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A platform for assessing outer segment fate in primary human fetal RPE cultures.

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Abstract

The daily shedding and renewal of photoreceptor outer segments (OS) is critical for maintaining vision. This process relies on the efficient uptake, degradation, and sorting of shed OS material by the retinal pigment epithelium (RPE). Poor OS degradation has been linked to retinal degenerations such as Stargardt disease and may contribute to macular degeneration. While primary human fetal RPE cultures have emerged as a valuable model of *in vivo* human RPE function, surprisingly few studies have utilized the model for tracking the degradation and fate of OS components in the RPE. Here, we establish an improved platform for studying this topic by modifying existing protocols and creating new methods. Our human fetal culture model facilitates studies of RPE secretion in response to OS ingestion, preserves RPE differentiation and polarization during live-cell imaging of OS phagocytosis, and minimizes costs. We optimize Mer tyrosine kinase-dependent OS phagocytosis assays specifically in human fetal cultures and provide a simple and accurate method for measuring total OS consumption by the RPE. Finally, we utilize chemical transfection, dextran labeling, and immunocytochemistry to evaluate key players in OS degradation, including lysosomes and autophagy proteins. To facilitate quantification of autophagy vesicles, we develop customized image analysis macros in the Fiji/ImageJ software environment. These protocols will facilitate a broad range of studies in human fetal RPE cultures aimed at determining the ultimate fate of OS components after ingestion, a critical step in understanding the pathogenesis of numerous retinal diseases.

Keywords

Retinal pigment epithelium; primary human fetal culture; outer segment phagocytosis; autophagy; lysosomes; microscopy; age-related macular degeneration

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1. Introduction

The retinal pigment epithelium (RPE), a cuboidal, pigmented cell layer that lies on top of Bruch's basement membrane and underneath retinal photoreceptors, is a critical participant in the renewal of photoreceptor outer segments (OS). Each RPE cell phagocytizes the shed OS tips of up to 30 photoreceptors each day (Volland et al., 2015). Once digested, the OS components can, among other possibilities, be incorporated into RPE membrane, converted into energetic starting material that sustains RPE and photoreceptor metabolism (Adjianto et al., 2014), or recycled back to the photoreceptors as building blocks to support new growth and function of OS (Anderson et al., 1992).

As RPE is largely post-mitotic, a single RPE cell might be responsible for phagocytizing over 800,000 OS during the life of an 80 year-old (calculated based on assumption that photoreceptors shed once daily (Kocaoglu et al., 2016) and an RPE cell is responsible for 30 overlying photoreceptors (Volland et al., 2015)). This makes the RPE the most phagocytically-burdened cell in the body (Mazzoni et al., 2014), and the incomplete degradation of phagocytosed OS may contribute, in part, to the accumulation autofluorescent intracellular waste, termed lipofuscin (Boulton, 2014). Accumulation of lipofuscin, in turn, is associated with several retinal degenerations, including Stargardt's disease.

Once OS are ingested, LC3 and certain other proteins normally associated with the intracellular catabolic process termed autophagy are co-opted onto the growing phagosome membrane, a process termed LC3-associated phagocytosis (LAP) (Frost et al., 2015; Kim et al., 2013). Subsequently, the phagosome is transported to the lysosome for OS degradation, and mutations in molecular motors responsible for this transport trigger retinal degeneration in both mouse models and humans (Gibbs et al., 2003; Jiang et al., 2015). Disturbance of the lysosome itself also leads to RPE dysfunction, frequently accompanied by lipofuscin-like accumulation (Guha et al., 2014), in diseases as wide ranging as hereditary spastic paraplegia (SPG15 or SPG11 gene defects), mucopolipidosis type IV (TRPML1 defect), and Danon disease (LAMP2 defect) (Chang et al., 2014; Riedel et al., 1985; Saijo et al., 2016; Tarantola et al., 2011; Thompson et al., 2016).

While the mechanisms for binding and initial engulfment of OS tips by the RPE have been well described (Mazzoni et al., 2014), fewer details are known about how each component of the OS is handled during subsequent degradation. Human fetal RPE (hfRPE) primary cultures, the most extensively validated and accepted *in vitro* model for recapitulating the *in vivo* phenotype of human RPE (Pfeffer and Philp, 2014), would seem a natural system for studies on OS degradation, but remarkably little has been published in this area. The long lead-time for confluent maturation of the cultures (>4–6 weeks), poor optical properties of the semi-permeable supports cultures are grown on, heavy melanization of hfRPE, and other factors have created significant technical hurdles.

Here, we describe a set of protocols to facilitate the study of OS degradation in hfRPE cultures. First, we have modified published hfRPE culture models to improve viability and decrease costs, allow for serum-free culturing when needed, and permit live-cell microscopy of RPE attached to semi-permeable supports. Next, we optimize phagocytosis protocols to

increase uptake specifically in hRPE cultures, improve labeling and tracking of ingested OS, and ascertain total OS consumption rates. Finally, we present live-cell and immunocytochemistry (ICC) methods to detect and quantify OS degradation, LC3-associated phagocytosis, and lysosomes during phagocytosis specifically in hRPE. These methods will facilitate studies determining the ultimate fate of OS components after ingestion, providing insight into an essential role of the RPE in preserving and maintaining retinal health.

