up and cutting experiments will need to be tailored to the particular instrument being used.

Materials

Carbon grids

Transmission electron microscope (Tecnai T12 electron microscope, FEI)

Ultramicrotome (Leica UCT)

Diamond knife (Diatome)

SEM stubs

Carbon cement

Serial block face-scanning electron microscope (Quanta 200 FEG ESEM; FEI) Digital microscope software

Fiji software

Reconstruct program

Block Preparation and Area Selection for Sectioning and Imaging

1. Shave the eye cup blocks down with a diamond knife blade to expose the edges of the eye cup (Figure 2A).
2. Cut the blocks with a microtome, and stain ultrathin (0.07 μm) sections with uranyl acetate and adsorb onto carbon grids
3. Image the carbon grids using an electron microscope to evaluate the block stain preparation, layer integrity, and areas amenable for serial sectioning. (We used a Tecnai T12 electron microscope operating at 120 kV with a tungsten filament).
4. Once the area of interest is identified, use an ultramicrotome and a diamond knife to trim the block so that only the resin-embedded tissue of the region of interest remains exposed.
5. Adhere the final tissue block to an aluminum SEM stub with conductive carbon cement to preserve conductivity and then place sample in an incubator at 37°C overnight.

Mounting Block on SEM Stub in the SBF-SEM

6. Position the SEM stub containing the block in the microtome (3View, Gatan) attached to the door of the SEM (Quanta 200 FEG ESEM) (Figure 2B).

- 7 Slowly raise the stub until it is nearly level with the top edge of the block (Figure 2C).
- 8 Initiate cutting in the evacuated specimen chamber. Course cuts should be initially taken until the block is approached and areas of interest are visible when imaged.

Initiating SBF-SEM automation

- 9 Use a low vacuum setting to prevent charging artifacts. (We used a chamber pressure of 0.23 torr).
- 10 Carry out imaging using the backscattered electron signal. (We used a beam voltage of 3 kV).
- 11 Identify and focus on areas of interest. Multiple high resolution image windows can be set-up to survey large surface areas. A 10% overlap should be maintained between adjacent fields of view so they can be stitched together for downstream image processing.
- 12 Initiate cutting with single sections ranging from 25 to 100 nm per section.
- 13 Using Digital Micrograph software interfaced to the SBF-SEM, automate the process so that the block is imaged, a section is cut and the new block face layer is imaged continuously to collect 300-1000 images based on experimental time.
- 14 Typically 3 μ s/pixel at 4096 x 4096 pixel resolution for each frame of view can be used for imaging.

Image Registration and Stack Creation

- 15 Since there can be a variable amount of drift during the course of automated cutting, images should be first registered and stacked to allow three-dimensional reconstructions.
- 16 Using Fiji (<http://fiji.sc/Fiji>), a free image processing software program comparable to ImageJ, from the Plugins menu choose the Registration tab and then Register Virtual Stack Slices.
- 17 If a virtual stack set up is used, images need not be opened to save computing power. Select the input image directory and the output directory and choose the Rigid Feature Extraction Model with Rigid Registration Model. Images will not be registered and placed in the chosen output folder.
- 18 If multiple fields of views are taken, they can be first stitched together and then registered (Figure 3A). If this option is chosen, simply choose the Stitching tab from the Plugins menu and select 2D Stitching along with the 1st and 2nd channels to stitch. If doing this after registration, simply choose the 3D Stitching option from the Stitching tab.

Three-Dimensional Reconstructions

- 19 Open the registered stack using the Reconstruct program (Fiala, 2005) by Opening a New Series.
- 20 Map the structural elements automatically by selecting Series Options and then the Auto Tracing tab according to the user's preferences. Alternatively, the structural elements can be mapped manually with the paint brush from the tool bar at the top left.
- 21 Visualize the three-dimensional reconstructions of structural elements by selecting the 3D scene tab from the Object tool bar (Figure 3B).

COMMENTARY

Background Information

Using mouse models of human disease and collaborative experimentation—

The protocols described herein outline the use of mouse tissue to identify retinal pathology related to human disease. The reader should be cautioned that phenotypic characterization of a retinal condition should also be substantiated with complementary genetic investigations to understand the relationship between the mouse model and the human disease. The genetics of mouse models used in this laboratory have been thoroughly characterized to understand their relationships to clinically relevant forms of human disease.

As the reader may be aware, this approach requires the collaboration of investigators with different types of expertise. These skills range from basic mouse husbandry and delicate tissue handling when preparing the blocks to the final steps of serial block face experiments which require user and operator training for optimal data acquisition. Success in this endeavor also relies on the cooperation of many different facilities and/or departments. Our initial experiments were done in close collaboration with the Electron Microscope Facility at the Friedrich Meischer Institute for Biomedical Research in Basel, Switzerland.

Critical Parameters and Troubleshooting

Experimental success will rely on the careful completion of the following two parameters:

1. Eye cup dissection should be carried out such that the retinal layers remain intact
2. The staining and washing steps must allow the best signal to noise ratio for the SEM

The initial eye cup dissection requires the experimenter to carefully dissect away the cornea to remove the lens, while not disrupting the retinal architecture or causing a retinal detachment. It is imperative that this step be done successfully, as an artificially induced retinal detachment can lead to the false impression that this is due to disease pathology. This also is a reason why multiple mouse eyes should be used in block preparation, to substantiate all phenotypic findings and provide statistical significance. Unfortunately, unless the retinal detachment is visibly apparent at the beginning, it may not be noted until the first imaging steps of block sections under the microscope.

