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Analysis of Photoreceptor Outer Segment Phagocytosis by RPE Cells in Culture

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Abstract

Retinal pigment epithelial (RPE) cells are among the most actively phagocytic cells in nature. Primary RPE and stable RPE cell lines provide experimental model systems that possess the same phagocytic machinery as RPE in situ. Upon experimental challenge with isolated photoreceptor outer segment fragments (POS), these cells promptly and efficiently recognize, bind, internalize, and digest POS. Here, we describe experimental procedures to isolate POS from porcine eyes and to feed POS to RPE cells in culture. Furthermore, we describe three different and complementary methods to quantify total POS uptake by RPE cells and to discriminate surface-bound from engulfed POS.

Keywords

Retinal pigment epithelium; Cell culture; Photoreceptor outer segments; Phagocytosis; Cell receptors; Engulfment; Recognition; Quantification; Immunofluorescence microscopy; Immunoblotting; Fluorescence scanning

1. Introduction

The retinal pigment epithelium (RPE) performs numerous functions in support of photoreceptor rod and cone neurons in the retina (1). Among them, the prompt and efficient clearance by receptor-mediated phagocytosis of photoreceptor outer segment fragments (POS) shed daily by photoreceptors in a diurnal rhythm is essential both for long-term viability and functionality of photoreceptors (2, 3). Although the role of the RPE in POS recognition and engulfment has long been recognized, the underlying molecular mechanisms are not fully understood. Comparison of POS phagosome load in RPE in experimental animals such as mice with different mutations is an important step towards identification of proteins that play a role in POS renewal (for methodology see Chapter 17 by Sethna and Finnemann). However, in situ POS phagocytosis analysis cannot pinpoint the specific contribution of an individual protein to the phagocytic process: (1) a candidate protein may act in photoreceptors or in the RPE or in both; (2) a candidate protein may promote a specific step of the phagocytic process, such as POS recognition, engulfment, or POS digestion. Analysis of POS uptake by RPE cells in culture allows discrimination of specific roles of proteins in the phagocytic process. Primary RPE cells as well as immortalized RPE cell lines have been shown to retain avid phagocytic activity toward isolated POS particles (4–8). Here, we present methodology to induce and quantify POS phagocytosis by RPE cells in culture. We discuss isolating POS particles from porcine eyes, challenging RPE cells in

culture with purified POS, and three different methods for analysis of POS uptake by the RPE, each one of which discriminates surface-bound from engulfed POS particles.

2. Materials

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

2.1. Solutions

2.1.1. POS Isolation Stock Solutions

1. 100 mM glucose: Dissolve 1.8 g glucose in ddH₂O; fill up to 100 mL in graduated cylinder. Store at 4°C.
2. 200 mM Tris/Acetate pH 7.2: Dissolve 12.1 g Tris Base in 400 mL ddH₂O; adjust pH to 7.2 with glacial acetic acid; fill up to 500 mL in graduated cylinder with ddH₂O.
3. 70% sucrose: Dissolve 175 g sucrose by gradually adding ddH₂O, be careful not to exceed final volume of 250 mL. Transfer to graduated cylinder and fill up to 250 mL with H₂O, rinsing original beaker. Store at 4°C.
4. 0.1 M Na₂CO₃ pH 11.5: Dissolve 1.06 g Na₂CO₃ in final volume of 100 mL ddH₂O.
5. 0.1 M NaHCO₃ pH 8.4: Dissolve 0.840 g NaHCO₃ in final volume of 100 mL ddH₂O.

2.1.2. POS Isolation Working Solutions—Prepare the following working solutions fresh on the day of the POS isolation using the stock solutions. Suggested volumes of all working solutions allow POS isolation from 75 fresh pig eyes.

