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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 epithelial (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.

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Keywords

Bruch's Membrane; Mouse Eye; Quick-Freeze/Deep-Etch; Transmission Electron Microscopy; Retinal Pigment Epithelium; Choriocapillaris

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.

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 comprised of 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; Saeidi et al., 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; Huang et al., 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; Johnson et al., 2007; Huang et al., 2008). 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 macro-morphology 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/6J 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 epithelial (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 modified Cressington CFE-40 freeze fracture system (Cressington, Watford, England (UK)) cooled to -170° C, or colder, and held at a minimum pressure of 10⁻⁶ torr. The tissue was then superficially freeze fractured (we will use just 'fracture' for the remainder of the paper) to a depth of 0 µm – 15 µm, depending on the desired view of the surface, with a cooled razor blade (-190° C). Once fractured, the samples were warmed to -100° C for periods of up to 1 hour to sublime the vitrified ice from the surface, exposing the ECM proteins and cellular components for subsequent shadowing. Following etching, the samples were recooled to ~ -130° C to reduce surface diffusion of the coating metal during evaporation. Next, the samples were rotary shadowed by evaporating 2-4 nm of platinum onto the rotating sample at a low angle to produce a 3D effect. They were then immediately backed with approximately 10nm of carbon at 90°. The samples were removed from the freeze fracture system and the replicas were isolated in diluted (25%) household bleach solution (5.25% sodium hypochlorite) to preserve replica integrity. After the tissues were digested from the metal, replicas were washed in DI water for over an hour, picked up on TEM grids then imaged on a JEOL JEM1010 TEM (JEOL USA, Inc., Peabody, MA) operating at 80 kV.

3. Results

3.1 Overview of the Posterior Pole of the Mouse with cTEM

A conventional TEM image of the posterior pole of a mouse eye is presented in Fig. 1, highlighting the cellular and ECM layers evaluated throughout this study with QFDE. The RPE is a cuboidal epithelial layer (Strauss, 2005) and forms part of the outer blood retina barrier (Bok, 1993; Rizzolo, 1997; Marmorstein, 2001). BrM, adjacent to the RPE, is a pentalaminar extracellular matrix just interior to the choriocapillaris. The five layers of BrM include the RPE basal lamina (RPE-BL), inner collagenous layer (ICL), elastic layer (EL), outer collagenous layer (OCL), and choriocapillaris basal lamina (CC-BL). The choriocapillaris (CC) is a fenestrated vascular pannus of anastomosed capillaries located between BrM and the choroid, acting as both a provider of nutrients/oxygen and remover of

waste for the retina (Wajer et al., 2000; McLeod et al., 2009), through the RPE. The choroid in mice is a melanosome-rich layer anterior to the sclera and constitutes the primary blood/nutrient supply for the outer retina (Linsenmeier et al., 1981; Bill et al., 1983; Linsenmeier and Braun, 1992). For a review on choroid functions see (Nickla and Wallman, 2010). Finally, the sclera, located at the outer aspects of the posterior pole, is composed of fibrillar type I collagen lamellae and provides structural and mechanical support to the fragile inner ocular tissues. Using the QFDE preparation method, we characterized and detailed *en face* views of each layer labeled in Fig. 1 with comparisons to cTEM, as explained below.

3.2 The Retinal Pigment Epithelium

To characterize the RPE cell layer with QFDE, we have imaged the RPE cell layer in three regions, apical, central and basal, identifying some of the main ultrastructural features in each plane.