Subsequent steps relate to the staining and washing of the eye cups. These steps are critical to achieve the backscattered SEM signal required for imaging since the block face is uncoated in plastic embedded tissue. The techniques have been optimized for mouse eye tissue, but translate well to larger eyes from species such as *Rattus norvegicus*. The user may find that staining incubation times can be adjusted to obtain the best signal depending on the SEM used and the parameters of imaging. The wash steps are essential to remove heavy metals from the tissue that provide a coarse appearance to the final image. Therefore, it is best to err in using more washing steps and longer periods than less.

Anticipated Results

Completion of an SBF-SEM experiment will result in a plethora of images that has both advantages and disadvantages. The advantage is that by reconstructing three-dimensional volumes of retinal regions, one can identify both common and rare phenotypic events that could be harbingers of more overt disease pathology. The major disadvantage is the large amount of time needed to analyze the different images and carry out the required tracing and reconstruction steps downstream after the data is collected. Without automated data processing software to date, this remains a very time intensive process. The anticipated end results will be highly dependent on the mouse model being studied. Some mouse models will display very dramatic phenotypic differences when compared to wild type controls, making the resulting imaging and reconstructions more robust. An example is work done showing the dysfunction in retinitis pigmentosa E150K opsin mice in which SBF-SEM revealed a global degenerative phenotype involving rod photoreceptor cell structure (Zhang et al., 2013). Examining and analyzing the data requires full attention to detail, especially to identify subtle differences such as those in mice exhibiting defective photoreceptor phagocytosis in a model of enhanced S-cone syndrome (Mustafi et al., 2011).

Time Considerations

The time line for Basic Protocol 1 is one week. The main limiting factor for Basic Protocol 1 is collection of the mouse eye cups which may require multiple litters to collect enough eye cups for statistically significant imaging. The time line for Basic Protocol 2 is variable. Cutting experiments generally take 1 to 2 days depending on the user settings for pixel resolution and time frame of pixel scanning. The time frame for the three dimensional reconstructions is highly variable as this will depend on the volume being reconstructed and the automation of the objects being traced versus the need for manual tracing.

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Literature Cited

- Chen Y, Palczewska G, Mustafi D, Golczak M, Dong Z, Sawada O, Maeda T, Maeda A, Palczewski K. Systems pharmacology identifies drug targets for Stargardt disease-associated retinal degeneration. *The Journal of clinical investigation*. 2013; 123:5119–5134. [PubMed: 24231350]
- Denk W, Horstmann H. Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. *PLoS biology*. 2004; 2:e329. [PubMed: 15514700]
- Du Y, Veenstra A, Palczewski K, Kern TS. Photoreceptor cells are major contributors to diabetes-induced oxidative stress and local inflammation in the retina. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:16586–16591. [PubMed: 24067647]
- Fiala JC. Reconstruct: a free editor for serial section microscopy. *Journal of microscopy*. 2005; 218:52–61. [PubMed: 15817063]
- Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature*. 2007; 450:56–62. [PubMed: 17972876]
- Mustafi D, Kevany BM, Genoud C, Bai X, Palczewski K. Photoreceptor phagocytosis is mediated by phosphoinositide signaling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2013; 27:4585–4595. [PubMed: 23913857]
- Mustafi D, Kevany BM, Genoud C, Okano K, Cideciyan AV, Sumaroka A, Roman AJ, Jacobson SG, Engel A, Adams MD, Palczewski K. Defective photoreceptor phagocytosis in a mouse model of enhanced S-cone syndrome causes progressive retinal degeneration. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2011; 25:3157–3176. [PubMed: 21659555]
- Mustafi D, Maeda T, Kohno H, Nadeau JH, Palczewski K. Inflammatory priming predisposes mice to age-related retinal degeneration. *The Journal of clinical investigation*. 2012; 122:2989–3001. [PubMed: 22797304]
- Nickell S, Park PS, Baumeister W, Palczewski K. Three-dimensional architecture of murine rod outer segments determined by cryoelectron tomography. *The Journal of cell biology*. 2007; 177:917–925. [PubMed: 17535966]
- Palczewski K. Chemistry and biology of vision. *The Journal of biological chemistry*. 2012; 287:1612–1619. [PubMed: 22074921]
- Zhang N, Kolesnikov AV, Jastrzebska B, Mustafi D, Sawada O, Maeda T, Genoud C, Engel A, Kefalov VJ, Palczewski K. Autosomal recessive retinitis pigmentosa E150K opsin mice exhibit photoreceptor disorganization. *The Journal of clinical investigation*. 2013; 123:121–137. [PubMed: 23221340]

Figure 1.

Dissection of mouse tissue for eye cup preparation. (A) After euthanization, position the mouse with its head tilted to expose the eye for enucleation. (B) The enucleation step should be done carefully so as not to put pressure on the eye and damage the retinal integrity. This can be done by gently cutting away the tissue around the eye with scissors and exposing it for removal. (C) Once the eye is removed, an eye cup can be made by making an incision with an 18½ gauge needle and then cutting away the cornea (denoted by yellow dotted circle) and removing the lens and vitreous inside.

Figure 2.

Block preparation for SBF-SEM experiments. **(A)** Blocks of eye cups after polymerization should be shaved with a diamond knife to expose the edges. A side and top view of a shaved block are shown. **(B)** After mounting on a SEM stub, the block should be positioned in the SBF-SEM. **(C)** The stub should then be raised slowly until it is nearly level with the microtome so that cutting can be initiated.

Figure 3.

Image analysis and three-dimensional reconstruction of acquired SBF-SEM data. **(A)** SEM experiments can be set up so that individual areas imaged at high resolution can then be stitched together in Fiji to carry out high-resolution reconstructions for better segmentation. **(B)** The resulting three-dimensional reconstructions, in this case for a RPE cell, can be carried out with Reconstruct software.