2. Materials and Supplies

For most protocols in our study, listed products represent the least expensive but fully efficacious alternative among several similar products tested head-to-head.

For RPE culture media, we used Premium Select, heat-inactivated fetal bovine serum from Atlanta Biologicals (#S11550H). Trans-epithelial resistance (TEER) was measured using an EVOM device with an STX2 electrode (World Precision Instruments).

Total RNA was extracted with a RNeasy micro kit (Qiagen, CAT#74004). mRNA expression levels of CRALBP, MERTK, and RPE65 were assessed with qPCR (4 biological samples, each sample amplified in triplicate) as described (Kurth et al., 2007) but using the CFX384™ Real-time System (Bio-rad) and β -actin as the normalization control. The following primers were used: MerTK-forward: 5'-ATCCTGGGGTCCAGAACCAT-3'; MerTK-reverse: 5'-TTCCGAACGTCAGGCAAAC-3' (Maruotti et al., 2013); CRALBP-forward: 5'-CTGGCAAAGTCAAGAAATCAC-3'; CRALBP-reverse: 5'-TGTCCACCATCTTCCTGAG-3' (Akrami et al., 2011); RPE65-forward: 5'-CGTCATAACAGAATTTGGCACC-3'; RPE65-reverse: 5'-GCCCCATTGACAGAGACATAG-3'; β -actin-forward: 5'-CAGGATGCAGAAGGAGATCAC-3'; β -actin-reverse: 5'-TGTCAAGAAAGGGTGTAAACGC-3'.

ZO-1 antibody clone #R40.76 was purchased from Millipore Sigma (#MABT11). Lamp1 antibody clone #H4A3 was developed by August, J.T. and Hildreth, J.E.K., purchased from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa. The rabbit polyclonal anti-Kir7.1 was developed by Hughes, B.A. (Yang et al., 2003). The rabbit anti-MCT3 is a generous gift from Nancy Philp and was raised against a C-terminal peptide of human MCT3 residues (Philp et al., 2003a). Phalloidin was used according to manufacturer instructions (Biotium, #00041). Western blotting of cell lysates utilized anti-rhodopsin (EnCor Biotech, #MCA-B630) and anti-GAPDH (EnCor Biotech, #MCA-1D4). Western blotting was performed on 8 μ L out of 200 μ L secreted supernatant using anti-PEDF (Abcam, #AB180711) and anti-TIMP-3 (Millipore, #AB6000). β -hydroxybutyrate (β -HB) was assayed on 50 μ L out of 200 μ L secreted media using an Amplitude fluorometric kit (AAT Bioquest, #13831), following manufacturer's protocol. mTor inhibitor torin 1 was from ApexBio (#A8312).

Fixed-cell microscopy was performed using a Lecia SP5 confocal TCS microscope with a 63X oil immersion lens (Leica, HCX PL APO). Live-cell microscopy in Supplementary

Figure 3 was performed on a Nikon Eclipse Ti inverted widefield microscope with a 60x water dipping objective lens (Nikon, CFI Fluor 60XW) using a set-up and custom software as described (Malik et al., 2018). Live-cell microscopy in Figure 4 and Supplementary Figure 4 was performed on a Nikon A1 confocal microscope in inverted configuration. Lamp1-mGFP plasmid was a gift from Esteban Dell'Angelica (Addgene, #34831), and mCherry-hLC3B plasmid was a gift from David Rubinsztein (Addgene, #40827) (Falcón-Pérez et al., 2005; Jahreiss et al., 2008).

Statistical analysis involved either paired or unpaired Student's T-tests unless indicated otherwise.

3. Detailed Methods

3.1 Primary Human Fetal RPE Cultures

3.1.1 Establishing Cultures—hfRPE cultures were established by modifying the protocol of (Maminishkis et al., 2006). We found that UV-curing during extracellular matrix coating of Transwells in the original Maminishkis et al. protocol led to more cell clumping and lower trans-epithelial electrical resistances (TEER) (Figure 1A). We therefore coat without UV-treatment. Using 24-well Transwells, we saved on media costs without sacrificing culture health by reducing media amounts per well (125 μ L apically, 600 μ L basolaterally) and changing media less frequently. We changed media twice a week during passage 0 (P0) plating and for approximately the first 5 weeks after passage 1 (P1) plating. Once RPE barrier function stabilized, as assessed by TEER at 5–7 weeks after P1 plating on Transwells, once- or twice-weekly media changes continued to preserve RPE barrier function (Supplementary Figure 1). For all experiments in this study, media changes of mature P1 hfRPE on Transwells were mostly performed twice-weekly, but occasional once-weekly media changes were employed during conferences or vacations. Given longer periods between media changes, we minimized the effects of evaporation by maintaining our incubator with a Rh% > 97% and placing autoclaved water in the voids between wells on the 24-well receiver plate. In addition, we avoid use of the four corner wells, as these have significantly higher evaporation rates. Our P1 cultures exhibit robust barrier function, a monolayer of cobblestone, pigmented cells outlined by both actin and ZO-1 (Figure 1A-B, 2A), expression of RPE markers RPE65, MERTK, and CRALBP (Figure 1C), and strong polarity (Figure 2C, Supplementary Figure 2B).

