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Analysis of Photoreceptor Rod Outer Segment Phagocytosis by RPE Cells In Situ

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Abstract

Counting rhodopsin-positive phagosomes residing in the retinal pigment epithelium (RPE) in the eye at different times of day allows a quantitative assessment of engulfment and digestion phases of diurnal RPE phagocytosis, which efficiently clears shed photoreceptor outer segment fragments (POS) from the neural retina. Comparing such activities among age- and background-matched experimental wild-type and mutant mice or rats serves to identify roles for specific proteins in the phagocytic process. Here, we describe experimental procedures for mouse eye harvest, embedding, sectioning, immunofluorescence labeling of rod POS phagosomes in RPE cells in sagittal eye sections, imaging of POS phagosomes in the RPE by laser scanning confocal microscopy, and POS quantification.

Keywords

Mouse eye; Retina; Retinal pigment epithelium; Diurnal photoreceptor outer segment phagocytosis; Rhodopsin-positive phagosome quantification; Immunofluorescence microscopy; Paraffin sectioning

1. Introduction

Prompt and complete clearance by phagocytosis of shed photoreceptor outer segment fragments (POS) by the underlying retinal pigment epithelium (RPE) is essential for life-long visual function (1, 2). In mammals, rod POS shedding and RPE phagocytosis are regulated and coordinated by the circadian rhythm such that they occur in a daily peak at light onset (3). Quantification of POS phagosome load of the RPE in eyes derived from experimental animals sacrificed at precise times before and after light onset therefore allows analysis of kinetics and capacity of POS phagocytosis of RPE cells in situ. Quantification of POS numbers per length of RPE in sagittal sections of fixed, paraffin-embedded eyecups has proven to be a reliable method to assess RPE phagosome load in individual eyes. Comparing POS load of the RPE in wild-type mice with POS load of RPE in age-matched mutant mice of the same genetic background is an efficient method to identify components and pathways relevant to POS uptake or digestion.

Transmission electron microscopy can identify engulfed POS in the RPE based on their morphological appearance (4, 5). This method is powerful but cost- and labor-intensive. Light microscopy can demonstrate POS in the RPE in frozen or paraffin eyecup sections stained with histological dyes (6). This method is fast and economical but it is only

appropriate for eyes derived from albino animals because RPE melanosomes may obscure phagosomes. Here, we describe the use of immunofluorescence laser scanning confocal microscopy to rapidly and unambiguously identify POS phagosomes in RPE in cross sections of paraffin-embedded mouse or rat eyes (7). We further discuss methodology to reliably quantify POS phagosome load per length of retina.

2. Materials

Prepare all solutions using double-distilled water (ddH₂O) or a similar quality water and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise.

2.1. Materials for Enucleation, Fixation, and Lens Removal of Mouse Eyes

1. Mice maintained in a strict 12-h dark/light cycle—at least four animals for each time point (see Note 1).
2. Curved forceps, No.11 blade, sharp edged forceps, microdis-section scissors.
3. Davidson's fixative (for 100 mL): Mix in this order: 33.5 mL H₂O, 33 mL 95% ethanol, 22 mL formaldehyde 37%, 11.5 mL glacial acetic acid (see Note 2).

2.2. Materials and Equipment for Eyecup Processing and Paraffin Embedding

Make ethanol dilutions using 95% ethanol and ddH₂O. Use 2 L plastic cylinders to make alcohol dilutions.

1. Automatic Tissue Processor (e.g., Leica TP1020).
2. Plastic processing cassettes and fitting metal molds.
3. 50% Ethanol.
4. 85% Ethanol.
5. 95% Ethanol.
6. 100% Ethanol (200 proof).
7. Histoclear II (Electron Microscopy Sciences).
8. Paraffin (e.g., Peel-A-Way® Micro-Cut Paraffin (Polysciences, Warrington, PA)).
9. Paraffin Embedding Center (e.g., Thermofisher Histostar).

2.3. Materials and Equipment for Paraffin Sectioning, Deparaffinization, RPE Pigment Bleaching, and Immunofluorescence Staining

1. Water bath set to 50°C, incubator set to 45°C, and hot plate set to 62°C.
2. Microtome (e.g., Thermofisher HM325) with low profile micro-tome blades (e. g. Accu-Edge, Sakura Finetek, Torrance, CA).
3. Superfrost® Plus slides and slide staining jars with slide rack (e.g., EasyDip Slide Staining System, Simport Plastics).

¹All animal procedures need to be reviewed and approved by authorities as appropriate. The procedure requires postmortem tissue harvest.

²Davidson's fixative maintains structural integrity of the retina very well and is hence the preferred fixative for this procedure. Use gloves while preparing and handling Davidson's fixative. White, fine precipitate may form after mixing or with time. This does not inactivate the solution. Remove precipitate by paper filtration before using.

