Journal of Visualized Experiments

Efficient dissection and culture of primary mouse retinal pigment epithelial cells.

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62228R1
Full Title:	Efficient dissection and culture of primary mouse retinal pigment epithelial cells.
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Additional Information:	
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TITLE:

Efficient dissection and culture of primary mouse retinal pigment epithelial cells

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KEYWORDS:

Mouse RPE, primary RPE, RPE cultures, RPE isolation, ocular disorders, polarized RPE, RPE on Transwells, mouse eye dissection.

SUMMARY:

This protocol, which was originally reported by Fernandez-Godino et al. in 2016[1], describes a method to efficiently isolate and culture mouse RPE cells, which form a functional and polarized RPE monolayer within one week on Transwell plates. The procedure takes approximately 3 hours.

ABSTRACT:

Eye disorders affect millions of people worldwide, but the limited availability of human tissues hinders their study. Mouse models are powerful tools to understand the pathophysiology of ocular diseases because of their similarities with human anatomy and physiology. Alterations in the retinal pigment epithelium (RPE), including changes in morphology and function, are common features shared by many ocular disorders. However, successful isolation and culture of primary mouse RPE cells is very challenging. This paper is an updated audiovisual version of the protocol previously published by Fernandez-Godino et al. to efficiently isolate and culture primary mouse RPE cells. This method is highly reproducible and results in robust cultures of highly polarized and pigmented RPE monolayers that can be maintained for several weeks on Transwells. This model opens new avenues for the study of the molecular and cellular mechanisms underlying eye diseases. Moreover, it provides a platform to test therapeutic approaches that can be used to treat important eye diseases with unmet medical needs, including inherited retinal disorders and macular degenerations.

INTRODUCTION:

This protocol, which was originally reported by Fernandez-Godino et al. in 2016[1], describes a method to efficiently isolate and culture mouse retinal pigment epithelium (RPE) cells, which form a functional and polarized RPE monolayer within one week on Transwell plates. The

retinal pigment epithelium (RPE) is a monolayer located in the eye between the neural retina and the Bruch's membrane. This single layer consists of highly polarized and pigmented epithelial cells joined by tight junctions, exhibiting a hexagonal shape that resembles a honeycomb[2]. Despite this apparent histological simplicity, the RPE performs a wide variety of functions critical to the retina and the normal visual cycle[2–4]. The main functions of the RPE monolayer include light absorption, nourishment and renewal of photoreceptors, removal of metabolic end products, control of the ion homeostasis in the subretinal space and maintenance of the blood–retinal barrier[2,3]. The RPE also has an important role in local modulation of the immune system in the eye[5–11]. Degeneration and/or dysfunction of the RPE are common features shared by many ocular disorders such as retinitis pigmentosa, Leber congenital amaurosis, albinism, diabetic retinopathy, and macular degeneration[12–15]. Unfortunately, the availability of human tissues is limited. Given their highly conserved genetic homology with humans, mouse models represent a suitable and useful tool for studying ocular disorders[16–19]. Furthermore, the use of cultured primary RPE cells provides advantages such as genetic manipulation and drug testing that can accelerate the development of new therapies for these vision-threatening disorders[9,11].

Existing methods available for mouse RPE isolation and culture lack reproducibly and do not recapitulate the RPE features in vivo with enough reliability. Cells tend to lose pigmentation, hexagonal shape and transepithelial electrical resistance (TER) within a few days in culture[13, 20]. Since establishing these primary RPE cell cultures from mice is a challenging process, this optimized protocol has been created based on other protocols to isolate RPE cells from rat and human eyes[21–23] to dissect the mouse eyes, collect the RPE and culture the mouse RPE cells in vitro.

PROTOCOL:

The guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed.

NOTE: This method has been proven successful with mice of different genetic backgrounds, including C57BL/6J, B10.D2-Hc^{o} H2^{d} H2-T18^{c}/oSnJ, and albino mice, at various ages. Preferably use 8 to 12-week-old mice to obtain RPE cells. RPE cells from older mice proliferate less in culture and younger mice have fewer and smaller cells, which requires pooling eyes from different animals to have viable cultures.

1. Preparation of reagents and the membrane inserts

1.1. Prepare the following reagents.

1.1.1. Prepare HBSS-H− (HBSS without calcium, without magnesium buffer + 10 mM HEPES): Add 1 mL of 1 M HEPES to 99 mL of HBSS– (without Ca/Mg) buffer. Store at 4 °C up to 1 month.

1.1.2. Prepare HBSS-H+ (HBSS with calcium, with magnesium buffer + 10 mM HEPES): Add 1 mL of 1 M HEPES to 99 mL of HBSS+ (with Ca/Mg) buffer. Store at 4 °C up to 1 month.

1.1.3. Prepare hyaluronidase solution (1 mg/mL): Add 10 mg of hyaluronidase to 10 mL of HBSS-H− and filter it with a 0.4 µm sterile syringe filter using a 10 mL syringe. Prepare fresh before use and leave it at room temperature (RT; 20–25 °C).