3.1.2 Serum-Free Culture Conditions—Our hfRPE cultures are grown in RPE complete media with 5% serum ("RPE Complete"), which has a base of α MEM (Maminishkis et al., 2006). However, to measure the secretion profile of certain lipids, proteins, and metabolites from the hfRPE in response to OS degradation, it is necessary to transfer mature Transwell cultures to serum-free growth conditions. We found that the combination of Neurobasal media without glutamine (Thermo, #21103049), supplemented with B27 (50x) (Thermo, #17504044), non-essential amino acids (100x) (Thermo, #11140050), penicillin/streptomycin (100x) (Thermo, #15140122), GlutaMax (100x) (Thermo, #35050061), and taurine (125mg/500mL of media final concentration) (Sigma, #T8691), which we term "NB+B27", resulted in stable cell cultures, as assessed over a one-

month period comparing TEER of hfRPE in “NB+B27” or in “RPE Complete” media (Figure 1D). When the serum-free media base is switched from Neurobasal to the α MEM base used in “RPE Complete” media, keeping all other components the same, TEER declines significantly. This indicates the Neurobasal base is specifically responsible for stabilized TEER in serum-free media (Figure 1D).

3.1.3 Live-Cell Microscopy Culture Conditions—Live-RPE imaging of OS phagocytosis must overcome the poor optical qualities of Transwell membranes. Prior live-RPE imaging has either involved growing cells on optically-clear glass (Hazim et al., 2017) or cutting and mounting RPE-lined Transwell membranes in a bath chamber (Jiang et al., 2015). Growing RPE cells on glass limits their polarization and cutting out a Transwell membrane from its well precludes future experimentation on the well after an imaging session. To image OS phagocytosis by highly polarized hfRPE without compromising our ability to perform future experiments on the imaged cells, we seeded cells on the basal side of the Transwell (“upside-down” plating) (Supplemental Document 1 for detailed protocol). This was achieved by first coating the basolateral side of the Transwell with human placental extracellular matrix (ECM) (Corning, #353808). After placing 75 μ L of media in the apical chamber, the Transwell was flipped upside down in a 6-well receiver plate and the basolateral surface was pre-incubated with 100 μ L of media. A split ratio between 1:1.5 – 1:3.5 from a P0 culture flask was used to make a cell suspension. After removal of the pre-incubation media, 100 μ L of the cell suspension was placed on the basolateral Transwell membrane to seed at a density of ~250k cells/cm², and the plate was then covered with the 6-well top. Plating densities were occasionally varied between 175k-400k cells/cm² depending on the health, pigmentation, and morphology of the P0 culture flask. After 12–24 hours in an incubator with ample humidity and water between the wells of the receiver plate, the Transwells were placed right side-up in a 24-well receiver plate. Placing 100 μ L of culture media in the apical chamber and 600 μ L of media in the basolateral chamber ensured that the basolateral column of media was higher than the apical column; this encouraged RPE attachment to the Transwell via positive hydrostatic pressure. At the time of imaging, Transwells were transferred into receiver plates with a 10 μ m-thick, transparent fluorocarbon film bottom (Coy Labs, #8602000, or Mobitec, #3231–20). Fluorocarbon film has a refractive index nearly identical to water, making the membrane optically transparent to a water-dipping objective. Morphology, pigmentation, TEER, and phagocytosis of “upside-down” cultured RPE were similar to standard RPE cultures (Figure 2A-B, Supplementary Figure 2A). High cell polarity, as assessed by the surface proteins MCT-3 (monocarboxylate transporter –3) and Kir7.1 (inward rectifying K⁺ channel), along with secreted factors PEDF (pigment epithelium-derived factor), TIMP-3 (tissue inhibitor of metalloproteinases –3), and the ketone body β -hydroxybutyrate (β -HB), was seen in both “upside-down” and standard RPE cultures (Figure 2C, Supplementary Figure 2B) (Philp et al., 2003b; Yang et al., 2003; Maminishkis et al., 2006; Galloway et al., 2017; Adjianto et al., 2014). The scheme for imaging “upside-down” RPE is demonstrated in Figure 2D.

3.2 OS Phagocytosis

3.2.1 Isolating and Labeling Outer Segments—We isolated bovine OS using a discontinuous sucrose gradient based on modifications to protocols published by the

Finnemann, Nandrot, and Papermaster groups (Mao and Finnemann, 2013; Papermaster, 1982; Parinot et al., 2014), obtaining dark-adapted, pre-dissected retinas from WL Lawson Company ([wllawsoncompany.com](http://www.wllawsoncompany.com), Omaha, NE). OS yield per bovine eye was $\sim 1.5 \times 10^8$ OS, with a total protein concentration of approximately 1–1.5 μg per 3.33×10^5 OS. For live-cell tracking of OS phagocytosis, we labeled freshly isolated OS prior to freezing with dyes that demonstrate higher photostability than fluorescein isothiocyanate (FITC). Utilizing manufacturer's instructions, we could label at least 1.5×10^9 OS with 100% labeling efficiency (# of OS with dye / total # of OS) using 1mg of DyLight 405 (Thermo, #PI46400) or DyLight 650 (Thermo, #PI62265). OS yield after labeling (# of OS after labeling / # of OS prior to labeling) was ~ 50 –70%. Supplementary Figure 3 demonstrates the use of DyLight 405-labeled OS with upside-down RPE cultures for live-cell tracking of OS phagocytosis over time using the scheme outlined in Figure 2D.