1. Homogenization solution (40 mL): 20% w/v sucrose, 20 mM Tris/Acetate pH 7.2, 2 mM MgCl₂, 10 mM glucose, 5 mM taurine.
2. 25% sucrose solution (80 mL): 25% sucrose, 20 mM Tris/Acetate pH 7.2, 10 mM glucose, 5 mM taurine.
3. 60% sucrose solution (80 mL): Dissolve 48 g sucrose in a solution with final concentration of 20 mM Tris–Acetate pH 7.2, 10 mM glucose, 5 mM taurine.
4. WASH 1 (100 mL): 20 mM Tris–Acetate pH 7.2, 5 mM taurine.
5. WASH 2 (50 mL): 10% sucrose, 20 mM Tris–Acetate pH 7.2, 5 mM taurine.
6. WASH 3 (100 mL): 10% sucrose, 20 mM phosphate buffer pH 7.2, 5 mM taurine.
7. DMEM: Use DMEM, 4.5 g/L glucose.
8. FITC stock solution: Dissolve 10 mg FITC isomer I (Invitrogen) in 5 mL 0.1 M Na-carbonate buffer, pH 9.5. Spin solution to remove any undissolved small particles before using supernatant solution to label POS.

2.1.3. Solutions for POS Phagocytosis Assays

1. PBS-CM: PBS supplemented with 1 mM MgCl₂, 0.2 mM CaCl₂.
2. PBS/EDTA: Add 1/500 v/v 0.5 M EDTA, pH 8.0, to PBS to yield PBS, 2 mM EDTA.

3. FITC-quenching solution: 0.4% trypan blue in PBS.
4. HNTG lysis buffer: 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl_2 , 1% Triton X-100.
5. Antibodies to opsin and to protein serving as loading control, e.g., porin.
6. DAPI nuclei staining solution: 10 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole DAPI in PBS.
7. Fluoromount G.

2.2. Equipment

1. Ultracentrifuge with swing-out rotor for tubes containing up to 30 mL that reaches $112,398 \times g$ (e.g., Beckman ultracentrifuge with rotor SW28); Refrigerated centrifuge that reaches $3,000 \times g$ for 30 mL tubes (e.g., Sorvall RC-2B with rotor SS-34); refrigerated microtube centrifuge.
2. Sensitive, fluorescence flatbed imager, (e.g., Typhoon Trio™, GE Healthcare).
3. Conventional setup for SDS-PAGE and immunoblotting.
4. Conventional epifluorescence or laser scanning confocal fluorescence microscopy system.

3. Methods

3.1. Isolation of POS from Porcine Eyes

This protocol was developed based on a published procedure by Molday and colleagues (9). Obtain 75 pig eyes from a slaughterhouse and process them as fresh as possible (see Note 1). Do not use frozen eyes. Chill eyes and keep them in the dark. Prechill all solutions on ice. Keep all materials ice-cold at all times.

1. Dissect pig eyes one by one under dim red light (see Notes 2 and 3): Take one eye firmly into your left hand. Poke front with edge of razor blade while holding eye away from you, to avoid splashing into your face. Use razor blade to cut anterior segment of eyeball into two halves. Remove lens. Flip the eyecup inside out. Use side of razor blade to gently dislodge retina, a pinkish layer, off the tapetum.
2. Collect the retina in 50 mL plastic tube containing 15 mL homogenization solution on ice.
3. Repeat until all 75 retinas are collected in the same tube.
4. Shake suspension gently for 2 min. Filter 3× through 1 layer gauze to remove large tissue fragments.
5. Use a gradient maker to prepare three 24 mL linear gradients of 25–60% sucrose, 20 mM Tris/Acetate pH 7.2, 10 mM glucose, 5 mM taurine in 30 mL ultracentrifuge tubes. Keep on ice until use. Use within 30 min of preparation.
6. In dim red light, use a 10 mL plastic pipet to gently overlay equal volumes of the crude retina isolate from step 4 over the three gradients. Balance tubes precisely using extra homogenization solution to equalize weights.
7. Spin immediately in a swing-out ultracentrifuge rotor at $112,398 \times g$ (i.e. 25,000 rpm in a Beckman SW-28 rotor) for 48 min at 4°C.
8. Collect sharp, single pink band in upper third of gradient (see Note 4). Discard the tube and the remaining tissue.