1.1.4. Prepare FBS solution: Mix 20% FBS with HBSS-H+. Add 2 mL of FBS to 8 mL of HBSS-H+. Prepare fresh before use.

1.1.5. Prepare RPE medium: N1 Medium Supplement 1/100 (v/v), glutamine 1/100 (v/v), penicillin–streptomycin 1/100 (v/v) and nonessential amino acid solution 1/100 (v/v), hydrocortisone (20 µg/L), taurine (250 mg/L) and triiodo-thyronin (0.013 µg/L) in alpha MEM + 5% FBS or without FBS[1,23,24]. If desired, FBS may be heat-inactivated; no differences have been observed. Prepare fresh for RPE isolation. For cell culture maintenance, store at 4 °C up to 1 month.

1.1.6. Prepare trypsin-EDTA: Prepare small single-use aliquots (~8 mL) of fresh trypsin-EDTA (0.25%) and freeze them at -20 °C. Thaw them at RT before each use. Avoid freeze-thaw cycles.

1.2. Prepare the membrane inserts (e.g., Transwell inserts).

1.2.1. Equilibrate the 6.5-mm membrane inserts with RPE medium at least 30 min at 37 °C, in a 5% CO_2-aerated incubator. Use one membrane insert to seed RPE cells from two mouse eyes.

1.2.2. After incubation, replace the medium of the lower compartment with 700 µL of PBS.

1.2.3. Remove the medium from the top compartment and coat the membrane insert with 100 µL of 10 µg/mL mouse laminin (in PBS) for at least 2 h at RT (shorter incubations may lead to poor attachment of the cells to the insert).

1.2.4. Coat an extra membrane insert to use as a blank for TER measurements.

2. Dissection and enucleation of the mouse eyes

2.1. Euthanize the mice by CO_2 asphyxiation by placing them in a CO_2 chamber and slowly releasing CO_2 at a fill rate of 30-70% of the chamber volume per minute.

2.2. Place the angled, serrated tips of micro-forceps on each side of the mouse eye and gently press to proptose the eyeball (enucleation).

2.3. Close the forceps placed around the eyeball. Then, pull gently while moving forward and backward to detach the whole eye with the optic nerve from the ocular muscles, ensuring that no connective tissue remains attached to the sclera.

2.4. Rinse the enucleated eyeball in 70% ethanol before placing them into one well of a six-well plate with 3 mL of HBSS-H− on ice.

2.5. Repeat steps 2.2 to 2.4 to remove the second eye of the mouse. Proceed with the next steps within 30 min.

NOTE: It is recommended enucleating/dissecting only two eyes at a time until some experience is acquired.

3. Collection of the RPE

NOTE: Perform the following steps under sterile conditions in a laminar flow hood. To avoid extended incubations on ice, which can result in RPE cell death, do not collect more than two eyes at a time.

3.1. Use a dissecting stereomicroscope, Dumont #5 forceps, and angled scissors to carefully clean away all the connective tissue, blood and muscles remaining attached to the eyeball without making any cuts in the sclera. Change the 3 mL of HBSS-H− buffer regularly, as needed, to keep the eye fresh and clean, and avoid the contamination of the RPE cultures.

3.2. Use the optic nerve as a handle to hold the eyeball and make a hole in the center of the cornea with a sharp carbon-steel #11 blade.

3.3. Use Dumont #5 scissors to make three incisions in the cornea through the aforementioned hole, ensuring that there is sufficient space to remove the lens.

3.4. Hold the optic nerve and apply slight pressure to the ora serrata with the base of the angled scissors until the lens comes completely out. Leave the iris epithelium in place to prevent the detachment of the neural retina and RPE during incubation. Place the eye in HBSS-H.

3.5. Repeat Steps 3.1–3.4 to dissect the second eye.

3.6. Incubate the eyes without lenses in hyaluronidase solution in a 12-well plate at 37 °C for 45 min in a 5% CO_2-aerated incubator (1.5 mL/well) to detach the neural retina from the RPE.

3.7. Place each eye in a new well and incubate it on ice for 30 min with 1.5 mL of cold HBSS-H+ buffer per well to stop the hyaluronidase activity. Do not extend the incubation for longer than 45 min.

3.8. Wash, and place each eye into a 35-mm culture dish with fresh HBSS-H+ buffer and cut the cornea through the original incisions until reaching the the ora serrata by using 8-cm Vannas scissors. Then, cut below the ora serrata to remove the iris epithelium and cornea.

3.9. Hold the eyecup edge/ora serrata with curved tweezers and, using angled microforceps, pull away the neural retina making sure that the RPE layer is not cut. Then, cut the internal attachment to the optic nerve. If some RPE cells remain attached to the neural retina, extend the incubation time but do not exceed 45 min total.

3.10. Cut the optic nerve and transfer each eyecup to a different 12-well plate containing 1.5 mL of fresh trypsin-EDTA per well. Ensure that the eyecups remain opened and completely submerged in the trypsin. Incubate the eyecups at 37 °C for 45 min in a 5% CO_2 incubator.

3.11. Collect each eyecup together with any RPE sheets detached during the trypsin incubation and transfer them into a 12-well plate containing 1.5 mL of FBS solution per well. If RPE sheets remain in the trypsin solution, use a micropipette to transfer them to the well with FBS solution.

4. Isolation of primary mouse RPE cells

4.1. Hold each eyecup by the optic nerve and shake it face down into the 12-well plate containing 1.5 mL of 20% FBS in HBSS-H+ until the complete detachment of the RPE sheets is achieved.