3.2.2 Optimizing Phagocytic Efficiency in hfRPE Cultures—For phagocytosis assays performed on hfRPE cultures in Transwells, we found a 30 – 120 min challenge with OS concentrations ranging from 4×10^6 OS/mL to 2×10^7 OS/mL produced results with the widest and most reliably quantifiable dynamic range. We delivered these concentrations in a total apical volume of 50 μL to 125 μL , always adjusting basolateral volumes to ensure a positive hydrostatic pressure on the cells. OS phagocytosis requires the presence of soluble bridging ligands to link phosphatidylserine moieties on OS membranes with the phagocytosis receptors $\alpha\text{v}\beta 5$ integrin and Mer tyrosine kinase (MerTK) on the RPE apical membrane (Law and Nandrot, 2012; Mazzoni et al., 2014). While bovine serum contains many of these key ligands, phagocytosis rates improved when we added isotypic human ligands milk fat globule-EGF factor –8 (MFG-E8) (1.5 $\mu\text{g}/\text{mL}$, Sino Biological, #10853-H08B) and Protein S (ProS) (4 $\mu\text{g}/\text{mL}$, Enzyme Research Laboratories, #HPS) to the media of human RPE cultures.

3.2.3 Measuring Total RPE Consumption Capacity During Phagocytosis—Classical phagocytosis assays involve a brief incubation of cells with OS (“pulse”) followed by wash-off of unbound OS and replacement of media for a “chase” period. As the chase period progresses, degradation of the internalized OS by the RPE results in diminishing levels of intact intracellular rhodopsin. The amount of rhodopsin remaining in the RPE is assessed by aspirating media, lysing cells directly with sample buffer for 30 minutes at room temperature, and probing the lysate for rhodopsin.

One issue with evaluating phagocytic rates using the pulse-chase method is that during the pulse period, both uptake and degradation of ingested OS occur. Thus, in the early chase period, cells with robust uptake and fast degradation of OS (high phagocytic efficiency cells) might contain the same amount of rhodopsin as cells with poor uptake and slower degradation of OS (low phagocytic efficiency cells). When we performed pulse-chase experiments in the presence or absence of the phagocytosis bridging ligands MFG-E8 and ProS, we found a seemingly paradoxical increase in the amount of rhodopsin contained in RPE cell lysates from the + bridging ligands group at early chase times (Figure 3A). In the absence of bridging ligands, fewer OS bind to the RPE surface. After washing off unbound OS, the group without bridging ligands will therefore have less rhodopsin early in the chase

period (Figure 3B). As the chase period continues, the bridging ligands group degrades the bound OS at a faster rate, leading to comparable rhodopsin levels between the groups at later timepoints. Without the *a priori* knowledge that bridging ligands should facilitate phagocytosis, however, an equally acceptable interpretation of the data in Figure 3A is that the experimental manipulation has no effect on OS binding, dramatically inhibits OS degradation early in the chase period, and loses its effect on degradation over time.

To eliminate the ambiguity in interpreting results from a classical phagocytosis pulse-chase setup, our revised phagocytosis assay accounts for all OS fed to a Transwell by avoiding the OS wash-off step. The method measures the number of non-degraded OS relative to the total number of OS fed to a Transwell. As seen in Figure 3C, this measure, which we term total consumptive capacity, unequivocally demonstrates the dramatic effect that addition of bridging ligands has on phagocytic efficiency.

To measure total consumptive capacity, we placed 50 μ L of media containing 4×10^6 OS/mL in the apical chamber of each Transwell. In contrast to the pulse-chase method used in classical phagocytosis assays, OS were added (“pulse”) but not washed off (“chase”). At various times after OS “pulse”, we added 50 μ L T-PER lysis buffer (Thermo, #78510) plus complete protease inhibitor mini-tab (Thermo, #PIA32955) to the apical chamber. The collected lysate thus included both the RPE cell layer as well as the media above, containing the non-consumed OS. Figure 3D schematically demonstrates how there are fewer non-consumed OS in the “supernatant + cell” lysate of the bridging ligands group compared to the group without bridging ligands. Once collected, the “supernatant + cell” lysate is pulled through a dot blot apparatus onto nitrocellulose membrane and blotted with a N-terminally directed anti-rhodopsin antibody (1:5000 dilution). Dot blots of these lysates revealed non-consumed OS, whether those OS were in the media, bound on the RPE cell surface, or internalized but incompletely degraded.

