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Advanced 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 provide experimental protocols to synchronize the POS binding and engulfment steps of phagocytosis. Finally, 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; Receptors; Uptake; 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 toward the identification of proteins that play a role in POS renewal. There are published protocols to quantify POS phagocytosis by the RPE in situ [4]. Yet, in situ POS phagocytosis analysis cannot pinpoint the specific contribution of an individual protein to the phagocytic process: (1) a candidate protein may function in photoreceptors, in the RPE, or in both, and (2) a candidate protein may play a role in one or several of the specific steps 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 and embryonic, adult, or induced pluripotent stem cell-derived RPE cells as well as immortalized RPE cell lines have been shown to retain avid phagocytic activity toward isolated POS particles [5–7]. Findings over the past 20 years from in vivo and cell culture

studies agree that the primary POS recognition/binding receptor of rat, mouse, and human RPE cells is the integrin receptor $\alpha v \beta 5$ [8, 9], while most RPE cells require and activate the TAM receptor MerTK for engulfment [10–12] although other TAM receptors may substitute [13]. Here, we present methodology to induce and quantify POS phagocytosis by RPE cells in culture and to separately analyze binding and engulfment steps in synchronized phagocytosis assays. We discuss isolating POS particles from porcine eyes, challenging RPE cells in culture with purified POS, and synchronizing POS binding and engulfment 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 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.

2.2 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 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.

2.3 Solutions for POS Phagocytosis Assays

1. DMEM with high glucose.
2. RPE phagocytic receptor ligand proteins MFG-E8 and Protein S.
3. PBS-CM: PBS supplemented with 1 mM MgCl_2 and 0.2 mM CaCl_2 .
4. PBS-EDTA: Add 1/500 v/v 0.5 M EDTA, pH 8.0, to PBS to yield PBS, 2 mM EDTA.
5. FITC-quenching solution: 0.4% trypan blue in PBS-CM.
6. HNTG lysis buffer: 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl_2 , 1% Triton X-100.
7. Antibodies to opsin and to protein serving as loading control, e.g., porin.
8. DAPI nuclei staining solution: 10 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole DAPI in PBS.
9. Fluoromount-G.

2.4 Equipment

1. Ultracentrifuge with swing-out rotor for tubes containing up to 30 mL that reaches $112,398 \times g$; refrigerated centrifuge that reaches $3000 \times g$ for 30 mL tubes; refrigerated microtube centrifuge.
2. Sensitive, fluorescence flatbed imager.
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 [14]. 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. Pre-chill 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 the front with the edge of a razor blade while holding the eye away from you, to avoid splashing into your face. Use razor blade to cut anterior segment of the eyeball into two halves. Remove lens. Flip the eyecup inside out. Use the side of the 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 pipette 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 $112,398 \times g$ for 48 min at 4 °C.
8. Collect sharp, single pink band in the upper third of gradient (*see* Note 4). Discard the tube and the remaining tissue.
9. Dilute with five volumes of ice-cold Wash 1.
10. Separate into as many tubes as needed for centrifugation in 30 mL tubes at $3000 \times g$ for 10 min.
11. Resuspend pellets in 10 mL Wash 2 each; combine pellets and spin 10 min at $3000 \times g$.
12. Resuspend pellet in 15 mL Wash 3; spin 10 min at $3000 \times g$.
 - a. Unlabeled POS stock: Resuspend POS in 10 mL 2.5% sucrose in DMEM. Dilute 10 μ L in 490 μ L DMEM (=1/50) and mix well by pipetting. Count diluted POS in a 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.
 - b. 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 label POS twice in Wash 3, twice in 2.5% sucrose in DMEM (spin at $3000 \times g$ for each wash), and then resuspend in 2.5% sucrose in DMEM. Count and store POS as described in **step 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 flat bottom low evaporation culture plates, respectively, for POS analysis by immunofluorescence microscopy; seed cells on 96-well flat bottom low evaporation culture plates without coverslips for POS quantification by immunoblotting. In each assay, test the samples in triplicate if possible. For POS quantification by fluorescence scanning or immunoblotting, set up two sets of triplicate samples for separate detection of the total and internalized POS quantification. Grow RPE cells in culture until desired density and differentiation (*see Note 5*).

3.3 Continuous POS Phagocytosis Assay

The procedure below describes POS phagocytosis assays during which POS feeding takes place continuously in the presence of ligands for both the primary POS recognition/binding receptor $\alpha v \beta 5$ integrin and the primary engulfment receptor MerTK (or related TAM receptor tyrosine kinases). In this assay, POS challenge is followed by POS uptake in an asynchronous fashion. Thus, POS binding and engulfment processes take place simultaneously such that individual cells may bind POS and engulf other POS at the same time.

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 ~10 POS particles per cell (*see Note 6*).
2. Thaw POS aliquots from -80°C in your hand. Spin POS for 5 min at $2400 \times g$ at RT in a microcentrifuge. Resuspend pellet immediately in serum-free DMEM by gently pipetting up and down. For each well of a 96-well or 24-well plate, POS should be resuspended in 40 μL or 300 μL DMEM, respectively.
3. Add specific recombinant ligands for $\alpha v \beta 5$ integrin and MerTK or heat-inactivated FBS (no more than 10% [15]) to POS suspension. Add any pharmacological agent if desired depending on the experimental question. Mix well by pipetting gently (*see Notes 7 and 8*).
4. 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 Notes 9 and 10*).
5. Proceed to one of the three methods to quantify phagocytosed POS as described in Subheadings 3.5–3.7.