4.2. Collect any RPE sheets and RPE clusters with a micropipette and place them in a 15-mL tube. Avoid any white pieces of sclera or choroid, which could contaminate the cultures. Pool two eyes from the same mouse in one tube.

4.3. Centrifuge the mixture at 340 x *g* for 2 min at RT and discard the supernatant.

4.4. Gently resuspend the RPE pellet in 1 mL of trypsin-EDTA (0.25%) and incubate the mixture for 1 min in a water bath at 37 °C to disaggregate the RPE sheets into single cells. After incubation, gently pipet up and down 10x with a micropipette, avoiding bubble formation while pipetting.

4.5. Add 9 mL of freshly prepared RPE medium to dilute and inactivate the trypsin and centrifuge the mixture at 340 x *g* for 2 min at RT.

4.6. Aspirate the supernatant and carefully resuspend the cell pellet in 150 µL of RPE medium with 5% FBS by using a micropipette, ensuring that cells are homogeneously resuspended and avoiding bubble formation while pipetting.

4.7. Take laminin-coated membrane insert from step 1.2, remove PBS from the bottom chamber and add 700 µL of RPE medium.

4.8. Remove the laminin from the upper chamber of the membrane insert and distribute the RPE cell suspension dropwise and uniformly to the center of the chamber, avoiding bubble formation while pipetting.

4.9. Place the membrane insert in a 5% CO_2 incubator at 37 °C and leave undisturbed for at least 24 h.

4.10. After 24 h, check the membrane insert under the microscope to make sure that most RPE cells are attached to the insert and the confluence is at least 50% (**Figure 1A**). It is critical not to change media during the first 72 h.

5. Culture of polarized RPE monolayers

5.1. Maintain the isolated RPE cells in culture for at least 72 h before refreshing the RPE medium to allow cell attachment. A cell confluence of 50% or more at seeding is fundamental for the formation of a suitable polarized RPE monolayer.

5.2. Change the culture medium twice a week using fresh and pre-warmed RPE medium (step 1.1.5) after the cells are attached. Serum can be removed from the culture medium after the first 72 h.

NOTE: After one week in culture, cells should be confluent, hexagonal, bi-nucleated, pigmented and polarized (**Figure 1B**), with expected cell numbers being around 50,000 cells per 6.5 mm Transwell insert. After two weeks in culture, RPE monolayers comprising of healthy cells are observed. RPE cultures display apical microvilli, basal infoldings, and tight junctions (**Figure 1C**).

6. TER measurement

NOTE: Perform TER measurements of the RPE cells after a minimum of 4 days in culture to ensure a good integrity and polarization of the RPE monolayer.

6.1. Clean the electrodes of the voltohmmeter with 70% ethanol and dry them carefully.

6.2. Remove the membrane inserts from the incubator and perform TER measurement within 3 min to avoid alterations due to temperature fluctuations. To measure TER, immerse the short electrode of the voltohmmeter in the upper chamber and the long electrode in the bottom chamber of the membrane insert. Avoid contact with the RPE monolayer to prevent cell detachment.

6.3. To calculate TER, deduct the value of the blank (membrane insert coated with laminin without cells) from the sample. Then multiply the obtained value (in ohms) by the surface area of the membrane insert (0.33 cm^2 in the case of 6.5-mm membrane insert). The product should be at least 200 $\Omega \cdot cm^2$ after 72 h in culture. TER values should measure above 400 $\Omega \cdot cm^2$ after two weeks. Discard RPE cultures with low TER values.

REPRESENTATIVE RESULTS:

This protocol has been used to isolate and culture RPE cells from genetically modified mice[1]. No differences have been observed between mouse strains or gender. The results have helped to understand some important aspects of the mechanism underlying ocular diseases such as age-related macular degeneration, which is the most common cause of vision loss among the elderly[9]. RPE cells isolated following this protocol were completely attached to the membrane insert 24 hours after seeding and showed the typical RPE size, morphology and pigmentation after 72 h[1]. After one week, a highly polarized RPE monolayer was formed by hexagonal pigmented RPE cells with two nuclei joined by tight junctions expressing ZO-1 (**Figures 1C, 2**). Transmission electron micrographs reveal the presence of apical microvilli and basal infoldings (**Figure 1C**). Polarization of the RPE monolayer was confirmed by TER values with average higher than 200 $\Omega \cdot cm^2$, which remained stable over time (**Figure 2**).

Functional validation of this method was performed though phagocytosis assays. Mouse RPE cells were cultured on membrane inserts and fed with FITC-labeled bovine POS. POS engulfment and digestion were demonstrated by fluorescent microscopy (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1. RPE cells at different time-points. (**A**) 10x brightfield micrographs of RPE cells 24 h post seeding on the membrane inserts, and (**B**) after one week. (**C**) Transmission electron micrographs display apical microvilli, basal infoldings, and melanin pigments (black spots) of the RPE cultured on the membrane inserts for two weeks. Scale bars: A, B: 100 µm, C: 2 µm.

Figure 2. TER over time. The transepithelial electrical resistance (TER) of ~60 primary mouse RPE cell cultures on membrane inserts was measured after 0.5 (n=33), 1 (n=58), 1.5 (n=54), and 2 (n=60) weeks. TER reaches over 200 $\Omega \cdot cm^2$ by 72 hours and remains stable for at least two weeks. Data represented as single values with average ± SD.