3.3 Assaying OS Degradative Machinery

3.3.1 Live-Cell Tracking of Degradative Machinery During Phagocytosis—We sought to transfect or transduce fluorescently-tagged autophagic and lysosomal markers in mature, confluent monolayers of hfRPE to facilitate tracking of OS degradation machinery in live cells. Unfortunately, published transfection methods such as electroporation (Deora et al., 2007) work on confluent cell lines such as ARPE-19, but have not been tested on hard-to-transfect primary cultures. Nucleofection protocols only work on dissociated primary RPE (Toops et al., 2014). Viral transduction is efficient in confluent, primary RPE cultures (Hansen et al., 2003) but settling on an optimal vector insert requires testing numerous constructs, which is expensive and time-consuming. Chemical transfection offers a faster throughput, lower cost alternative to viral transduction during construct optimization. As there are no reports of moderate efficiency chemical transfection of confluent, primary hfRPE cultures in the literature, we developed a protocol. Briefly, we pre-incubated cells in our standard primary hfRPE cell culture media *without* antibiotics. For each Transwell, we added 167ng plasmid DNA and 0.666 μ L Viafect reagent (Promega, #E4981) to 12.5 μ L Opti-MEM (Thermo, #31985070) in a microcentrifuge tube using manufacturer’s instructions. Standard hfRPE culture media *without* antibiotics was then added to this mixture to a final

volume of 125 μ L. Each Transwell was aspirated and the transfection mixture was then added and incubated for 4 hours before washoff. The amount of DNA used, the kit used to purify the DNA (NucleoBond Xtra Maxi Plus EF (Clontech, #740426)), the ratio of transfection reagent to DNA, the incubation time with the transfection reagent, the use of RPE media without antibiotics prior and during transfection, and the type of plasmid promoter were all critical for successful transfection. Details of the method are available in Supplementary Document 2.

With our optimized protocol, we were able to transfect months-old, mature, confluent primary hRPE cultures with Lamp1-mGFP and mCherry-hLC3B plasmids. After adding DyLight 650-labelled OS, we could identify initial LC3 colocalization with the OS, indicative of LC3-associated phagocytosis. This was followed by colocalization of OS with Lamp1 and subsequent disappearance of the OS, revealing delivery to the lysosome and efficient degradation (Figure 4A). An additional example of simultaneous live-cell tracking of OS, LC3, and Lamp1 in a hRPE cell with lower expression of the transfected plasmids is shown in Supplementary Figure 4.

As an alternative to labeling via transfection, lysosomes can also be tracked for long periods of time in living cells via dextran labeling. Dextran, which will mark lysosomes in all cells rather than just transfected cells, can be conjugated with dyes across the visible spectrum and persist for weeks as a lysosomal marker in culture, in contrast to the ~ one hour long duration of lysosomal labeling with typically used Lysosensor or LysoTracker probes (Thermo-Fischer). In addition, Lysosensor and LysoTracker, along with related lysosomotropic dyes, work by becoming protonated upon entry into acidic organelles, potentially causing lysosomal alkalization over time (Pierzyńska-Mach et al., 2014). Thus, observing outer segment degradation using Lysosensor or LysoTracker theoretically risks impairing the efficiency of the lysosomes themselves.

To label hRPE lysosomes, we incubated Cascade Blue dextrans (2mg/mL, Thermo, #D1976) or tetramethylrhodamine (TMR) dextrans (1mg/mL, Thermo, #D1817) in Transwells for 24 hours, followed by at least 24 hours of washout (Figure 4B). Protocol steps are available in Supplementary Document 3.

3.3.2 ICC Staining of Autophagic and Lysosomal Markers that Facilitate

Phagocytosis—To complement live-cell imaging of OS degradative machinery, we also optimized ICC protocols for lysosomal and LC3 staining in the heavily pigmented hRPE. From a review of the literature, we found no references for Lamp1 immunofluorescence staining in P0, P1, or P2 pigmented hRPE cultures grown on Transwell supports where differentiation and polarity, and therefore pigmentation, is likely to be maximal. Similarly, we found only 1 reference for LC3 staining (Frost et al., 2015) under the above culture conditions. We were unable to recapitulate the LC3 staining from this reference in our own hands. The paucity of literature references despite the ubiquity of the hRPE culture system likely reflects the same difficulty we experienced finding antibodies and staining protocols robust enough to fluoresce through the heavy pigmentation of highly differentiated hRPE (personal communication, Arvydas Maminishkis). Bleaching of melanin has been used in the literature to enhance immunostaining of heavily pigmented cells (personal

communication, Arvydas Maminishkis; Kim and Assawachananont, 2016; Nandrot et al., 2007), but we found these bleaching protocols also destroyed antigenicity of the epitopes we were attempting to stain.

Lysosomal staining with Lamp1 (antibody H4A3, 1mL supernatant, Developmental Studies Hybridoma Bank, 1:10 dilution) required the use of paraformaldehyde fixation, 0.2% saponin permeabilization for 10–15 min, and 1 hour room temperature incubation in a 0.1% saponin solution for the primary antibody (Figure 5A). LC3 staining required co-incubation with an LC3B antibody (Cell Signaling Technologies, #3868, 1:100 dilution) and an LC3A/B antibody (Cell Signaling Technologies, #12741, 1:100 dilution) at 4°C overnight in a 0.45% Triton-100 solution, preceded by ice-cold methanol fixation. LC3 puncta were more abundant when autophagy was induced using 1μM of the pan-mTor inhibitor Torin1 (Figure 5B). These puncta also occasionally localized with OS, confirming our ability to detect LAP (Figure 5C). ICC protocols are available in Supplementary Document 4.

