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Quick-freeze/deep-etch electron microscopy visualization of the mouse posterior pole

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ABSTRACT

The mouse is one of the most commonly used mammalian systems to study human diseases. In particular it has been an invaluable tool to model a multitude of ocular pathologies affecting the posterior pole. The aim of this study was to create a comprehensive map of the ultrastructure of the mouse posterior pole using the quick-freeze/deep-etch method (QFDE). QFDE can produce detailed three-dimensional images of tissue structure and macromolecular moieties, without many of the artifacts introduced by structure-altering post-processing methods necessary to perform conventional transmission electron microscopy (cTEM). A total of 18 eyes from aged C57BL6/J mice were enucleated and the posterior poles were processed, either intact or with the retinal pigment epithelium (RPE) cell layer removed, for imaging by either QFDE or cTEM. QFDE images were correlated with cTEM cross-sections and en face images through the outer retina. Nicely preserved outer retinal architecture was observed with both methods, however, QFDE provided excellent high magnification imaging, with greater detail, of the apical, central, and basal planes of the RPE. Furthermore, key landmarks within Bruch's membrane, choriocapillaris, choroid and sclera were characterized and identified. In this study we developed methods for preparing the outer retina of the mouse for evaluation with QFDE and provide a map of the ultrastructure and cellular composition of the outer posterior pole. This technique should be applicable for morphological evaluation of mouse models, in which detailed visualization of subtle ocular structural changes is needed or in cases where post-processing methods introduce unacceptable artifacts.

1. Introduction

For almost a century and a half, mice have been used to test various hypotheses regarding the nature of mammalian genotypes and phenotypes. They have emerged as the principal model organism used for comparative human studies due to their genetic similarities to humans (Wagner et al., 1981; Mouse Genome Sequencing et al., 2002; Paigen, 2003), and their minimal cost relative to other mammals. Mice have been particularly useful in our understanding of basic ocular biology, pathobiology of ocular diseases, and identification of therapeutic targets (Edwards and Malek, 2007). Furthermore, many mouse models have been used in testing potential new therapies and in preclinical studies, providing support for drug applications in the clinic. The ability to evaluate phenotypic changes at various scales (e.g. micro and nanoscale) is critical to the development of a mouse model that both recapitulates the pathobiology of disease and allows a deeper understanding of how the disease affects the tissue. Precise imaging modalities unencumbered by artifacts introduced due to tissue post-processing methods can greatly enhance the fidelity of data obtained in an experimental animal model such as the mouse. Current high magnification imaging of post-mortem specimens using conventional transmission electron microscopy (cTEM) requires several tissue processing steps that result in artifactual changes in the tissue architecture (Chandler, 1984; Hobot et al., 1985; Paul and Beveridge, 1992), and though it is a valuable tool for morphologists and cell biologists, cTEM can be supplemented with more sensitive and precise methods of evaluating the ultrastructure of post-mortem tissues.

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Abbreviations

BIM	Basal Infolding Membrane
BrM	Bruch's Membrane
CC	Choriocapillaris
CC-BL	Choriocapillaris Basal Lamina
CMel	Choroidal Melanosomes
Mcyte	Melanocytes
cTEM	Conventional Transmission Electron Microscopy
ECM	Extracellular Matrix
EL	Elastic Layer
ICL	Inner Collagenous Layer
ICM	Intracellular Matrix
OCL	Outer Collagenous Layer
QFDE	Quick-freeze/deep etch
RMel	RPE Melanosomes
RPE	Retinal Pigment Epithelium
RPE-BL	RPE Basal Lamina
MV	Microvilli