4. Histoclear II, 100% ethanol, 95% ethanol, 70% ethanol, prepared as in Subheading 2.2.
5. PBS: Make a 5× PBS stock: Dissolve 40 g NaCl, 1 g KCl, 1 g KH₂PO₄, 10.8 g Na₂HPO₄ in 500 mL dH₂O. Adjust pH to 7.2 and fill to 1 L with ddH₂O. Dilute fivefold with ddH₂O to make a 1× PBS working solution.
6. Pigment bleaching solution: Weigh 1 g sodium borohydride and dissolve in 100 mL ddH₂O, prepare fresh daily.
7. Pap pen, kimwipes, slide staining chamber.
8. 1% Bovine serum albumin in PBS: Add 1 mL 30% BSA stock to 29 mL PBS. Store at 4°C, bring to room temperature before use.
9. Mouse anti-rhodopsin antibody (see Note 3).
10. AlexaFluor488-conjugated donkey anti-mouse IgG.
11. DAPI nuclei staining solution: 10 µg/mL 4',6-diamidino-2-phenylindole DAPI in PBS.
12. Glass cover glasses (24 mm × 50 mm size).
13. Vectashield (Vector Laboratories, Burlingame, CA).
14. Nail polish.

2.4. Equipment and Software for POS Phagosome Imaging and Counting

1. Laser scanning confocal microscope.
2. Image J Software (NIH—<http://rsb.info.nih.gov/ij/download.html>).

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Enucleation, Fixation, and Lens Removal of Mouse Eyes

1. For each mouse to be sacrificed, prepare two 2 mL flat-bottom microtubes with 1.5 mL each Davidson's fixative. Assign numbers or codes to mice and label tubes accordingly. Record genotype, precise time of sacrifice, and other relevant information.
2. At selected times of day (e.g., 1 h before, 30 min and 8 h after light onset), sacrifice mice using a CO₂ chamber or other suitable and approved method (see Notes 4 and 5).

³A number of rhodopsin monoclonal antibodies are commercially available and many can be used to label early POS phagosomes that still contain intact opsin protein. However, depending on the epitope recognized, antibodies may differ in their recognition of partially digested opsin that is present in phagosomes at later time points. Choice of the appropriate opsin antibody therefore depends on the scientific objective of the experiment.

⁴It is imperative to maintain the dark/light cycle precisely for at least 3 weeks before the experiment to ensure that mice are entrained to a specific rhythm. RPE phagocytosis is regulated by the circadian rhythm and influenced by the light cycle in mice and other higher vertebrates. If the light cycle is not maintained precisely, results will be ambiguous. Aspects to consider include access of personnel to animal rooms at dark times, which may cause light on/off at inappropriate times, and changes associated with daylight savings time.

⁵If testing mice at time points before light onset, place the required mice in a cage and into an open cardboard box near the door of the animal room the night before the experiment. The day of the experiment, enter the animal room in the dark and place a black photography cloth over the box before moving the box to where euthanization and tissue harvest take place. Sacrifice the mice and remove eyes in the dark under dim red light provided by a darkroom red lamp and/or a red flashlight.

3. Promptly following sacrifice, enucleate eyes using a curved forceps and place into separate tubes filled with fixative. Make sure that eyes are completely immersed in fixative (see Note 6).
4. Leave eyes at room temperature for 30 min to 12 h before storing fixed eyes at 4°C for at least another 12 h.
5. 24–48 h after sacrifice, dissect cornea and lens from fixed eyes one at a time: Carefully transfer eye into 3 cm tissue culture dish filled with fixative. Under a dissecting microscope, use a sharp-edged forceps to hold the eye in place via the optic nerve, and with the other hand use a No.11 blade to create a 3–5 mm incision in the cornea. Use microdissection scissors to enlarge the incision by cutting in the shape of a cross, ultimately generating an opening in the cornea that is large enough to allow the lens to float out of the eyeball (see Note 7). Place the eyecup back in its tube with fixative for at least 30 min or until all eyes are processed.

