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Preservation of Photoreceptor Nanostructure for Electron Tomography Using Transcardiac Perfusion Followed by High-Pressure Freezing and Freeze-Substitution

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Abstract

The phototransductive membrane disks of a vertebrate photoreceptor outer segment (OS) are highly susceptible to perturbations during preservation for electron microscopy. To optimize their preservation for nanostructural studies, such as with electron tomography (ET), we developed a protocol, using a combination of chemical and physical fixation approaches, including transcardiac perfusion, high-pressure freezing, and freeze-substitution.

Keywords

Photoreceptor; Electron tomography; High-pressure freezing; Freeze-substitution; Disk membrane morphogenesis

73.1 Introduction

Phototransduction takes place in stacked membrane disks contained within the OS of the vertebrate photoreceptor. These disks are turned over by the shedding of older disks at the OS tip and the formation of new disks at the base of the OS (Young 1967). Because disk morphogenesis involves complex membrane-shaping mechanisms, it is necessary to first define the membrane organization at the base of the OS. To achieve this goal, we studied the disk membrane nanostructure by using ET (Volland et al. 2015). For these studies tissue preservation is paramount, but also challenging. The disk membranes, especially the nascent disk membranes, seem to be particularly susceptible to anoxia and mechanical damage, which readily leads to artifacts during tissue preparation.

Chemical fixation methods, such as those involving transcardiac perfusion, allow quick and effective delivery of fixative to target organs in situ and can help stabilize delicate tissue, prior to dissection (Sosinsky et al. 2008). On the other hand, high-pressure freezing (HPF) and other physical fixation approaches (e.g., slam freezing) are preferable to chemical fixation as they allow fixation within milliseconds, rather than seconds to minutes for chemical fixation. But they pose their own set of limitations (Studer et al. 2001). Only small tissue samples of about 300 μm in thickness can be frozen without ice crystal damage (McDonald and Auer 2006), which means that tissues like the retina need to be exposed by ocular enucleation and dissection prior to HPF. Previous reports have shown that a combination of chemical and physical fixation methods can yield superior results when sensitive tissues are being processed (Murk et al. 2003; Sosinsky et al. 2008). Here, we provide a detailed description of a combination protocol that was optimized for the preservation of photoreceptor disk membranes. This protocol was used to study the nanostructure of these membranes by ET.

73.2 Transcardiac Perfusion

Wild-type mice of the strain C57BL/6J were treated in accordance with the appropriate institutional guidelines. Animals were kept under a 12 h light/12 h dark cycle and used at an age of 4–8 weeks. The mice were deeply anesthetized before an abdominal incision and thoracotomy were performed to expose the heart. A G20 needle, attached to a catheter, was inserted into the left ventricle, and the right atrium was cut open. Chilled (4 °C) 0.1 M phosphate buffered saline was then passed through the catheter, using gravity to drive the perfusion. After 1 min, the perfusate was switched to chilled (4 °C) Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer). Each mouse was then perfused with ~20 ml fixative before the eyes were enucleated and dissected quickly. Small pieces of retinal tissue (~1 mm $\times$ 1 mm) were kept in fixative solution on ice until they were frozen under high pressure within 1 h of initial fixation.

73.3 High-Pressure Freezing and Freeze-substitution

Each piece of retinal tissue was placed in a Leica flat specimen carrier (diameter, 1.5 mm; depth, 200 μm), with additional 0.1 M phosphate buffer, and transferred to a freezer holder where the specimen chamber was sealed with a diamond screw. The holder was attached to a handle and placed in an EMPACT2 high-pressure freezer (Leica Microsystems, Wetzlar, Germany). The tissue was then frozen within milliseconds under high pressure of >2000 bar, allowing samples of up to 300 μm in thickness to be frozen without the perturbation of cellular ultrastructure from ice crystal formation. After freezing, samples were collected and transferred in liquid nitrogen to an EM AFS2 automatic freeze-substitution unit (Leica Microsystems, Wetzlar, Germany). The specimen carriers containing the tissue were quickly placed in 1.5 ml Eppendorf tubes filled with 1% osmium tetroxide and 0.1% uranyl acetate in acetone at –90 °C, within the freeze-substitution unit. Over the course of 88 h, the temperature was gradually raised from –90 °C to room temperature (20 °C) while the fixative slowly replaced the water in the samples. After freeze-substitution, samples were washed 3 $\times$ for 5 min in acetone and propylene oxide consecutively, before they were infiltrated with 1:2 and 2:1 parts Araldite 502 resin (Electron Microscopy Sciences, Hatfield,