Figure 3. Phagocytosis assays. 40x fluorescent micrographs of primary mouse RPE cultures fed with FITC-labeled bovine POS (green) for two hours and fixed with methanol[25]. Smaller fragments correspond to digested POS inside the cytoplasm of the cell. Visualization of tight junctions (red) was facilitated by immunostaining with antibodies anti-ZO1 as previously described[1,9]. DAPI (blue) was used to stain nuclei. Typical mouse RPE cells with two nuclei can be observed in the center of the image. Scale bar: 50 µm.

DISCUSSION:

While several methods for mouse RPE cell isolation and culture had been developed before[1,13,20,22,26,27], Fernandez-Godino's method first used membrane inserts allowing the efficient growth of the RPE cells in culture for weeks[1,9]. Another major change in their protocol[1,9] was the use of enzymatic solutions instead of mechanical peeling to dissociate the RPE cells[1,13,20,26]. The lenses were removed through an incision in the cornea, leaving the iris epithelium intact and preserving the integrity of the retina during the first incubation. The gentle isolation of the RPE cells combined with the use of membrane inserts and specific RPE

medium[23] results in an improved cell survival, enhancing the formation of a functional confluent monolayer which mimics the physiological conditions of the RPE within days in culture[2]. Typically, primary mouse RPE cells cultured with this method display hexagonal morphology, polarization, pigmentation, barrier properties, and proliferation. Moreover, the RPE can be cultured in the absence of serum from day three up to several weeks, which is important to study complement-associated RPE pathologies[9].

A limitation of this protocol is that primary mouse RPE cells cultured on membrane inserts cannot be expanded. Passaging primary mouse RPE cells with enzymatic solutions or culturing them for a long time results in loss of pigmentation, de-differentiation, and decreased TER, thereby losing the properties that resemble in vivo features. The limited amount of RPE cells obtained from one animal obligates to pool samples from different animals for transcriptomic and proteomic analyses, where relatively large amounts of starting material are required. It is not recommended to scale up the RPE cultures by pooling more eyes into a larger membrane insert, as it can result in a higher percentage of cell death and multilayered RPE cultures.

There are some critical steps to the protocol as well. No more than two eyes should be harvested at the same time, since a prolonged dissection time results in a higher percentage of cell death. The optic nerve must be cut away only before the incubation with trypsin. It is critical to handle the eyecup without disturbing the RPE cells, but the optic nerve gets digested by trypsin, which leads to impurities in the RPE cultures. Peeling off the ocular muscles must be performed with caution. If the sclera is perforated and the neural retina pops out, the eye must be discarded because the RPE cells will be damaged and will not survive. Minimal pressure should be applied to remove the lenses. It is preferable to perform a larger corneal incision. It is critical that the eyecup is open and completely submerged in trypsin so that all the RPE cells are exposed to the solution. The RPE cells must be collected through gentle shaking of the eyecup, and never by spraying media or peeling them mechanically.

ACKNOWLEDGMENTS:

This work was supported by the Ocular Genomics Institute at Massachusetts Eye and Ear.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