9. Dilute with 5 volumes of ice-cold WASH 1.
10. Separate into as many tubes as needed for centrifugation in 30 mL tubes at $3,000 \times g$ for 10 min.
11. Resuspend pellets in 10 mL WASH 2 each, combine pellets and spin 10 min at $3,000 \times g$.
12. Resuspend pellet in 15 mL WASH 3, spin 10 min at $3,000 \times g$.
 - (12a) Unlabeled POS stock: Resuspend POS in 10 mL 2.5% sucrose in DMEM. Dilute 10 μ L plus 490 μ L DMEM (=1/50) and mix well by pipetting. Count diluted POS in cell counting chamber. Calculate yield and concentration. Dilute to $\sim 1 \times 10^8$ POS/mL with 2.5% sucrose in DMEM. Store POS at -80°C in aliquots appropriate for single phagocytosis assays.
 - (12b) FITC-labeled POS stock: Resuspend POS in 5 mL WASH 3, and add 1.5 mL FITC stock solution. Rotate 1 h at RT in the dark. Wash labeled POS twice in WASH 3, twice in 2.5% sucrose in DMEM (spin at $3,000 \times g$ for each wash), then resuspend in 2.5% sucrose in DMEM. Count and store POS as described in (12a).

3.2. Plating of RPE Cells for Phagocytosis Assays

Seed RPE cells on multi-well culture plates depending on the method chosen for POS quantification. Use 96-well clear bottom black plates for fluorescence imaging; seed cells on 5 mm or 1.2 cm coverslips in 96- or 24-well plates, respectively, for POS analysis by immunofluorescence microscopy; seed cells on regular 96-well flat bottom low evaporation culture plates for POS quantification by immunoblotting. In each assay, test samples in triplicate. For POS quantification by fluorescence scanning or immunoblotting, set up two sets of triplicate samples for separate detection of total and internalized POS quantification. Grow RPE cells in culture until desired density and differentiation (see Note 5).

3.3. POS Phagocytosis Assay

1. Calculate the amount of POS needed based on the number of RPE cells per well and number of samples. Confluent, polarized RPE cells should be challenged with ten POS particles per cell (see Note 6).
2. Thaw POS aliquots from -80°C in your hand. Spin POS for 5 min at $2,400 \times g$ at RT in a microcentrifuge. Resuspend pellet immediately in serum free DMEM by gently pipetting up and down. Add pharmacological agents, recombinant proteins, or heat-inactivated FBS (no more than 10% (4)) to the POS suspension as desired. Mix well by pipetting gently.
3. Aspirate medium from RPE cells completely and add POS suspension immediately. Avoid introducing air bubbles. Incubate RPE cells in the tissue culture incubator with POS for the appropriate time depending on your experiment and on your choice of RPE cells (see Note 7).
4. Proceed to one of three methods to quantify phagocytosed POS as outlined below.

3.4. Quantification of Phagocytosed POS

There are three ways to detect and quantify opsin.

3.4.1. Fluorescence Scanning

1. Use FITC-POS for the phagocytosis assay. To terminate POS challenge aspirate POS completely. Wash cells 3×1 min with PBS-CM at room temperature. For multi-well plates, use a plastic squirt bottle to quickly dispense PBS-CM. Avoid damage to the RPE cell layer by adding PBS-CM to the side of wells (see Note 8).
2. Divide wells into two sets: One for detection of total (bound plus internal) POS, the other one for detection of internalized POS. Both sets should allow testing samples in triplicate.
3. To detect internalized POS only, aspirate PBS-CM and incubate cells with FITC-quenching solution for 10 min at room temperature. The other set of wells for total POS quantification remains in PBS-CM during this time. After FITC-quenching, aspirate solution and rinse $2 \times$ with PBS-CM. No wait is required between washes.
4. Aspirate solutions from all wells. Fix cells by filling wells with ice-cold methanol. Let sit for 5 min at room temperature.
5. Aspirate methanol and immediately rehydrate cells by filling wells with PBS-CM and incubating overnight at 4°C (see Note 9).
6. Scan the plate with a fluorescence flatbed scanner using setting appropriate for FITC detection (for a Typhoon TrioTM use excitation 488 nm, detection 520 nm, medium sensitivity). A representative image of the resulting scan is shown in Fig. 1.
7. Quantify the intensity of fluorescence signals in representative areas of each well using ImageQuantTM TL software (GE Healthcare). Calculate relative bound POS by subtracting average internal POS values from average total POS values for each sample.