Quick-freeze/deep-etch electron microscopy (QFDE), is a technique, initially developed by Hans Moor (Moor et al., 1961) and further advanced by John Heuser (Heuser and Salpeter, 1979), that permits nearly pristine tissue preservation and produces high resolution (~2 nm), dimensionally-stable, metallic replicas of the surface topography of cells, cell membranes, and extracellular matrix (Heuser and Kirschner, 1980; Hirokawa and Heuser, 1981; Souto-Padron et al., 1984; Gong et al., 2002). The replicas produced are observable by transmission electron microscopy and give a pseudo 3D appearance of the sample surface. As such, QFDE is capable of revealing intracellular matrices comprising networks which can include intermediate filaments, actin filaments and microtubules, cell membranes, basement membrane, collagenous extracellular matrix (ECM) and lipids, in great structural detail (Heuser and Kirschner, 1980; Hirokawa and Heuser, 1981; Souto-Padron et al., 1984; Yamabayashi et al., 1991; Ou et al., 1995; Hirsch et al., 1999; Tamminen et al., 1999; Gong et al., 2002; Ruberti et al., 2003; Brown et al., 2004; Huang et al., 2007; Saeidi et al., 2012a, 2012b). The high resolution, dimensional stability, and preservation quality increases the potential to capture subtle structural changes as well as macromolecular events such as those related to lipid transport and processing, which are difficult to image with a cTEM preparation (Takayama et al., 1999; Haberland et al., 2001; Brown et al., 2004; Huang et al., 2007, 2008). To date, the QFDE method (Heuser, 2011) has been used to successfully image neural synapses (Heuser and Salpeter, 1979), muscle actin/myosin interactions (Heuser and Cooke, 1983), intra/inter cellular structures of intestinal epithelial cells (Hirokawa and Heuser, 1981), and lipid accumulation in the aorta of rabbits (Frank and Fogelman, 1989; Haberland et al., 2001) and mice (Tamminen et al., 1999; Brown et al., 2004). Of particular importance to vision researchers, it has also been used to evaluate ocular tissues including the cornea (Yamabayashi et al., 1991; Hirsch et al., 1999), the trabecular meshwork in human eyes (Gong et al., 2002), the outer retinal vasculature and the pentalaminar extracellular matrix of Bruch's membrane, in aged human eyes (Ruberti et al., 2003; Huang et al., 2007, 2008; Johnson et al., 2007). The latter studies resulted in revealing a novel aging phenomenon, namely the accumulation of lipoprotein particles within human Bruch's membrane as a function of age (Ruberti et al., 2003; Huang et al., 2007), highlighting the value of this imaging technique.

Herein, we present a comprehensive view of the ultrastructure of the posterior pole of 'aged' wild-type mice using QFDE. Our goal is to demonstrate the value of this imaging technique in tandem with cTEM for a more thorough appreciation of the macromorphology of the mouse eye. We choose to focus on the posterior pole of older mice given its vulnerability in several retinal diseases including age-related macular degeneration (Bird et al., 1995; Curcio et al., 1998; Davis et al., 2005; Ferris et al., 2005), macular edema (Andrews et al., 2014; Lally et al., 2016), and choroidopathies (Smiddy et al., 1988; Ramrattan et al., 1994; Spraul et al., 1999). This first step in mapping the ultrastructure of the posterior pole of mice with a 'normal' outer retinal architecture, is necessary in order to better characterize the pathology of potential mouse models of retinal diseases and/or gain insight into the effects of disease modifying risks, such as environmental and genetic factors, on the outer retina. To our knowledge, this is the first study to employ the QFDE technique in evaluating the morphology of the posterior pole of the mouse eye.

2. Methods

2.1. Eye tissue collection

All animals were handled in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. The cohort of animals from which the tissue was obtained for this survey were part of a control group for a larger investigation into the pathobiology of murine age-related retinal degeneration. Aged C57BL/6 J mice (12–20 months; n = 18 eyes from 3 male and 6 female mice) were transcardially perfused prior to enucleation. Eyes were enucleated and the anterior segment removed. Post-processing of the eyes involved either removal of the neural retina or removal of both the neural retina and the retinal pigment epithelium (RPE) cell layer. To achieve the latter, following dissection of the anterior segment and the retina, radial cuts were made in a posterior pole (RPE, choroid and sclera) flatmount placed on a glass slide. Next, the RPE cell layer was removed by gently tapping the wholemount of the posterior globe, with a PBS moistened Weck-cel sponge. The tissue was then fixed in 2% glutaraldehyde/2% paraformaldehyde/cacodylate buffer mix (Karnovsky fix).

2.2. Tissue preparation for conventional transmission electron microscopy (cTEM)

Eyes were post-fixed in 1% osmium tetroxide and embedded in Spurr's resin, as previously described (Hu et al., 2013). Morphologies of the retinal pigment epithelium (RPE)/Bruch's membrane (BrM)/choroid/sclera (n = 4) or BrM/choroid/sclera (n = 2) were examined in toluidine blue stained semithin sections and uranyl acetate/lead citrate stained ultra-thin sections, using a light and electron microscope, respectively.

2.3. Tissue preparation for quick freeze/deep etch transmission electron microscopy (QFDE)

Following the steps outlined in "Eye Tissue Collection", 12 of the fixed eyes were used for the QFDE imaging. The eyes were dissected into smaller sections in phosphate buffer saline (PBS), approximately 1 mm × 1 mm, such that an intact replica could easily fit on a single TEM grid. Sections were mounted, with the RPE or Bruch's membrane side up onto laponite clay (Southern Clay Products, Gonzales, TX) then impact frozen on a liquid nitrogen cooled pure copper block using our custom two step *Touch Freezer* to minimize distortion. Sections were stored under liquid nitrogen if not used immediately, otherwise the preserved tissue was transferred to a