3.2.1 Apical RPE—To image the apical (inner most) region of the RPE we did not fracture the samples, rather we removed the retina first prior to freezing, leaving the RPE/BrM/CC/Sclera complex intact (Fig. 2B-E). The two main identifiable features observed included the apical microvilli and melanosomes (Fig. 2B/C). The apical microvilli connecting the photoreceptors to the RPE, are long and look “flattened” rather than vertical, as they would appear when attached to the retina. The elongated, conical shaped melanosomes (RMel) (Fig. 2B) are present throughout the RPE cells but are also observable between the apical microvilli as well as in the apical to central regions of RPE cells (Fig. 2A). Additionally, in Fig. 2B we observed flat, empty areas around the RMels. Given the location, the literature suggests two possible features: 1) a mucopolysaccharide/proteoglycan layer (Hall et al., 1965; Goldbaum and Madden, 1982) at the microvilli-RPE junction or 2) the surface membrane of the RPE cell. However, we cannot conclude definitively, based on our data, what these structures represent. Figure 2A shows a comparative cTEM *en face* view of this same area. Typical RPE melanosomes (RMel, Fig. 2D) are prolate spheroid shaped with a high aspect ratio, distinct in comparison to choroidal melanosomes (CMel, Fig. 2E), which are ellipsoidal but do not have a high aspect ratio. RMels appear to vary in size to a much greater extent than CMels. RMels can be as short as 0.8 μm or longer than 3 μm with varying diameters. They are not always straight and may contain subtle bends or distinct kinks. Qualitatively, CMels are distinguishable from RMels as they tend to cluster in the choroid and form extended continuous sheets, hence forth referred to as melanosome bed. The majority of CMels are generally uniform in shape and size; but can be larger (in length and diameter) and may have an appearance similar to RMels (though these are few in number). In cTEM (Fig. 2A) RMels that are circular in cross-section are possibly sectioned perpendicular to their long axis. These sections make their full shape difficult to determine, but the abundance of elliptical sections suggests that most of the RMels are prolate spheroids with high aspect ratios.

3.2.2 Central RPE—The central RPE is where most of the organelles of the cell appear to be concentrated as well as the location of the nucleus (Fig. 1). While identification of specific organelles with QFDE is challenging, Fig. 3A demonstrates organelles exposed on a bed of cytoskeletal filaments in the intracellular matrix (ICM). The QFDE process exposes

the 3D nature of structures. The fine structure of the filaments of the ICM are observable in great detail as is their interface with the nucleus in this cell and other organelles. The nucleus appears to be wrapped in a filamentous “basket”. The ICM fibers approach the nucleus tangentially and then appear to continue around rather than directly penetrate the nuclear membrane. Supplementary Fig. 1 is a similar image of the central RPE cellular content captured via QFDE but in this case, there is an impression where the nucleus was pulled from the ICM. Surrounding the nucleus impression there are structures and organelles both above and within the ICM filaments. An unusual invagination is seen on the surface of the nucleus, similar to that reported by others (Malhas et al., 2011; Jorgens et al., 2017). Jorgens *et al.* showed using human mammary epithelial cells that these invaginations are linked to the cell’s cytoskeleton and continue to connect to the plasma membrane (Jorgens et al., 2017), which we observed in the RPE as well. We can see this same cytoskeletal network extending far beyond the immediate vicinity of the nucleus creating the impression of a fully invested and connected intracellular network, linking the nucleus to the membrane and potentially to the surrounding RPE cells and to the ECM. Some organelles and structures are readily identified, such as RMels and the endoplasmic reticulum, based on their shapes and vicinity to the nucleus. Fig. 3B shows a corresponding cTEM *en face* image in the central RPE.

3.2.3 Basal RPE—Two approaches were used to image the basal aspects of the RPE: (1) fracturing into the basal RPE plane or (2) gently removing the RPE cells prior to freezing with no fracturing (as described in the methods). Fig. 4A shows a transverse section cTEM of BrM-CC with the RPE removed and Fig. 4B is an oblique section cTEM image, which runs from the basal infolding membrane (BIM) through BrM to the choriocapillaris, with the RPE intact. The RPE removal method was used to ease the process of imaging the basal RPE and Bruch’s membrane, as well as to ensure that what we believed to be RPE-basal lamina (RPE-BL) and BIM while fracturing was, in fact, just that structure. Figures 4C and 4D show that fracturing or removing the RPE, respectively, does not distort the RPE-BL or other underlying structures. A prominent feature of the basal RPE is the BIM, which is brought out in strong relief with the QFDE method. Fig. 4E shows a more detailed structure of the BIM in a non-fractured sample displaying clearly periodic features along the infolding structures. Fig. 4B, E provides a comparison of QFDE and cTEM for the BIM and RPE-BL. We note in Fig 4B that in BrM there are areas where the OCL expands substantially, constricting the subjacent CC. The expansion primarily comprises a sparse OCL rather than a uniformly enlarged BrM. This type of BrM OCL expansion is also often seen between two capillaries (not shown here).