3.3.3 Quantification of Autophagic Puncta—Despite our optimized LC3 ICC protocol, automated quantification of LC3 puncta number was still challenging. The vesicles were less defined than the puncta seen with Lamp1 staining, and when we employed simple, built-in background subtraction methods in the Fiji open-source image analysis environment (Schindelin et al., 2012), clumps of background staining were frequently counted as true puncta. To improve automated quantification of LC3 puncta, we developed a customized program in the Fiji macro programming language.

We first defined LC3 puncta as: (a) brighter than local background, (b) solid with well-defined borders, and (c) at least 0.5μm size. We then performed background subtraction on our images employing NanoTrackJ (NTJ) (Wagner et al., 2014). The Nano Track Background Removal Tool weighs the local intensity environment around objects to select subtraction parameters. Puncta brighter than their immediate surroundings, but not bright enough to be captured by simple thresholds, are preserved. Conversely, areas of bright background without a distinct local maxima are eliminated. We also found utility in the Difference of Gaussians (DoG) approach to object recognition. DoG involves subtracting a blurred version of the original image from a less blurred version using Gaussian kernels of varying sizes. The resulting band-pass filter enhances edges of objects out of the background haze.

Individually, however, the DoG and NTJ Background Removal methods were unable to identify LC3 puncta in the same way across different experiments (Figure 6A). Thus, we turned to averaging the puncta count from the NTJ Background Removal and DoG algorithms. Specifically, after a conservative simple background subtraction, we applied the DoG algorithm to each slice of a z-stack of LC3 puncta and summed the segmented puncta from all slices (3D DoG). We also applied the NTJ Background Removal tool and DoG algorithm to separate instances of the 2D maximum projection of the stack and tallied puncta counts from each algorithm (NTJ and 2D DoG). Finally, we calculated a definitive puncta count by equal-weight averaging the counts from all 3 methods: 3D DoG, 2D DoG, and NTJ. This average count significantly improved accuracy and precision, providing results comparable to manual counting, but with substantially less user effort and time

(Figure 6B). Our macro codes and user manuals are available as Supplementary Document 5 and Supplementary Files 1–3.

4. Potential Pitfalls and Troubleshooting

4.1 Poor or Uneven Growth of Cultures and Long-Term Maintenance of Upside-Down Cultures

Poor growth, fibroblastic transformation, or excessive floating (dead) cells in hFRPE cultures may be attributable to plating densities that are too low, mycoplasma contamination, and variability between donor eyes. Ensure the cell culture incubator is maintaining a stable temperature and adequate humidity (Rh% >95 after door has been shut for 4 hours or more) by using a portable external device that measures both properties. Maintain CO₂ concentration in the incubator such that the pH of culture media is between 7.25 and 7.45. To check pH, place a thin layer of media into a tissue culture dish in the incubator for at least 24 hours, before transferring to a conical tube for pH testing. Poor growth can also be attributable to variability between lots of serum. Attempt to lot match serum when purchasing a new lot or test a new lot before committing to purchase it. Poor RPE density in the center of the Transwell may occur due to meniscus formation with clearing of the central portion of the Transwell during coating with too low a volume of human placental ECM. Increase volume of ECM per Transwell if this occurs. Uneven plating can also occur due to vibrations from the hood or incubator that induce a standing wave pattern of cell density in the culture just after plating. For upside-down cultures, the period immediately after flipping Transwells right-side up involves some sick but viable cells falling off the Transwell and to the surface of the 24-well receiver plate. These compromised cells become fibroblastic and can grow up the receiver plate to reach and attach to the Transwell membrane. To eliminate this problem, transfer upside-down Transwells to new receiver plates once the Transwells are mature. If fibroblastic regrowth on the bottom of the new receiver plate again becomes exuberant, the Transwells may need to be retransferred to another receiver plate.

4.2 Poor Phagocytic Efficiency

We have found that more than one freeze-thaw of OS drastically increases OS clumping and reduces their uptake. In serum-free media, the lack of undefined bridging ligands contained in the serum may hinder very high phagocytic rates even when the media is supplemented with exogenous purified human MFG-E8 and ProS.

4.3 Transfections

To improve transfection efficiency, consider increasing the ratio of transfection reagent to plasmid DNA, the absolute concentration of DNA per well, or the incubation time of the transfection reagent solution on the Transwell. Promoters such as that contained in the GW1 plasmid (Miller et al., 2010) have high expression in hFRPE and will also increase the number of cells with detectable transfected gene. Pairing CombiMag (OZ Biosciences, #CM20100) with Lipofectamine 3000 (Thermo, #L3000001), TransIT-X2 (Mirus Bio, #MIR 6003), or Viafect may significantly increase transfection efficiency but also toxicity. Cultures with improvements in transfection efficiency should be monitored for any concomitant increase in toxicity by tracking TEER.