3.4.2. Opsin Immunoblotting

1. Use unlabeled POS or FITC-POS for the assay. Terminate phagocytosis by washing $3 \times$ with PBS-CM and designating triplicate samples as described in Subheading 3.4.1, steps 1 and 2.
2. To detect internalized POS only, rinse washed cells $1 \times$ with PBS, then incubate cells with PBS-EDTA for 5–10 min. Samples designated for total POS detection remain in PBS-CM.
3. Aspirate PBS-EDTA and wash wells $3 \times$ with PBS. No wait required between washes.
4. Aspirate PBS-CM from all wells and lyse cells with HNTG buffer freshly supplemented with protease inhibitor cocktail. Analyze phagocytosed POS content of samples by SDS-PAGE and opsin immunoblotting (see Notes 10 and 11). Numerous opsin antibodies are commercially available and will work well. Analyze triplicates on the same blot membrane determine sample-to-sample variability.
5. Re-probe the same membranes for proteins unrelated to the phagocytic pathway to control for equal cell load in each sample (see Note 12). Figure 2 shows an example immunoblot detection of phagocytosed POS opsin and of the mitochondrial protein porin as a loading control.

6. Quantify bands representing internal or total POS and normalize POS content of individual samples for cell load as indicated by the loading control protein blot. Average triplicates.

3.4.3. Fluorescence Microscopy—Carry out all steps at room temperature.

1. Use FITC-OS for the phagocytosis assay. Terminate phagocytosis by washing 3× with PBS-CM as described in Subheading 3.4.1.
2. Fix cells with 4% PFA in PBS-CM for 20 min.
3. Quench remaining fixative by incubating cells in 50 mM NH₄Cl in PBS-CM for 20 min.
4. Block with 1% BSA in PBS-CM at RT for 10 min.
5. Incubate cells with opsin antibody diluted in 1% BSA in PBS-CM for 25 min (see Note 13).
6. Wash wells 2× with PBS-CM and 1× with 1% BSA in PBS-CM for 5 min each.
7. Incubate cells with appropriate secondary antibody conjugated with a fluorophore that does not conflict with FITC (e.g., AlexaFluor 568, 596, 647).
8. Wash wells 2× with PBS-CM for 5 min each, 1× with DAPI nuclei stain for 10 min (optional), and 1× with PBS-CM for 5 min.
9. Mount coverslips on microscopy slides with Fluoromount G.
10. Image FITC- and secondary antibody-derived fluorescence signals. Internalized POS will appear in the FITC image only. Surface-bound POS will appear both in the FITC image and in the secondary antibody image. In a color overlay of FITC (green) with secondary antibody (red), internal POS will appear green and surface-bound POS will appear yellow. The example in Fig. 3 shows images of the same field showing surface POS (left) and total FITC-POS (right).
11. Count total and surface-bound POS and cell nuclei in representative areas of at least 50 cells in each sample. Calculate bound POS/cell, total POS/cell, and internal POS/cell (by subtracting bound from total POS/cell). Average counts per cell obtained from triplicates.

4. Notes

1. 25 Cow eyes or 35 calf eyes may be substituted for 75 pig eyes.
2. This is a messy procedure. Cover bench with absorbent pads and prepare 15 cm dishes or plastic trays to collect tissue waste, wear double lab coat and gloves.
3. Handle eyes and crude retinal isolate in dim red light only to avoid bleaching rhodopsin in POS fragments. The pink color of unbleached rhodopsin will allow easy identification of the tissue fraction containing the POS following gradient separation.
4. Depending on the quality of the gradient the band containing the POS particles may be more or less diffuse. Identify the right band by observing the bleaching of rhodopsin, which is visible as a color change from pink to beige. Mark band location on the centrifuge tube using permanent marker while observing the bleaching to ensure collection of the correct tissue fraction.
5. Most if not all RPE cells in culture retain phagocytic activity towards isolated POS. However, phagocytic capacity, pathways and mechanisms used may differ