3.2. Tissue Processing and Paraffin Embedding

1. Place each eyecup into a separate plastic cassette labeled clearly with pencil and close with plastic lid (see Note 8).
2. Incubate eyecups with solutions in a tissue processor with vacuum using the following protocol:
 - a. 50% Ethanol—1 h.
 - b. 50% Ethanol—1.30 h.
 - c. 85% Ethanol—1.30 h.
 - d. 2 × 95% Ethanol—1.30 h each.
 - e. 2 × 100% Ethanol—1.30 h each.
 - f. 2 × HistoClear II—1.30 h each.
 - g. 2 × Paraffin—2 h each.
3. Remove plastic cassettes from the tissue processor container and place them in the designated preheated area of the Tissue Embedding Center.
4. Embed each eyecup individually as follows. Pour just enough liquid paraffin to cover the bottom of a preheated metal mold (see Note 9). Move mold to cold area of processor. As paraffin hardens, use sharp-tipped forceps to place the eyecup in the center of the mold. Handle the eyecup using the optic nerve only. The eyecup should be placed such that the optic nerve is parallel to the bottom of the mold. Move the mold to the heated area, and wait for the hardened paraffin in the mold to melt slightly before adding more liquid paraffin until the mold is filled completely. Move the mold to the cold area and immediately remove any bubbles that may be trapped in or around the eyecup using the forceps. Place the numbered plastic cassette on top of the metal mold before the paraffin solidifies. Add more paraffin until the plastic cassette is filled completely. Place the entire assembly in the

⁶Keep the optic nerve intact. Handle the eye grasping only the optic nerve to prevent distorting and damaging the retina. The optic nerve will also be useful to orient the eye during embedding.

⁷Make a large enough incision such that the lens floats out. Do not squeeze the eye to remove the lens; this may damage the retina. Trim off any excess connective tissue and muscles on the outside of the eyeball.

⁸Write clearly and with a pencil as subsequent alcohol treatments will not have an effect on pencil but will remove permanent markers and ball pen writing.

⁹Make sure that there is no paraffin in the mold from previous use. If there is any paraffin, use a Kimwipe to clean it off.

cooling area. Once the paraffin has hardened completely, remove the paraffin block containing the embedded eye along with the plastic mold and store at room temperature.

3.3. Paraffin Sectioning, Deparaffinization, Pigment Removal, and Immunofluorescence Staining

1. Bring water bath temperature to 50°C. Label eight slides with the coded number for each eyecup to be sectioned and consecutive slide numbers.
2. With a razor blade remove excess paraffin surrounding the eyecup, such that a ~1 cm × 1 cm cube is left around the eyecup. Securely mount one paraffin block with eyecup embedded with plastic cassette attached to the microtome. Set section thickness to 25 µm and cut the block until excess paraffin is trimmed. Once the sectioning reaches eyecup tissue reduce section thickness to 7 µm and cut sections as a ribbon and transfer ribbons consisting of 3–4 sections onto the water surface with a paint-brush (No. 6 or smaller) (see Note 10). Collect ribbons on labeled slides. Check under a light microscope for proper alignment after completing two slides (see Note 11). If tissue orientation is correct (Fig. 1), continue sectioning to generate ribbons of 5–7 sections, completing eight slides per eyecup. If not, adjust orientation of tissue block on the microtome, cut one slide, and check again. Repeat checking until orientation is correct, and then cut eight slides.
3. Dry slides at 45°C for 1 h.
4. For phagosome count, use slides numbered 1, 4, and 7.
5. Heat slides on a hot plate set to 62°C for 30 min immediately before tissue deparaffinization.
6. Deparaffinize slides by sequential incubation in slide staining boxes in the following: HistoClear II, 3 × 3 min, 100% ethanol 3 × 1 min, 95% ethanol 2 × 1 min, 70% ethanol 1 × 1 min, ddH₂O 1 × 1 min, and PBS 1 × 1 min.
7. Let PBS run off slides, dry areas far away from tissue with Kimwipe, and mark tissue fields to be stained with Pap pen. Transfer slides to humidified chamber and cover sections with PBS. Keep tissue sections covered with solutions at all times for the rest of the procedure. Change solutions by carefully aspirating one solution, immediately replacing it with the next.
8. To remove RPE pigment, incubate in fresh 1% sodium borohydride solution for 2 min. Rinse with PBS 4 × 1 min (see Note 12).
9. To block the sections, incubate with 1% BSA in PBS for 20 min (see Note 13).
10. Incubate sections in opsin monoclonal antibody in PBS overnight at 4°C (see Note 14).

¹⁰If the blade is no longer sharp, sections bunch up or wrinkle, which should be avoided. If the mounted blade fails to cut sections properly, move it sideways to use a different blade area or, eventually, replace blade. Use the paintbrush to smooth out minor wrinkles before placing section ribbons in water bath. Smooth out wrinkles further there if needed, but beware of sections sticking to bristles.

¹¹Check the tissue for proper orientation where specifically RPE cells are cut perpendicular to Bruch's membrane. The RPE should be visible as a pigmented layer of uniform thickness where pigment is oriented mostly toward the outer segments while the basal aspect of the RPE faces Bruch's membrane, which should appear as a thin, clear, shiny line (Fig. 1). Incorrect orientation of the section will result in incorrect POS phagosome counts. If needed, adjust the orientation of the paraffin tissue block and repeat checking the orientation until it is correct and a uniform RPE layer can be seen under light microscope.