Lower transfection efficiencies will also occur if cells undergo a concurrent endocytically-intensive process, such as transfection and dextran loading or transfection and outer segment feeding. Finally, poor efficiencies are seen in cultures containing fibroblastic RPE nests, as these nests will be transfected preferentially over cobblestone areas.

4.4 Dextrans

TMR-dextrans lose their localization (and intensity) during permeabilization, and while Cascade Blue-dextrans are also susceptible to this permeabilization-induced diffusion, the loss is less dramatic than with TMR-dextrans. During fixation, expect dextran-labeled lysosomes to be more dimly and diffusely labeled than during live-cell imaging.

Imaging of Cascade Blue-dextrans near the junction of the basolateral RPE and Transwell membrane is difficult both because melanin effectively blocks this fluorophore and because the membrane strongly auto-fluoresces in the blue spectrum. Thus, if imaging dextrans basolaterally in cultures, consider TMR-dextrans instead.

Dextran loading and chemical transfection in close succession leads to decreased efficiency for both processes, possibly via endocytic competition. If both protocols are to be performed on a Transwell, dextrans should be loaded 4–5 days prior to carrying out transfections.

4.5 Immunocytochemistry

As melanin in hRPE blocks immunofluorescence, pigment bleaching prior to ICC may improve staining intensity in certain cases. However, bleaching can also weaken or destroy the epitope of interest. Thus, trials of bleaching for each antibody are required. For antibodies with weak signal in hRPE, consider switching to a secondary antibody with a red or far-red dye conjugated to it, which can bypass the major absorptive bands of melanin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

In highly polarized, mature, confluent primary human fetal RPE cultures, we develop or improve methods for:

- Live-cell imaging of OS phagocytosis by RPE directly on semi-permeable supports
- Culturing in serum-free media to facilitate measurement of secreted metabolites
- Efficient OS phagocytosis and accurate assessment of OS degradation
- Tracking lysosomes and autophagy during phagocytosis despite heavy cell pigmentation

Figure 1:

Culture Protocols for Preserving Highly-Differentiated hfRPE in Serum-Free Media and During Live-Cell Imaging. **(A)** Trans-epithelial electrical resistance (TEER) of RPE cultures seeded onto UV-cured ECM was significantly lower than cultures seeded onto ECM without UV curing. RPE seeded onto UV-cured ECM were also more rounded and less confluent one day after plating. $n=11$. Scalebar = 50 μm **(B)** Cultures demonstrate pigmented, cobblestone morphology as assessed by actin-staining (phalloidin - green) and brightfield visualization of melanin. Scalebar = 10 μm . **(C)** qPCR of RPE-specific markers RPE65, MerTK, and CRALBP in the ARPE-19 cell line, hfRPE cultures, and fresh adult RPE/choroid tissue obtained from human cadaveric eyes. $n=4$. **(D)** Serum-free media with Neurobasal base + B27 supplement + other components results in a similar TEER as RPE complete media with α MEM base and 5% serum ("RPE Complete") (Maminishkis et al., 2006). Switching serum-free media base to α MEM (" α MEM+B27") results in reduced TEER. $n=3$. P values

for entire figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars for entire figure = SE. All cultures in figure 1 are right-side up.

Figure 2:

Upside-Down Plated RPE Cultures Demonstrate Similar Differentiation and Polarity as Rightside-Up Cultures and Facilitate Long-Term, Repeated Live-Cell Imaging on Transwells. **(A)** Morphology and tight-junctions of upside-down RPE wells is similar to that of rightside-up wells, as assessed by ZO-1 staining of junctional complexes (green) and TEER. X-Z confocal imaging shows that RPE grew as a monolayer. Scalebar = 20µm for X-Y images, 10µm for X-Z images. TEER n=6. **(B)** Phagocytosis efficiency, as assessed by classic pulse (1hr) then chase assay with rhodopsin normalized to GAPDH, is similar between upside-down and rightside-up cultures. n=3. **(C)** Polarity, as assessed by cell surface proteins, is also similar between upside-down and rightside-up cultures. The inward rectifier potassium channel Kir7.1 (green) is predominately apical, whereas the monocarboxylate transporter MCT-3 is predominately basolateral (green). Phalloidin staining of actin outlines apical microvilli and cell borders (pink). DAPI stains nuclei (blue).

Images (from top to bottom) represent X-Y view through an apical slice (top), X-Y view through a basolateral slice (middle), and X-Z view (bottom). Scalebar = 10 μ m. Graphs underneath quantify the relative intensity of Kir7.1, MCT-3, phalloidin, and DAPI at different Z-slices through the cell, normalized to the slice with maximum intensity. Phalloidin has an apical staining bias given the density of actin within apical microvilli, and Kir7.1 peak intensity overlaps with phalloidin. DAPI has a basolateral staining bias as nuclei are basolaterally oriented in polarized RPE. The peak of MCT-3 staining overlaps with DAPI. Quantification from n=6 randomly chosen cells. **(D)** Live-cell imaging of hRPE attached to Transwells is possible by plating RPE on the bottom side of the Transwell **(i)**, thereby eliminating the Transwell membrane from the imaging path. The Transwells are placed in a receiver plate whose bottom consists of a 10 μ m thick fluorocarbon membrane, which has the same refractive index as water. This allows a water-dipping lens to view the RPE without any refractive index mismatches. **(ii)** To ensure the Transwells were as close to the bottom of the receiver plate as possible without touching the fluorocarbon film, we engineered a shim to sit on top of the fluorocarbon plate, thereby spacing the Transwell membrane 200–500 μ m above the fluorocarbon film. This spacing ensured media flow on both the apical and basolateral sides of the Transwell, but kept the Transwell close enough to the bottom of the plate to stay within the working distance of a high-resolution water-dipping objective. * p<0.05, ** p<0.01. Error bars for entire figure = SE.