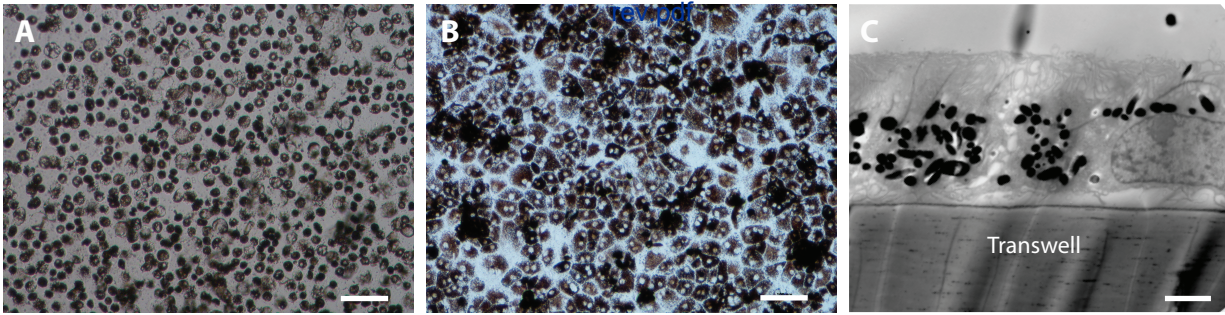


Figure 2

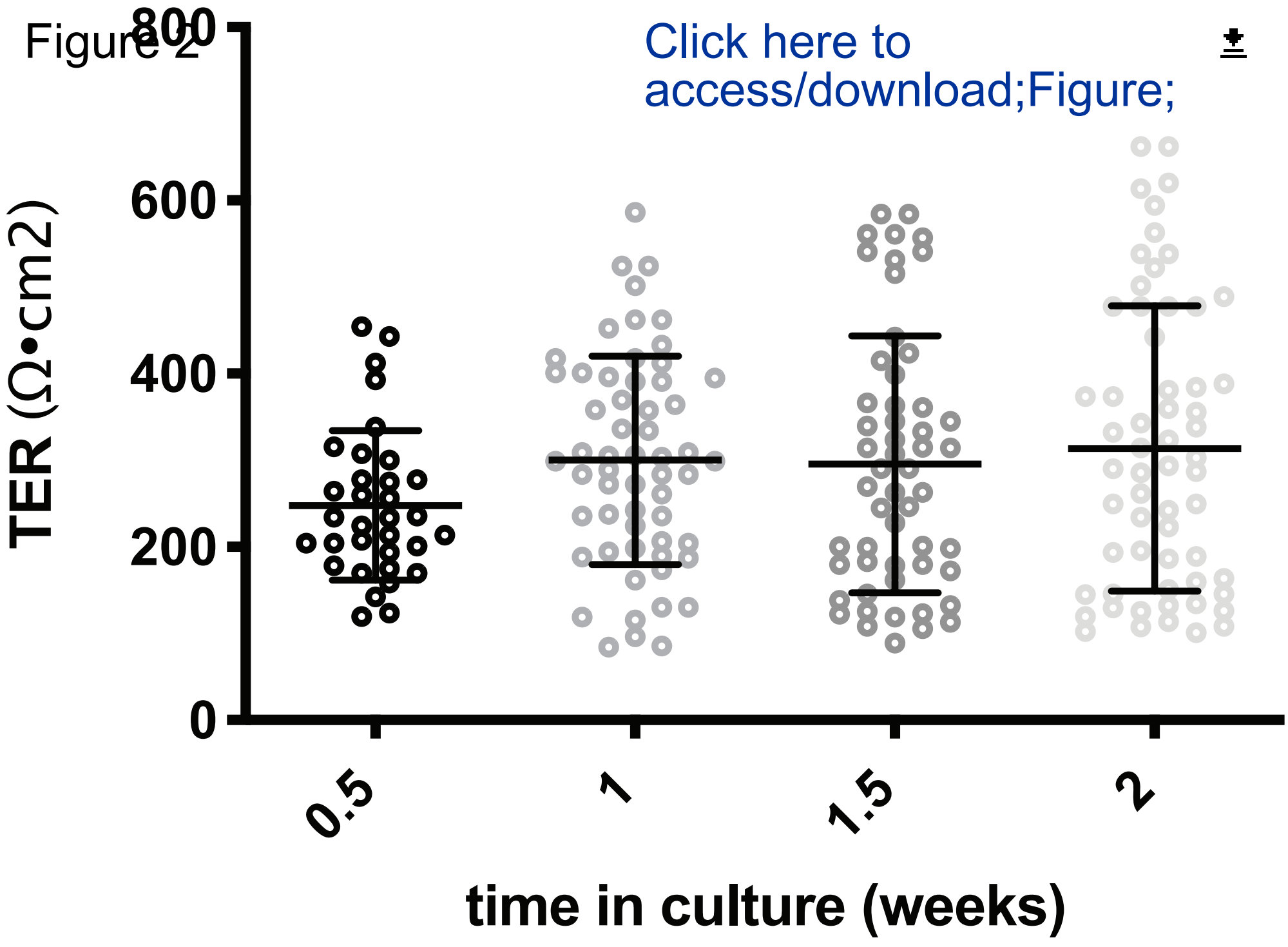
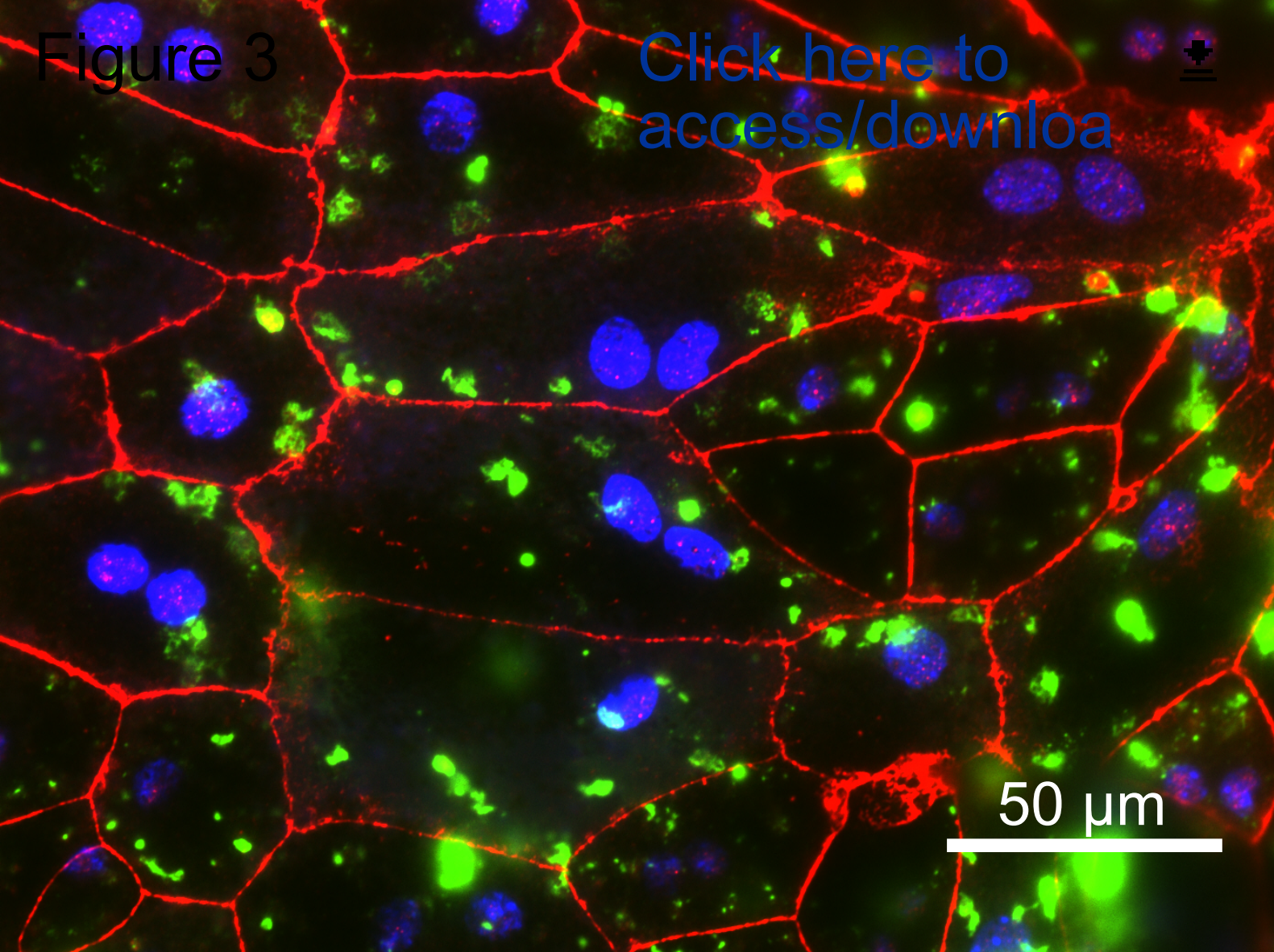