3.4 Discontinuous POS Phagocytosis Assay with Synchronized Binding and Engulfment Steps

This two-step phagocytosis assay separates the recognition/binding and engulfment phases of the phagocytic process to yield important complementary insight to results obtained from the continuous phagocytosis assay described in Subheading 3.3. Of particular importance, it

allows pharmacological manipulation of the engulfment step alone, subsequent to POS binding. The synchronization is based on (1) the sequential addition of recombinant proteins that serve as ligands for recognition and engulfment receptors of the RPE; (2) the incubation first at the restrictive temperature of 20°C, at which RPE cells bind POS but do not internalize POS; (3) the removal of excess unbound POS to prevent additional POS binding during the engulfment phase; and (4) a second incubation at the permissive temperature of 30–37°C (depending on cell model), which triggers engulfment of surface-tethered POS (*see* Fig. 1).

1. Follow **steps 1 and 2** of Subheading 3.3.
2. Add MFG-E8 as ligand for the recognition/binding receptor $\alpha v\beta 5$ integrin to the POS suspension. Criteria for ligand choice and concentration are discussed in Subheading 3.3 (*see* Notes 7 and 8). Mix well by gently pipetting.
3. Aspirate medium from RPE cells, and rinse cells once with serum-free DMEM at RT; add 40 μ L or 300 μ L freshly mixed POS suspension per well of 96-well or 24-well plate, respectively, and incubate cells for 1 h at 20°C (*see* Note 11).
4. Prepare 40 μ L or 300 μ L DMEM with Protein S per well of 96-well or 24-well plate, respectively, for samples that will be shifted in temperature to induce engulfment of surface-bound POS.
5. Aspirate excess unbound POS and wash cells 3 $\times$ for 2 min each with serum-free DMEM at RT. At this time, select samples must be washed 3 $\times$ with PBS-CM, fixed or lysed and prepared for POS quantification as described in Subheadings 3.5–3.7; only these samples allow later accurate quantification of bound POS (*see* Notes 12 and 13).
6. Add DMEM with Protein S to the remaining cells, and incubate at 37°C for select time period/s (*see* Note 9).
7. Terminate the phagocytosis process by aspirating the supernatant medium followed 3 $\times$ 2-min wash with PBS-CM and sample fixation or lysis as required for the POS quantification procedure of choice. Process all samples further by following one of the protocols to quantify phagocytosed POS that are described in Subheadings 3.5–3.7. Figure 1 summarizes the steps of synchronized POS phagocytosis assays including sample analysis by surface POS immunofluorescence labeling as described in Subheading 3.7.

3.5 Quantification of Phagocytosed POS by Fluorescence Scanning

1. Use FITC-POS for the phagocytosis assay.
2. Designate samples for detection of either total (bound plus internal) POS or only internalized POS. Test samples in triplicates if possible. For discontinuous POS uptake assays, terminate select cells in triplicates after the binding step.
3. To quantify internalized POS only, aspirate PBS-CM, and incubate live cells with FITC-quenching solution for 10 min at RT. The remaining wells designated for total POS quantification are kept in PBS-CM at RT during this time. After FITC-

quenching, aspirate solution and rinse 2× 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 RT.
5. Aspirate methanol and immediately rehydrate cells by filling wells with PBS-CM and incubating overnight at 4 °C (*see* Note 14).
6. Scan the plate with a fluorescence flatbed scanner using setting appropriate for FITC detection. A representative image of a resulting scan is shown in Fig. 2.
7. Quantify the intensity of fluorescence signals in representative areas of each well. Calculate relative bound POS by subtracting average internal POS values from average total POS values for each sample.

3.6 Quantification of Phagocytosed POS by Opsin Immunoblotting

1. Use unlabeled POS or FITC-POS for the assay. Designate triplicate samples for detection of either total POS or only internalized POS as described in **step 2** of Subheading 3.5. For discontinuous POS phagocytosis assays, when terminating the binding step, quickly rinse select samples in triplicates 2× with PBS-CM at RT. Lyse cells in ice-cold lysis buffer (*see* **step 4** below); collect and freeze lysates until SDS-PAGE analysis.
2. To detect only internalized POS, rinse washed cells 1× with PBS; then incubate cells with PBS-EDTA for 5–10 min at RT, depending on the cell model. Samples designated for total POS detection remain in PBS-CM at RT during this incubation.
3. Aspirate PBS-EDTA and wash wells 3× with PBS at RT. No wait required between washes.
4. Aspirate PBS/PBS-CM from all wells and chill cells on ice. Lyse cells with ice-cold HNTG buffer freshly supplemented with protease inhibitor cocktail (*see* Note 15).
5. Analyze phagocytosed POS content of samples by SDS-PAGE and opsin immunoblotting (*see* Notes 16–18). Numerous opsin antibodies are commercially available and will work well. Analyze triplicates on the same blot membrane to determine sample-to-sample variability.
6. Re-probe the same membranes for proteins unrelated to the phagocytic pathway to control for equal cell load in each sample (*see* Note 19). Figure 3 shows an example of immunoblot detection of phagocytosed POS opsin and of the mitochondrial protein porin as a loading control.
7. 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.