3.3 BrM’s Pentalaminar Structure

BrM in mouse eyes is much thinner (0.5-1 μm in thickness) than in humans, making it difficult to “hit” with a randomly wandering fracture plane. Additionally, the borders of the pentalaminar structure are not always distinctly separate. When the fracture plane did traverse BrM, it often did so very steeply or remained within one of the central layers. In Figures 5A (QFDE) & 5B (cTEM) the fracture plane and thin section are primarily within the basal RPE layer. However, there is a section penetrating below the RPE, which provides a window “into” BrM. In most cases, particularly with QFDE, the ICL was either not easily

identified as ICL, not visible, or appeared as short curled fibril ends (Fig. 5A, QFDE, Fig. 5B cTEM). In QFDE images that are *en face* of the EL, we observed elastin in two main forms: 1) as a knobby continuous sheet with many small protrusions and 2) as a fibrous and interrupted sheet (Fig. 5C). When the fracture plane crossed BrM at a steep angle, providing an oblique view, the EL appears as a dense layer (Fig 5D).

3.4 The Choriocapillaris

The choriocapillaris (CC), a fenestrated vascular pannus, which provides perfusion support to the outer retina, displays fenestrations on the endothelial cell surface on the BrM/RPE side. The fenestrations are easily recognized in cTEM transverse or oblique sections (*vide supra*: Fig. 4B). Fig. 6A is the result of a remarkable fracture plane, exposing the full RPE/BL-BrM-CC complex, which captures two capillaries and an expanse of ECM between them. In the center of the image, the fracture plane descends from the RPE-BL through the upper layers of BrM revealing a substantial OCL between the capillaries. On either side, the fracture plane traverses the pentalaminar structure of the entire Bruch's membrane, revealing the CC-BL and the fenestrations on the surface of the CC endothelial cells. The fracture further progresses into the capillary lumens, revealing what appears to be the capillary lumen posterior wall. Upon first inspection, on the capillary surface, fenestrations appear as clusters of openings with no internal structures (likely an artifact of fracture pull-out). However, at higher magnification, the classic wagon wheel structure of the capillary fenestration lumen is readily appreciated in some of the fenestrations (Fig. 6B,C). In sections traversing through BrM, though it was not often clear which collagen fibrils were ICL versus OCL, we venture that the denser collagen fibrils are in the ICL and the sparser fibrils belong to the OCL (Fig. 6D), paralleling the architecture observed in human eyes (Goldbaum and Madden, 1982). Fig. 6E, taken at the posterior choriocapillaris shows the posterior portion of a capillary surrounded by pigment. The loosely organized fenestrations observed do not have the wagon wheel structure.

3.5 The Choroid

There are three main (non-vascular) features observed in the choroid: melanocytes (Mcyte), choroid melanosomes (CMels), and collagen. The infrachoroid, inner regions of the choroid (including Sattler's layer), adjacent to the choriocapillaris, appears to have numerous Mcytes with relatively fewer CMels, and the collagen fibrils are locally highly aligned (Fig. 7; SI Fig 2). The QFDE image in Fig. 7A is part of a larger mosaic (SI Fig. 3), in which choroidal collagen structures appear disorganized and form highly-aligned bundles. Immediately next to this initial area in the choroid we see few Mcytes but a heavily dense population of CMels, and almost no apparent collagen structures (Fig. 8A). The central choroid has more collagen structures than the infrachoroid but not large bundles, and has more Mcytes between the large CMel beds. While imaging this layer we were able to dislodge areas of CMels exposing their matrix ultrastructure (Fig. 8B). It appears that the CMels strongly interact with a continuous sheet-like structure resembling basement membrane. The outer regions of the choroid (including the Haller's layer), closest to the sclera, has fewer Mcytes and fewer CMels than the center, but is traversed by bundles of highly aligned collagen fibers, some of which seem to merge into the sclera (Fig. 8C).