Figure 3



Name of Material/Equipment	Company	Catalog Number	Comments/Description
10 ml BD Luer-Lok tip syringe, disposable	BD Biosciences	309604	
15 ml centrifuge tube	VWR International	21008-103	
50 ml centrifuge tube	VWR International	21008-951	
Alpha Minimum Essential Medium	Sigma-Aldrich	M4526-500ML	
Angled micro forceps	WPI	501727	
Bench-top centrifuge	any		
CO2 incubator	Thermo	HERA VIOS 160I CO2 SST TC 120V	
Dissecting microscope	Any		
Dulbecco's Phospate Buffered Saline no Calcium, no Magnesium	Gibco	14190144	
Dumont #5 45° Medical Biology tweezers, 0.05 x 0.01 mm tip, 11 cm length	WPI	14101	
Ethanol	Sigma-Aldrich	E7023-500ML	
Falcon Easy-Grip Clear Polystyrene Cell Culture Dish, 35mm	BD Biosciences	353001	
Fetal Bovine Serum	Hyclone	SH30071.03	Heat inactivated.
Hank's Balanced Salt Solution plus Calcium and Magnesium, no Phenol Red	Life Technologies	14175095	
Hank's Balanced Salt Solution plus Calcium and Magnesium, no Phenol Red B6	Life Technologies	14025092	
HEPES 1M	Gibco	15630106	
Hyaluronidase	Sigma-Aldrich	H-3506 1G	

Hydrocortisone	Sigma-Aldrich	H-0396	
Laminar flow hood	Thermo	CLASS II A2 4 115V PACKAGECLA	
Laminin 1mg/ml	Sigma-Aldrich	L2020-1 MG	Dilute in PBS at 37C to 1mg/ml
McPherson-Vannas Micro Scissors 8 cm long	WPI	503216	
Non-essential amino acids 100X	Gibco	11140050	
N1 Supplement 100X	Sigma-Aldrich	N6530-5ML	
Penicillin-Streptomycin	Gibco	15140-148	
Sterile Bard-Parker Carbon steel surgical blade size 11	Fisher-Scientific	08-914B	
Taurine	Sigma-Aldrich	T-0625	
Tissue culture treated 12-well plate	Fisher-Scientific	08-772-29	
Tissue culture treated 6-well plates	Fisher-Scientific	14-832-11	
Transwell supports 6.5 mm	Sigma-Aldrich	CLS3470-48EA	
Triiodo-thyronin	Sigma-Aldrich	T-5516	
Trypsin-EDTA (0.25%), phenol red	Gibco	25200056	
Tweezer, Dumont #5 Medical Biology 11 cm, curved, stainless steel 0.02 x 0.06 mm Mod tips	WPI	500232	
Vannas Scissors 8cm long, stainless steel	WPI	501790	
Whatman Puradisc 25mm Syringe Filters 0.45μm pore size	Fisher-Scientific	6780-2504	

Resubmission manuscript JoVE6222

Dear editor,

We have resubmitted a revised version of our manuscript, entitled ***"Efficient dissection and culture of primary mouse retinal pigment epithelial cells".*** We appreciate the editor's and reviewers' comments and their insight into improving our article. We think that the article is now ready for publication in the Journal of Visualized Experiments.

Editorial comments:
As requested, we have proofread the manuscript for grammar issues and we have removed the used of pronouns. We have also added a short summary describing the protocol.

Part of the text (pages 5-7) has been highlighted in the manuscript to identify the essential steps for the video.

