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".