3.6 The Sclera

The sclera is characterized by polydisperse arrays of type I collagen fibrils arranged into lamellae, which form the principal load-bearing structure of the posterior ocular tunic (Fig. 9A). Since the sclera is relatively deep within our tissue sample sections, which begin at the RPE, we are approaching the limits of our freezing technique attributable to poor vitrification, which may begin at ~20 μm from the impact surface (Vanharreveld and Crowell, 1964; Schwabe and Terracio, 1980; Hirsch et al., 1982). Nonetheless, with careful freezing we are able to observe regions, which were clearly vitrified such that we can appreciate the proper structure of the sclera (Fig. 9B).

4. Discussion

Here presented for the first time is an atlas of the ultra-structures of the posterior pole of the mouse eye obtained with QFDE and supported by corroborating cTEM images. Historically, the development of this freeze-fracture/freez-etch method was in part a response to the need for a technique that would reduce artifacts associated with tissue processing while revealing protein/matrix structures in three dimensions (Heuser and Kirschner, 1980). The advent of QFDE led to visualization of ultra-structural components of tissues previously not appreciated (Kirchhausen et al., 1986). The goal of the current study, was to focus on imaging the outer retina including the RPE, BrM and choroid using QFDE, since pathological changes in these regions are prominent features of several retinal diseases including age-related macular degeneration (Bird et al., 1995; Curcio et al., 1998; Davis et al., 2005; Ferris et al., 2005), retinitis pigmentosa (Hartong et al., 2006), choroidopathies (Smiddy et al., 1988; Ramrattan et al., 1994; Spraul et al., 1999), and Best vitelliform macular dystrophy (MacDonald and Lee, 1993) to name a few. As such, creating a compendium of the morphology of the 'normal' outer retina with QFDE is the necessary first step to appreciating the utility of this technique, as a reference for future studies intended on characterizing the pathology of mouse models of ocular diseases and/or effect of potential therapies in mouse models. Given that QFDE had not been used to examine the mouse eye prior to this study, the major challenge was identifying the location of the fracture plane in the RPE-BrM-CC complex. To identify the regions being imaged and specifically sub-cellular features, we applied different methods to tissue collection, including processing the entire RPE/BrM/choroid/sclera complex or sloughing off the RPE and processing only the BrM/choroid/sclera complex. Removal of the RPE followed by etching without fracture served to help us positively identify landmark 3D features of the posterior pole. For example reference to the structures revealed when the RPE was removed followed by etching, confirmed for us that structures in the fractured section of Fig. 4E are indeed BIM, exhibiting a unique repeating ridge structure. Overall, by examining many freeze fracture planes, countless classical intracellular and extracellular features of the RPE, BrM and choroid could be positively identified by QFDE, and with greater detail, providing a unique perspective when compared to cTEM and standard scanning electron microscopy.

It is noteworthy that morphological differences exist between mouse and human BrM. For example, the thickness of BrM in mice is approximately 0.5-1 μm versus 4-9 μm in humans (Curcio and Johnson, 2013). The scale dissimilarity is to be expected given the difference in

the sizes of the eyes, however, it presents a particular challenge for QFDE because the fracture plane traverses tissues in an uncontrolled manner. Thus, the signal-to-noise ratio in our replicas was often very low. In addition to the scale difference, there were also structural differences. While ICL in human BrM is continuous and can be as thick as 1 micron, in mouse eyes, the ICL often appeared to be discontinuous and at most a few collagen fibrils thick (SI Fig. 4). This was corroborated in cTEM images in which the collagen fibrils in this layer were sparse, often revealing an “empty” space between the RPE-BL and the EL. Similar to the human BrM, the largest layer in the mouse BrM is the OCL, comprising of over 50% of the total cross-section of this pentalaminar extracellular matrix. Its thickness ranges from 0.3 μm to multiple times the thickness of the rest of BrM in instances where it fills the inter-capillary pillars in the CC or when a capillary thins in some regions permitting the OCL to expand into that space. It could be said that this excess area is no longer BrM but since it appears as a continuous structure, and there is no interruption by a basal lamina, we find no reason to not include it as part of BrM. The collagen fibrils in the OCL run mostly parallel to the basal lamina and the EL with some spanning the thickness of BrM. There are also areas where there is no EL, and the OCL and ICL appear as one layer.