Revised lines to avoid auto-plagiarism:

34-35: The main functions of the RPE monolayer include light absorption, nourishment and renewal of photoreceptors, removal of metabolic end products, control of the ion homeostasis in the subretinal space and maintenance of the blood–retinal barrier.

90-94 (4.1 to 4.4.):

- Euthanize mice by CO_2 asphyxiation.
- Place the animal in a CO_2 chamber and slowly flow CO2 at a fill rate of 30-70% of the chamber volume per minute.
- Place the angled serrated tips of microforceps on each side of the mouse eye and gently press to proptose the eyeball (enucleation).
- Close the forceps placed around the eyeball. Then pull gently while moving forward and backward to detach the whole eye with the optic nerve from the ocular muscles, ensuring that no connective tissue remains attached to the sclera.

95: The two enucleated eyeballs must be rinsed in 70% ethanol before placing them into one well of a six-well plate with 3 mL of HBSS-H− on ice.

104-107: Utilize a dissecting stereomicroscope, Dumont #5 forceps and angled scissors to carefully clean away all the connective tissue, blood and muscles remaining attached to the eyeball without making any cuts in the sclera. Change the 3 mL of HBSS-H− buffer regularly, as needed, to keep the eye fresh and clean, and avoid the contamination of the RPE cultures.

108-116 (5.2 to 5.6):

- The optic nerve will be used as a handle to hold the eyeball while a hole is made in the center of the cornea with a sharp carbon-steel #11 blade.
- Use Dumont #5 scissors to make three incisions in the cornea through the aforementioned hole. Make sure there is sufficient space to remove the lens.

- Hold the optic nerve and apply slight pressure to the *ora serrata* with the base of the angled scissors until the lens comes completely out. By leaving the iris epithelium in place, detachment of the neural retina and RPE during incubation will be prevented.
- Repeat Steps 5.1–5.4 to dissect the second eye.
- To detach the neural retina from the RPE, eyes without lenses will be incubated in hyaluronidase solution in a 12-well plate at 37 °C for 45 min in a 5% CO_2-aerated incubator (1.5 mL/well).

118-124 (5.7 to 5.8):

- Place each eye into a new 12-well and incubate them on ice for 30 min with 1.5 mL of cold HBSS-H+ buffer per well to stop the hyaluronidase activity. Do not extend the incubation for longer than 45 min.
- After washing, place each eye into a 35-mm culture dish with fresh HBSS-H+ buffer and cut the cornea through the original incisions until reaching the the *ora serrate* by using 8-cm Vannas scissors. Then cut below the *ora serrata* to remove the iris epithelium and cornea.

136-138 (6.1): Hold each eyecup by the optic nerve and shake it face down into the 12-well plate containing 1.5 mL of 20% FBS in HBSS-H+ until achieving the complete detachment of the RPE sheets.

148-154 (6.6-6.8):

- Aspirate the supernatant and then carefully resuspend the cell pellet in 150 µl of RPE medium with 5% FBS by using a p200 micropipette. Make sure that cells are homogeneously resuspended and avoid bubbling while pipetting.
- Take laminin-coated transwells from Step 3, remove PBS from the bottom chamber and add 700 µl of RPE medium instead.
- Remove the laminin from the upper chamber of the transwell and distribute the RPE cells suspension dropwise and uniformly to the center of the chamber. Avoid making bubbles while pipetting.

162-164 (7.1): Maintain the isolated RPE cells in culture for at least 72 h before refreshing the RPE medium to allow cell attachment. A cell confluence of 50% or more at seeding is fundamental for the formation of a suitable polarized RPE monolayer.

176-182 (8.2-8.4):

- *Note: Perform TER measurements of the RPE cells after a minimum of 4 days in culture to ensure a good integrity and polarization of the RPE monolayer.*
- Clean the electrodes of the voltohmmeter with 70% ethanol and dry them carefully.
- Take the transwells from the incubator and perform TER measurement within 3 min to avoid alterations due to temperature fluctuations. To measure TER, immerse the short electrode of the voltohmmeter in the upper chamber and the longest in the bottom chamber of the transwell. Avoid contact with the RPE monolayer to prevent cell detachment.
- To calculate TER, deduct the value of the blank (Transwell coated with laminin without cells) from the sample. Then multiply the obtained value (in ohms) by the surface area of the Transwell (0.33 cm^2 in the case of 6.5-mm transwells). The product should be at least 200 Ω • cm^2 after 72 h in culture. TER values should measure above 400 Ω • cm^2 after two weeks. RPE cultures with low TER values should be discarded.

We have included an ethics statement before the numbered protocol steps.
Line 83: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL. *Done.*
Line 89: Please include how do you perform euthanasia in your experiment. *This information has been added.*
Line 106: Please mention the volume of HBSS-H- buffer. How regularly is the buffer changed? *This information has been added.*
Line 162-163: Please include the details of the medium used. Please define the confluency. *This information has been added.*
Figure 1: Please ensure that the scale bars are visible in all the images. *Done.*
Figure 2: Please insert a scale bar and define it. Please define what the blue color represents in the Figure Legends. *Done.*
Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. *Done.*

Reviewer #1

Major Concerns:
1. The protocol seems to be an example of self-plagiarism from the 2016 Nature Protocols publication. Many sentences have not even been rephrased and were copied exactly as they were published before.
We have solved this by rewording the text as described above.