Figure 3:

Optimized OS Phagocytosis Methods in hRPE Allow Accurate Assessment of Total OS Consumptive Capacity. **(A)** Quantification of rhodopsin dot blot (below) from classical pulse-chase phagocytosis assay illustrates how a higher amount of rhodopsin in the bridging ligands group at early chase times could lead to the false conclusion of poor phagocytic efficiency rather than improved uptake. $n=3$. **(B)** Schematic representation of classical pulse-chase phagocytosis assay setup. Lysates from the group with bridging ligands have more rhodopsin due to larger populations of both bound and internalized OS than wells without ligands. **(C)** Quantification of rhodopsin dot blot (below) from pulse-only phagocytosis assay. Lysates include cell layer plus supernatant containing unbound OS. The bridging ligands group consistently has less rhodopsin, unambiguously showing improved phagocytic efficiency across the full duration of the assay. $n=3$. **(D)** Schematic representation of the new total consumptive capacity (pulse-only) assay setup. Lysates from the group with bridging

ligands have less rhodopsin due to smaller populations of unbound, unconsumed OS than wells without ligands. * $p < 0.05$. Error bars for entire figure = SE. All cultures in figure 3 are right-side up.

Figure 4:

Live-Cell Tracking of hRPE Degradation Machinery During OS Ingestion. (A) Lamp1-mGFP and mCherry-hLC3B transfected RPE, cultured upside-down, were incubated with DyLight 650-labelled OS, after which OS were washed off and cells were imaged every 30 minutes post-chase. An OS (red) tracked by the white arrowhead is first visible at time 0 and colocalizes with both LC3 (green) and Lamp1 (cyan) 30 minutes later. At 60 minutes post-chase, the OS colocalizes with Lamp1 but not LC3, and by 90 minutes, the OS is degraded (circle). Yellow arrowhead tracks an OS that colocalizes with LC3 and Lamp1 for an hour after which it splits into two fragments, each also labelled with LC3 and Lamp1. The large OS marked with a pink arrowhead is apical to the cell. A small bud from the larger OS undergoes phagocytosis at 60 minutes, colocalizing with LC3 and Lamp1 at 90 minutes. Scalebar = 5µm. (B) Robust tetramethylrhodamine (TMR)-dextran labeling of lysosomes (red) allows long-term longitudinal live-cell tracking of the degradation of ingested material.

The actin cytoskeleton is marked by phalloidin (green) and outlines both RPE microvilli and points of cell-cell contact. Right-side up culture fixed 5 days after dextran washout, imaged on confocal microscope. Scalebar = 25 μ m.

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Figure 5:

Immunofluorescent Staining of Autophagic and Lysosomal Markers that Facilitate OS Phagocytosis. **(A)** Lamp1 immunocytochemistry of hfrPE lysosomes. Scalebar = 25 μ m. **(B)** LC3 immunocytochemistry in hfrPE cultures in which autophagy has been induced with the mTOR inhibitor Torin1 at 1 μ M, assessed 22 hours after Torin1 addition. Scalebar = 20 μ m. Error bars = SE. * $p < 0.05$. $n = 3$. **(C)** LC3 (green) colocalization with DyLight 405-labeled outer segments (red) is consistent with LC3-associated phagocytosis. ZO-1 (pink) outlines the RPE cell border. Central ZO-1 puncta appear to be an artifact of methanol fixation and have been seen in prior publications (Low et al., 2002). Scalebar = 2.5 μ m. All cultures in figure 5 are right-side up.

Figure 6:

Quantifying LC3 Puncta Using Automated Methods in ImageJ. **(A)** Difference of Gaussian (DoG)-based segmentation of LC3 puncta is handled properly in some images (see pair on left) and poorly in others (see pair on right). Correctly segmented puncta circled in yellow; “false puncta” circled in blue. **(B)** Macro for consistent LC3 puncta quantification begins with use of the core background subtraction tool from ImageJ/Fiji. Applying the NanoTrackJ and DoG algorithms to separate instances of the background-subtracted image each improve counts, but both algorithms overcount and undercount puncta in different ways (bottom left and right images). Blue circles represent areas of bright background successfully avoided. Yellow circles represent correctly segmented LC3 puncta. Red arrows indicate LC3 puncta correctly counted by DoG method but missed by the NTA method. White arrow marks background incorrectly counted by DoG method (single small pixel) and correctly omitted by NTA method. (Graph) Averaging results from DoG and NanoTrackJ

Background Subtraction analysis provides puncta counts proportional to counts obtained manually. Error bars = SE. n=10 images per condition.