In spite of the improved preservation of tissue and the reduced artifacts associated with fixation and processing, QFDE has its own unique set of limitations. Some of these are easy to appreciate. For example, fracturing the sample can pull structures from the surface, as we observed with the capillary fenestrations in Fig 6A/B/C. Ice crystals are also readily identified where there is bad freezing. Other artifacts are not so clear, which is why it is instructive to evaluate corroborating cTEM images to aid in structure verification. For instance, we observed what appeared to be “pores” in the CC-BL that we initially associated with underlying CC fenestrations (SI Fig. 5), as the diameters are similar. In support of this, directly adjacent to this section of the TEM grid we observed a lamellar layer of collagen that is often present subjacent to the CC (SI Fig. 2A). However, because we observed the same structures over melanosomes in the same replica and because we were not able to identify these structures in cTEMs, we concluded that the pores may be an artifact. Still, it is tantalizing to imagine that the choriocapillaris basement membrane is more permeable by design, and that the enhanced porosity is a true feature of the CC-BL, raising the possibility that whatever mechanism causes the fenestrations in the endothelium to form may also affects its basal lamina.

QFDE in the mouse eye also highlighted interesting differences in the collagen structures in the choroid/scleral transition. Collagen fibrillar organization was variable in that fibrils could be found in both small and large aligned bundles, and as single wandering fibrils (Fig. 8A/B & SI Fig. 3). Comparison of multiple QFDE with cTEM images suggests that collagen organization transitions steadily from globally disorganized, but locally aligned, lamellae in the choroid to increased global organization as the sclera is approached. Though it remains to be determined what may be the underlying purpose of this variability in collagen organization in the posterior eye, it is plausible that collagen bundles that invade the choroid are extensions or transitions, which remain from the formation of sclera. They may also serve to positively attach the choroid to the sclera.

In conclusion, this is the first catalogue of the intracellular and extracellular landmarks of the posterior pole of the mouse using QFDE. Our examination included the RPE, BrM, choroid and sclera, areas that may be particularly vulnerable and prone to structural alterations in retinal diseases, such as age-related macular degeneration. Previously, QFDE of human donor tissue from aged individuals revealed macromolecular components such as lipoprotein particles within BrM and ultra-structural features such as BrM inclusions, both characteristics of aging and age-related macular degeneration (Ruberti et al., 2003; Huang et al., 2007; Johnson et al., 2007; Huang et al., 2008). These features are not easily identified using conventional imaging techniques nor is it possible to manipulate human tissue to produce disease states in a controlled manner. Therefore, QFDE imaging of post-mortem tissue along with mouse models of eye diseases, is a potentially powerful combination to evaluate the effects of therapies on pathological features such as lipoprotein accumulation, BrM, RPE, and choroidal structural changes. Future studies aimed at examining the QFDE profile of young versus older mouse eyes, focusing on the morphology of the retina and/or designed to take advantage of emerging technical methods combining QFDE with immunohistochemical labeling (Rash and Yasumura, 1999) may be able to further expand our understanding of structure and function not only in mouse eyes and mouse models of ocular disease but in human donor tissues as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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

Highlights

- A compendium of the morphology of the posterior mouse eye with QFDE is presented.
- Ultrastructural landmarks of the RPE, Bruch's membrane, choroid and sclera, as visualized with QFDE, are characterized.
- QFDE provides finer details of the ultrastructure of murine ocular tissue structures compared to cTEM.
- QFDE is a powerful alternative to traditional post-mortem imaging techniques.

Fig 1. cTEM of RPE-BrM-Choroid complex with attached sclera

Cross-section of the complex architecture of the posterior pole of a wild type mouse eye including the RPE, Bruch's membrane, choroid and subjacent sclera. Regions of pigment infiltration into the OCL and expanded regions of the OCL into the CC are marked by black arrows. The interface and boundaries between the CC and Bruch's membrane are not linear. Scale bar: 5 μ m.