USA) to propylene oxide, respectively, for 30 min each. Finally, samples were transferred to pure Araldite 502 resin for another 30 min and then placed in flat molds and polymerized at 60 °C for 48 h. Sections, 300 nm thick, were collected on formvar-coated, copper, slot grids and stained for contrast with 10% uranyl acetate in methanol and 0.4% lead citrate in 0.4% sodium hydroxide for 10 min each. Gold fiducials (Ted Pella, Redding, USA) of 15 and 20 nm diameters were then placed on opposite sides (top and bottom) of the slot grids. At this stage grids can be carbon coated to stabilize the sections in the electron beam during image acquisition, but we usually didn't find this to be necessary.

73.4 Electron Tomography and Data Processing

ET is an extension of conventional transmission electron microscopy, with the difference that the sections are thicker and imaged at higher acceleration voltages. We performed ET on mouse retinal sections, using an FEI Tecnai TF20, operated at 200 kV, and FEI's "batch tomography" software (Hillsboro, USA). A series of images were recorded as the section was tilted from -70° to $+70^{\circ}$, with 2° increments at the lower tilt angles (range $\pm 40^{\circ}$) and 1° increments above $+40^{\circ}$ and below -40° . The images were collected with a 16-megapixel CCD camera (TVIPS) at magnifications between 14,500 $\times$ and 19,000 $\times$ and with an under-focus of approximately -1 to $3 \mu\text{m}$. To obtain dual-tilt data sets, the grids were rotated by 90° on the x-y axis after the acquisition of a first tilt series, and a second tilt axis was recorded, centered on the same point.

We used "eTomo" in the IMOD software package (Boulder, USA) to generate tomographic reconstructions of the acquired double-tilt series through fiducial-based alignment (Fig. 73.1a, b). A median filter of n4 was used to improve the reconstruction appearance if deemed necessary (Fig. 73.1a). After tomogram reconstruction, segmentation and subsequent image processing were conducted using "3dmod" (IMOD, Boulder, USA). Every 3–10 z-slices of the tomographic reconstruction were traced manually to create a 3-D model of the membrane disks at the photoreceptor OS base (Fig. 73.1c). The photoreceptors OS plasma membrane and membranes continuous with it were modeled in light green, in contrast to mature disks, modeled in dark blue (Fig. 73.1b, c). Videos of 3-D models were generated using Chimera (UCSF, San Francisco, USA), while videos of tomogram reconstructions were generated using 3dmod (IMOD, Boulder, USA) and Fiji (NIH), an open-source processing package (e.g., Suppl. Movies in Volland et al. 2015).

73.5 Conclusions

ET is a powerful tool to visualize the 3-D ultrastructure of cells and, in particular, allows insight into complex membrane organization (McDonald and Auer 2006). In order to obtain optimal images from mouse photoreceptors, we used a combination of chemical and physical fixation approaches, including transcardiac perfusion, high-pressure freezing, and freeze-substitution. These samples were well suited for ET and enabled us to study the formation of nascent disks using 3-D visualization. Overall, we were able to show that a combination of different fixation methods allows for better preservation than either method can on its own.

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Fig. 73.1.

Photoreceptor morphology after the combined fixation approach, using transcardiac perfusion and HPF (modified from Volland et al. 2015). **(a)** Z-slice from a tomographic reconstruction of the basal area of a mouse rod OS (median filter n4). **(b)** Z-slice from the same tomogram (no filter), with 3-D renderings, tracing the connecting cilium, nascent disks and the OS plasma membrane in light green, and mature disks in dark blue. **(c)** Overview of a 3-D model of the rod OS basal area, based on the tomogram shown in **(a)** and **(b)**. Scale bar 300 μm