3.7 Quantification of Phagocytosed POS by Fluorescence Microscopy

Carry out all steps at room temperature.

1. Use FITC-OS for the phagocytosis assay.
2. Fix washed cells with freshly prepared 4% PFA in PBS-CM for 20 min (*see Note 20*).
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 21*).
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., Alexa Fluor 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 principle of immunolabeling of fluorescent POS that are surface-bound for discrimination of bound from engulfed POS is shown in Fig. 1. Figure 4 shows two images of the same example field with 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 are obtained from triplicates.

4 Notes

1. Twenty-five cow eyes or 35 calf eyes may substitute 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 coats 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 toward 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, polarized RPE monolayers for phagocytosis assays that have assembled the known components of the POS phagocytic machinery at their apical surface [6]. We also recommend developing and adhering to stringent RPE cell culture preparation, seeding, passaging (if appropriate), and feeding protocols to improve reproducibility of results among repeat assays.
6. POS should not be re-frozen.
7. MFG-E8 is the physiological ligand for the POS recognition receptor $\alpha v \beta 5$ integrin [16]. Protein S is one of the retinal ligands capable of stimulating MerTK or related TAM POS engulfment receptors [17]. Both ligands are available in purified form from several commercial sources.
8. The capacity of ligands to stimulate their receptors is concentration dependent. As ligands acts as bridge proteins between POS and RPE surface receptors, both too little and too much ligand may inhibit POS recognition or binding [16]. Effective ligand concentrations depend on the density of ligand binding sites (e.g., phosphatidylserine) on POS and on receptor availability at the exposed apical surface of RPE cells [18]. They must be established experimentally.
9. 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 and human fetal and stem cell-derived RPE cells, 2–5 h [8]; unpassaged primary rat or mouse RPE cells, 20 min to 2 h [9].
10. Aspirate solution completely for every change of solution by holding the plate at a 45° angle. Keep aspirator tip steady and reach to the bottom of the well. Aspirate from the same side of the well for all steps to minimize cell layer damage.
11. 20 °C is a restrictive temperature that allows POS binding via MFG-E8/ $\alpha v \beta 5$ integrin but not POS internalization [19, 20].

12. For large numbers of samples in 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.
13. Select samples must be harvested after the 20 °C incubation for accurate quantification of bound POS; one cannot assume that bound POS persist during the engulfment period if pharmacological agents are added to the second incubation.
14. 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.
15. Use at least 40 µL lysis buffer for wells of 96-well plates and at least 160 µL for wells of 24-well plates to ensure complete lysis; volume may be increased as appropriate depending on blotting antibody detection sensitivity.
16. Apply samples to gels immediately after addition of reducing SDS sample buffer. Boiling samples causes the formation of opsin multimers making opsin quantification ambiguous.
17. It is important to consider the stability of epitopes recognized to lysosomal protein degradation when choosing an opsin antibody for detection of phagocytosed POS. This is particularly relevant when studying late phases of POS phagocytosis during which POS opsin will be degraded [21, 22].
18. With extended duration of incubation at the permissive temperature in discontinuous POS phagocytosis assays, engulfed POS will be degraded by the cells leading to disappearance of opsin bands or bands representing partially degraded opsin. Testing different incubation times is important when testing POS phagocytosis in new cell models. Results may also vary depending on opsin antibody used (see Note 18).
19. Proteins chosen as loading controls need to be detectable in unboiled samples and must not fluctuate with POS phagocytosis or phagocytosis receptor stimulation. Examples include VDAC/porin mitochondrial protein and α - or β -tubulins. Predetermine that all primary antibodies recognize their antigen in unboiled samples. Actin is not an ideal loading control because it is directly involved in POS phagocytosis, and its level or stability may change during the experiment.
20. To fix samples at the end of the binding period, leave coverslips in wells. Following washes, incubate with 4% PFA in PBS-CM in the incubator while remaining cells start engulfment phase. Seal wells with strips of parafilm during fixation to avoid dissipation of fixative vapors to adjacent wells. After 15 min, replace fixative with PBS-CM, but leave fixed wells covered with Parafilm until the end of the experiment.

21. 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.

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Fig. 1.

Summary scheme outlining steps of synchronized POS phagocytosis assays as described in Subheading 3.4 and labeling of surface-bound POS as described in Subheading 3.7

Fig. 2.

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 3.5. 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. 3.

Immunoblotting analysis of POS phagocytosis. Confluent, polarized RPE-J cells were challenged with unlabeled POS for 1 h or 3 h before processing as described in Subheading 3.6. 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

Fig. 4.

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 3.7. The left panel shows immunodetection of surface-bound (external) POS with rhodopsin antibody B6–30 [23] and Alexa Fluor594-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