Fig 2. Correlative imaging of apical RPE with cTEM and QFDE

A) An *en face* cTEM section of apical RPE. Remnants of microvilli can be seen on the left side of the image (MV). RMels of varying shapes and sizes appear discretely or as clusters. RPE nuclei are readily identified (N). B) QFDE of a non-fractured, etched surface of the apical RPE with the retina removed. The flat regions marked by * (top and left sides) are either on the surface of the RPE cell under the retina where the microvilli were completely removed or the open space within the cytoplasm of the RPE cells. As is typical at the apical RPE there are clusters of both vertical (white arrows, inset a) and horizontal (black arrows, inset b) RMels. The areas marked with “B” are areas of torn replica. C) QFDE of the non-fractured, etched surface of the apical RPE with the retina removed. Black arrows point to microvilli that are collapsed, lying flat (normally upright when intact and in contact with the retina). The white arrow on the right points to a lightly outlined microvilli process. D) & (E)

compare the typical shapes of RPE melanosomes (RMel, D), and choroidal melanosomes (CMel, E). Black arrows point to collagen fibrils in (E). Scale bar: (A) 5 μm ; (B) 10 μm (inset a = 2 μm ; inset b = 2 μm ; (C) 15 μm ; (D) 400 nm; (E) 400 nm.

Fig 3. Correlative imaging of central RPE with cTEM and QFDE

A) QFDE image of an intact nucleus (N, inset (b) shows filaments on the surface) surrounded by organelles (ER is labeled *). The background material in the image surrounding the nucleus and the organelles is the cytoskeleton of the cell, most likely composed of intermediate filaments (insert (a) shows higher mag). B) cTEM thin-section of the central area of the RPE showing two adjacent cells. RMels are visible in abundance in this plane through the RPE as are numerous other small dense structures (*). Scale bar: 6 μm ; inset a = 1 μm ; inset b = 2 μm .

Fig 4. Correlative imaging of basal infolding membrane and RPE basal lamina with cTEM and QFDE

A) cTEM cross-section of BrM, CC, Choroid and Sclera with the RPE removed, leaving the RPE-BL as the most apical structure along with occasional remnants of BIM (not shown here). B) cTEM *en face* view of the BIM, all layers of BrM [RPE-BL, ICL (not visible), EL, OCL, and CC-BL], and the CC with its fenestrations (bottom left inset highlights the OCL-CC interface). The section depth of this image moves from the top right (RPE BIM) to the bottom left (choroid) at a relatively shallow slope. The RPE-BL appears as a grey diffuse layer. The ICL should follow, but due to its thinness in the mouse eye is difficult to appreciate in oblique and cross sections. The prominent collagen layer that occupies the majority of the figure is OCL. The EL is the dark, semi-fibrous layer comprising randomly oriented short, straight, fibers located between the OCL and the RPE-BL. The capillaries of the CC are visible with their characteristic fenestrations (arrows). C) & D) Show RPE-BL using two methods of pre-freezing QFDE preparations. In (C) the RPE was left intact prior to freezing then freeze fractured to expose this layer. In (D) the RPE was gently removed (same as A) prior to freezing and no fracturing was used. E) QFDE of non-fractured basal RPE structures following RPE removal prior to freezing. The image shows significant amounts of BIM with a periodic appearance, attached to a dense field of RPE-BL. Scale bar: (A) 6 μ m; (B) 9 μ m (inset: 500 nm); (C) 2 μ m; (D) 2 μ m; (E) 2 μ m.

Fig 5. en face side-by-side comparison of views into BrM from the RPE BIM

A) QFDE: In the center of the image the fracture plane descends briefly into BrM revealing the RPE-BL and numerous fibrous structures (EL and OCL). The ICL is difficult to observe in the mouse, but is likely indicated by short curly fibril ends protruding from the RPE-BL (white arrow). “Below” the OCL there is a flat surface, which is likely the unetched ice front. The EL is shown in the inset, arrows pointing to elastin fibers. The mesh like structure surrounding the view into BrM is etched BIM. B) cTEM: The RPE-BL is traversed (black arrows) over a short distance exposing the dark, fibrous structure of the EL (white arrows) and the collagen fibrils of the OCL (black arrow heads). The ICL cannot be readily discerned in this image, but may be indicated by the short fibrils protruding from the

basement membrane of the RPE (white arrowheads). (C) & (D) show two aspects of the EL. C) The elastin, in enface section, appears as knobby fibers that are densely packed but still distinct. This is observed when the fracture plane remains in the EL. D) EL is a flat, continuous sheet (arrows) with occasional “bumps” or protrusions. This is observed when the fracture plane descends rapidly and obliquely through the EL. Scale bar: (A) 2 μm (inset: 500 nm); (B) 2 μm ; (C) 2 μm ; (D) 1 μm .