¹²Omitting this step will increase the immunofluorescence staining. However, pigment interference may make phagosome counts unreliable.

¹³5% Normal donkey or goat serum in PBS can be substituted for blocking.

¹⁴Some primary antibodies will yield stronger staining if they are incubated in the presence of 0.1% Triton X-100.

11. Wash with PBS 2×5 min and with 1% BSA–PBS 1×5 min.
12. Incubate sections in freshly diluted secondary antibody AlexaFluor488-conjugated donkey anti-mouse IgG (1:250 in PBS) for 2 h.
13. Wash with PBS 2×5 min.
14. Counterstain with DAPI nuclear stain for 30 min. Wash with PBS for 5 min.
15. Mount in Vectashield, cover with cover glass, aspirate excess Vectashield, and seal with nail polish. Store slides in the dark at room temperature for up to 1 week or at 4°C for up to 1 month.

3.4. Microscopy and Quantification of POS Phagosomes

1. Set up a sequential scan with first scan acquisition parameters excitation being at 488 nm, emission at 490–540 nm, and second scan parameters excitation being at 405 nm, emission at 410–480 nm (see Note 15). Use a 40 $\times$ objective and set zoom such that about half of the length of RPE in a section is visible in the field. Image the central 50% of each eye section.
2. For the 488 scan, increase gain, offset, and laser power until RPE phagosomes are clearly distinguishable. This setting will result in overexposing the opsin signal of the intact outer segment area. Adjust 403 nm scan setting similarly such that RPE nuclei are visible. For each field, acquire an x – y image stack of exactly 5 μ m height with 0.25 μ m distance between individual x – y scans (see Note 16). Acquire at least two 5 μ m-stacks per slide, for a total for at least six stacks per sample. Once stacks are acquired, add 100 μ m scale bars and save maximal projections of channel overlays (see Fig. 2 for example of maximal projection of opsin stain only). Export maximal projections as “tiff” format files.
3. For measuring the length of RPE surveyed in each scan, use Image J software. Open the image, click on “line” tool, and draw a line exactly over the scale bar. Under the “Analyze” menu, click on “set scale.” Change “known distance” to 100 and “unit of length” to “ μ m.” Click “OK.”
4. Draw another line covering at least 100 μ m, just above the RPE layer from which phagosomes will be counted. Under the analyze menu, click on “Measure.” The software will give out readout in μ m. If there is no continuous area of 100 μ m of intact RPE to count, measure and count two smaller stretches totaling at least 100 μ m. Count phagosomes under the line and transfer values to an excel file along with the length measurement. Divide phagosome count by length and multiply by 100 to obtain phagosome count per 100 μ m length of RPE. Count phagosomes of all images acquired for a total for six times per mouse eyecup sample. Average the six values to obtain the mean number of POS phagosomes residing in 100 μ m RPE for that eyecup. Repeat for all eyecup samples.
5. For each sample type (identical age, genotype, and time collected) analyze at least one eye each of four different mice. Calculate average $\pm$ SD to obtain average phagosome count for each sample.

¹⁵The exact acquisition parameters will differ depending on the instrumentation and quality of immunofluorescence staining. It is imperative to only compare samples obtained following identical experimental procedures.

¹⁶Laser scanning confocal microscopy and collapsing image stacks representing a precise tissue volume are imperative. Acquiring POS phagosome signals from an x – y image stack of precise thickness that is identical for all samples is necessary to ensure that sample counts represent load of phagosomes in the same tissue volume. Individual x – y scans or images obtained by epifluorescence (wide-field) microscopy cannot yield such normalized phagosome counts that represent POS phagosome load in the same volume of RPE.

Acknowledgments

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Fig. 1.

Bright field image of a 7- μ m thick section of a paraffin embedded eyecup of a 2-month-old mouse at the correct orientation. *Arrows* indicate the apical side of the thin, pigmented RPE layer. Note the uniform thickness and appearance of the RPE layer throughout the section. *Inset* shows a magnified image showing the RPE cell layer residing on Bruch's membrane, which is visible as a clear line. Note that most pigment granules localize to the apical aspect of RPE cells. *Scale bars* are 100 μ m.

Fig. 2. Maximum projection of a 5- μm stack of x - y images acquired at 0.25- μm interval of a paraffin section of an eyecup of a 2-month-old mouse stained with monoclonal opsin antibody clone B6-30 (8). The overexposed, white tissue area is the rod outer segment layer of the mouse retina, while the *arrows* indicate individual POS phagosomes in the RPE. *Scale bar* is 20 μm .