depending on the differentiation state of RPE cells. Different RPE cell lines and primary RPE cells take different periods of time to differentiate into an epithelial phenotype that best resembles RPE in the eye and to reach polarity in culture. Furthermore, RPE cells in culture may dedifferentiate with time and passage especially if seeded at low confluence after split. We strongly recommend only using post-confluent, mature RPE monolayers for phagocytosis assays that have assembled the known components of the POS phagocytic machinery at their apical surface. It is useful to establish a successful protocol that ensures only high-quality RPE cells with reproducible phagocytic activity are used and to strictly adhere to the protocol for all studies. We also recommend using primary RPE that is unpassaged to maximize their resemblance to the RPE in the eye and to split stable RPE cells at low ratio (e.g., split ARPE-19 cells 1:2 every 2–4 weeks, split RPE-J cells 1:4 exactly 1× per week) to allow the cells to maintain an epithelial phenotype at all times.

6. POS should not be refrozen.
7. Different RPE cell models bind and engulf POS with different kinetics. Furthermore, time of phagocytic challenge may be chosen to represent primarily active POS binding (early phase of uptake), ongoing binding and internalization, or primarily active internalization of bound POS (late phase of uptake). Suggested times of POS challenge based on our work: RPE cell lines, 2–5 h (8); unpassaged primary rat or mouse RPE cells, 30 min to 2 h (3, 10).
8. Aspirate solution completely for every change of solution by holding the plate at a 45° angle. Keep aspirator tip steady, reaching into the bottom of the well and facing the same side of the well for all steps to minimize cell layer damage.
9. After rehydration, cell nuclei may also be stained, e.g., with DAPI. However, note that trypan blue quenching will cause high red background fluorescence emission. This precludes using red fluorescing nuclei counterstains such as propidium iodide for trypan blue treated samples.
10. Apply samples to gels immediately following addition of reducing SDS-sample buffer. Boiling samples cause formation of opsin multimers making opsin quantification ambiguous.
11. It is important to consider stability of epitopes recognized to lysosomal protein degradation when choosing an opsin antibody for the detection of phagocytosed POS. This is particularly relevant when studying late phases of POS phagocytosis during which POS opsin will be degraded.
12. Proteins chosen as loading controls need to be detectable in unboiled samples and must not fluctuate with POS phagocytosis. Examples include VDAC/porin mitochondrial protein and α - or β -tubulins. Actin should not be used because it is directly involved in POS phagocytosis and its level or stability may change during the experiment.
13. Without permeabilization, only external opsin will be detected if incubation with primary antibody is short. After incubation with primary antibody overnight at 4°C some antibody may enter cells causing ambiguous results.

Acknowledgments

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Fig. 1.

Fluorescence scanning analysis of POS phagocytosis. Confluent, polarized RPE-J cells were challenged with FITC-POS for 5 h before processing as described in Subheading 3.4.1. The fluorescence scan was obtained using a Trio™ Scanner. Triplicate wells of a 96-well plate were used to detect total (bound plus internal) and internal (trypan blue-quenched) FITC signal (+POS, as indicated). Wells with cells but not fed with POS (–POS) were used to subtract background fluorescence. Cells in these wells were washed and fixed exactly like POS-treated cells.

Fig. 2.

Immunoblotting analysis of POS phagocytosis. Confluent, polarized RPE-J cells were challenged with unlabeled POS for 1 or 3 h before processing as described in Subheading 3.4.2. The same blot membrane was probed for porin as loading control and for opsin to indicate POS content as indicated. *int* cells treated with EDTA before lysis, samples show internalized POS opsin, *tot* cells lysed without EDTA treatment, samples show total (bound plus internal) POS opsin. As expected, cells bind and internalize increased numbers of POS with time.

opsin: bound POS

FITC: total POS

Fig. 3.

Microscopy analysis of POS phagocytosis. Unpassaged, polarized Long Evans (wild type) rat primary RPE cells were challenged with FITC-POS for 1 h before processing and imaging as described in Subheading 3.4.3. The *left panel* shows immunodetection of surface-bound (external) POS with rhodopsin antibody B6-30 (11) and AlexaFluor594-conjugated donkey anti-mouse IgG. The *right panel* shows the FITC fluorescence signal of total phagocytosed (bound plus internalized) POS in the same field. *Arrow* indicates bound POS, which is visible in both images. *Arrowhead* indicates internalized POS, which are only visible in the FITC image. Scale bar, 10 μ m.