Fig 6. QFDE of OCL and Choriocapillaris complex

A) Fracture plane traverses the BrM of the RPE and proceeds obliquely to the capillaries of the choriocapillaris complex. The replica reveals the principal structures that separate the outer retina from its primary blood supply. Among the many structures revealed in this image, there are two features that stand out: 1) thickly populated OCL and 2) clear arrays of capillary fenestrations (Fen). (B), (C), & (D) are magnifications of the boxed regions in panel A. B) A high magnification of the CC basal lamina, fenestrations of the endothelium, as well as the inner layers of BrM. The fracture plane descends rapidly from the BrM (top, right) through the RPE-BL/ICL/EL (arrow) and the OCL, which is mostly fractured away except to the far right of the image (OCL). The asterisk indicates an area of the CC-BL with both the lamina rara and densa. The fenestrated endothelium of the choriocapillaris can be readily identified by the clustering of small “holes” adjacent to an open space (capillary lumen). C) High magnification of the fenestrations found in panel B. Two structures indicating the presence of fenestrations were found. The classic wagon wheel was present in what we assume are intact fenestra (black arrows) and ‘holes’ where, presumably, the internal structure of the “wagon wheel” has been pulled away (white arrows). D) Dense

collagen networks of the OCL decorated with proteoglycans, fine filaments and remnants of the EL (arrows). E) A fractured CC vessel is surrounded by CMels. white arrows point to small groups of loosely organized fenestrations which we believe are on the posterior side of the capillary. The mesh material in the center is likely to be etched, fixed, plasma proteins within the exposed capillary lumen. Scale bar: (A) 4 μm ; (B) 1 μm ; (C) 500nm; (D) 1 μm ; (E) 2 μm .

Fig 7. Correlative imaging of the choroid with QFDE and cTEM

A) QFDE and B) cTEM. In the post-CC area, discrete groups of CMels are observed (unlike the infrachoroid where larger areas include CMels) separated by melanocytes (Mcytes) and semi-organized collagen fibrils. This QFDE image is part of a larger mosaic (SI Fig. 5), in which collagen structures appear as both disorganized and highly-aligned bundles. Scale bars: 2 μm .

Fig 8. QFDE of the infra, central, and outer choroid

A) Shows the typical structure the infrachoroid, posterior to the choriocapillaris, comprising large clusters of CMels with few collagenous structures apparent. Myocyte membranous surfaces are visible in this area and a displaced red blood cell (RBC) can be seen (possibly an artifact of perfusion during tissue processing), indicating proximity to the CC. B) The central choroid is composed of small collagen strands in abundant ECM. In some areas, CMels have been dislodged exposing their encapsulating extra-cellular matrix. The right half of the image comprises mostly of dislodged melanosomes while in the left side the CMels are intact. The left inset is a higher magnification of a melanosome secure in its ECM while the right inset shows empty supporting matrix (arrows surround the object in each). C) As the choroid approaches the sclera we begin to observe larger and more frequent aligned collagen bundles. In this central-outer layer, the collagen appears in fiber like bundles while still fully surrounded by CMels. The insert shows a cTEM image of a cross-section of a region similar to, but orthogonal in orientation, to the corresponding QFDE. Scale bar: (A) 10 μ m (inset: 1 μ m); (B) 5 μ m (insets: 500 nm); (C) 2 μ m.

Fig 9. Correlative imaging of scleral lamellar collagen layers with QFDE and cTEM

A) Cross section through the sclera, illustrating the lamellar arrangement of collagen fibrils with cTEM. B) Deeper fracture with vitrified ice, allowing further imaging of the sclera with QFDE. In this image, we can appreciate layers of differentially oriented collagen lamellae, in which some fibrils have been fractured and recoiled into curved shapes (a common, mild artifact of fracturing collagen). On the right side of the image (*) is an area of partially etched, vitrified ice. Scale bar: (A) 3 μm ; (B) 2 μm .