In the discussion, the authors state: "To date, a few methods for RPE isolation and culture from mice have been developed [references, including the 2016 paper]. The advantage of our method…" Further, there is a statement "The major modification with respect to other protocols is the use of enzymatic dissociation instead of forcepts… [references, including the 2016 paper]." This is extremely misleading because the 2016 paper already had that sentence: "Another key modification with respect to previous protocols is the use of enzymatic digestion to separate the RPE from the Bruch's membrane". I believe that it would be fair to make a very clear statement in the introduction or even in the abstract that this current protocol follows that earlier publication (and highlight modifications FROM THAT ONE, if any).
We have changed the discussion to clarify that this is an updated version of the Nat 2016 paper. The first paragraph of the discussion is now focused on the advantages and differences of this protocol compared to others. We have also added a sentence in the abstract and summary.

2. Since the TER is explained so well in the protocol part of the paper, it would be logical to include the graph depicting the change in TER over time in the results.

We have added a new figure (Figure 2) and have renamed old figure 2 as figure 3 accordingly.

3. Lines 217-218. Is it possible to provide any data supporting this statement: "Gentle enzymatic dissociation results in a higher percentage of cell survival which accelerates the formation of a functional confluent monolayer" (both in terms of survival and acceleration)?

In our experience, RPE cells exposed to mechanical stress die more than RPE monolayers detached with trypsin. Cell growth is enhanced by the higher confluence resulted from better cell survival. This is an observation based on hundreds of dissections and cultures, but we have not performed precise measurements, thus, we do not have data supporting this statement. We have mentioned the advantages of these aspects of the protocol in the discussion.

Minor Concerns:

1. Lines 42-44. You state that the other methods are not reproducible and reliable. Here would be a good place to briefly discuss what is the difference of your method and what modifications lead to an increased reproducibility and reliability.

We have discussed this in the first paragraph of the discussion.

2. Line 83 - why PBS, but not the medium? Maybe explain?

This is a standard coating procedure to minimize contamination, using cell culture media would probably not make a difference.

3. Looks like the dissection method that you are proposing is different from the method presented by Shang et al (JOVE, 2018). Could you discuss that in the discussion section - what is the advantage (if any) of cutting the cornea and gently squeezing the lens out compared to cutting along the ora serrata?

We have discussed this as follows: "The major modification with respect to other protocols is the use enzymatic dissociation instead of forceps to separate the RPE from the Bruch's membrane[12, 19, 20, 24]." (Reference 24 corresponds to the JOVE 2018).The major advantage of leaving the ora serrata intact during the first incubation is that the neural retina and the RPE cells do not start detaching, but they remain in place protected by the iris epithelium.

4. L233. The statement that the RPE would not survive if the sclera is perforated sounds a bit exaggerated. Can you, please, explain, how a local cut and retina protrusion can affect ALL the RPE, but not only the cells that are surrounding the affected area?

Using this protocol, the RPE monolayer usually detaches as large RPE sheets. In every single dissection where the sclera was pierced and the neural retina popped out, it was impossible to obtain large sheets. Instead, single RPE cells detached here and there, and the yield was too low. The resulting RPE cell showed abnormal morphology and the cultures were not viable (never reached confluence). Based on experience, it is preferable to stop the dissection and discard the eye once the sclera is pierced.

5. Figure 1C. Is it possible to change the orientation of the TEM image so that it's not diagonal (transwell should be on the bottom, RPE's apical side on top)?

We have now reoriented the figure 1C.

6. N1 supplement is missing from the list of material and equipment. Also, include culture hood and CO_2 incubator.

We have added this information to the list.

Reviewer #2

Major Concerns:
the article does not mention that the procedure is based on prior published protocols: Bonilha et al JCB 1999 PMC2174247 described a principally similar two step enzyme digestion / dissection procedure for rat RPE. This procedure was then adapted to yield primary mouse RPE with very similar appearance (phagocytosis, ZO1) by Nandrot et al J Exp Med 2004. These two papers should be acknowledged and cited.

We have now acknowledged those two papers (lines 63-64 revised manuscript) and added them to the discussion.

Section 5.7.it is not clear how one can determine at this stage whether or not some RPE remains attached, as separation of neural retina follows only in section 5.9. please clarify when and how specifically to assess completion of hyaluronidase treatment.

We have modified the text for clarification. The recommendation about extended incubation time is now in section 5.9.

Line 76: please specify which strains of mice have been used successfully for the described method. statement provided is unnecessarily vague.

We have added this information to the mouse selection section.

Section 5.11. is there a difference between "FBS solution" and "20% FBS in HBSS=-H+". please clarify.

There is no difference, a consensus has been made in the text.

Section 6.4. it is not clear when and how pipetting occurs, before/ after the 1 min incubation? how much pipetting (strokes) and with micropipette or serological pipette?
section 3/ section 6.7: what is the expected yield: how many 6.5 mm transwells will be seeded from the 2 mouse eyes?

We have added this information to the sections 6.4, 6.6, and 7.3

Minor Concerns:
Abstract, line 20: please revise to remove "correct", which implies that other protocols are not correct. there are / may be other protocols that yield high quality mouse RPE

We have changed the abstract accordingly." This paper is an updated audiovisual version of the protocol previously published by Fernandez-Godino et al. to efficiently isolate and culture primary mouse RPE cells".