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Title: Efficient dissection and culture of primary mouse retinal pigment epithelial cells

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   No

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Leica S9 stereomicroscope

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 16
Number of Shots: 47

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Blanca Chinchilla:** This protocol allows for the efficient isolation and culture of mouse RPE cells. Healthy primary mouse RPE cultures are valuable models to understand the mechanisms of underlying eye diseases.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Blanca Chinchilla:** Confluent and viable RPE cultures are obtained within 3 days and can be grown for weeks.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Heran Getachew:** This protocol has been successfully used to understand the mechanisms underlying early age-related macular degeneration, which is the leading cause of blindness among the elderly in developed countries.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Rosario Fernandez-Godino:** It is important to visualize the dissection steps. Details are critical for the success of the protocol. For instance, how to handle the eyeball so that the sclera isn't punched, where exactly cuts should be performed, and how to embed the eyecups in trypsin so that they stay open.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

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

Protocol

2. Collection of RPE

2.1. Begin with preparing all the necessary reagents for the protocol **[1].** Then, start cleaning the eyeball by carefully removing all connective tissue, blood, and muscles using Dumont number 5 forceps and angled scissors, without making any cuts in the sclera **[2]**.

2.1.1. WIDE: Shot of the labelled containers of reagents, sitting on the lab bench.

2.1.2. SCOPE: Talent cleaning the eyeball under the microscope.

2.2. Transfer the eyeball to a fresh dish containing 3 milliliters of HBSS-H− buffer *(pronounce hepes free HBSS buffer)* regularly to keep the eye fresh and clean and avoid any contamination **[1]**.

2.2.1. Talent transferring the eyeball to a fresh dish.

2.3. Use the optic nerve as a handle to hold the eyeball **[1]** and make a hole in the center of the cornea with a sharp carbon-steel number 11 blade **[2]**. Make three incisions in the cornea through the hole with Dumont number 5 scissors, ensuring that there is sufficient space to remove the lens **[3]**. *Videographer: This step is important!*

2.3.1. SCOPE: Talent holding the eyeball with optic nerve. NOTE: 2.3.1 – 2.4.1 filmed in one shot

2.3.2. SCOPE: Talent making hole in the cornea with a blade.

2.3.3. SCOPE: Talent making incisions in the cornea.

2.4. Hold the optic nerve and apply slight pressure to the ora serrata with the base of the angled scissors until the lens comes out completely **[1]**. Keep the iris epithelium intact to prevent the detachment of the neural retina and RPE during incubation **[1]**. Place the eye in HBSS-H- buffer **[2]**. *Videographer: This step is important!*

2.4.1. SCOPE: Talent applying pressure to the ora serrata until the lens comes out.

2.4.2. Talent placing the eye in a buffer.

2.5. Incubate the eyes without lenses in hyaluronidase solution at 37 degrees Celsius for 45 minutes in a 5% carbon dioxide-aerated incubator to detach the neural retina from the RPE **[1-TXT]**. Place each eye in a new well with 1.5 milliliters of cold HBSS-H+ buffer *(HBSS-hepes buffer)* per well **[2]** and incubate it on ice for 30 minutes **[3]**.

2.5.1. Talent placing well plate in the incubator. **TEXT: hyaluronidase solution:1.5 mL/well in a 12-well plate**

2.5.2. Talent placing eye in new well. NOTE: 2.5.2 – 2.5.3 filmed in one shot

2.5.3. Talent placing well plate on ice.

2.6. After washing, place each eye into a 35-millimeter culture dish with fresh HBSS-H+ buffer *(HBSS-hepes buffer)* **[1]**. Cut the cornea through the original incisions with 8-centimeter Vannas scissors until the ora serrata is reached **[2]**, then cut below the ora serrata to remove the iris epithelium and cornea **[3]**. *Videographer: This step is difficult and important!*

2.6.1. Talent placing eye in the culture dish.

2.6.2. SCOPE: Talent cutting cornea with scissors.

2.6.3. SCOPE: Talent cutting below ora serrata to remove iris epithelium and cornea.

2.7. Hold the ora serrata with curved tweezers **[1]** and, using angled microforceps, pull away the neural retina, making sure that the RPE layer is not cut **[2]**. Then, cut the internal attachment to the optic nerve **[3]**.

2.7.1. SCOPE: Talent holding ora serata with tweezers. NOTE: 2.7.1 – 2.8.1 filmed in one shot

2.7.2. SCOPE: Talent pulling away neural retina.

2.7.3. SCOPE: Talent cutting the internal attachment to the optic nerve.

2.8. Cut the optic nerve [**1**] and transfer each eyecup to a different 12-well plate containing 1.5 milliliters of fresh trypsin-EDTA per well **[2]**. Ensure that the eyecups remain opened and completely submerged in the trypsin **[3]**. Incubate the eyecups at 37 degrees Celsius or 45 minutes in a 5% carbon dioxide incubator **[4]**. *Videographer: This step is difficult and important!*

2.8.1. SCOPE: Talent cutting optic nerve.

2.8.2. Talent transferring eyecup to fresh well plate. NOTE: 2.8.2 – 2.8.3 filmed in one shot

2.8.3. Eyecups, open and submerged in liquid in a well.

2.8.4. Talent placing well plate in an incubator.

2.9. Collect each eyecup together with any RPE sheets detached during the trypsin incubation **[1]** and transfer them into a 12-well plate containing 1.5 milliliters of FBS solution per well **[2]**. If RPE sheets remain in the trypsin solution, use a micropipette to transfer them to the well with FBS solution **[3]**.

2.9.1. Talent collecting eyecups. NOTE: 2.9.1 – 2.9.2 filmed in one shot

2.9.2. Talent transferring eyecups into a well plate.

2.9.3. Talent transferring RPE sheets with micropipette.

3. Isolation of primary mouse RPE cells

3.1. Hold each eyecup by the optic nerve **[1]** and shake it face down into the 12-well plate containing 1.5 milliliters of 20% FBS in HBSS-H+ *(HBSS-hepes buffer)* until complete detachment of the RPE sheets is achieved **[2]**. *Videographer: This step is important!*

3.1.1. Talent holding eyecup by the optic nerve. NOTE: 3.1.1 – 3.1.3 filmed in one shot

3.1.2. Talent shaking the eyecup into a well plate.

3.2. Collect any RPE sheets and RPE clusters with a micropipette, avoiding any white pieces of sclera or choroid, which could contaminate the cultures **[1]** and place them in a 15-milliliter tube. Pool two eyes from the same mouse in one tube **[2]**.

3.2.1. Talent collecting RPE sheets with micropipette. NOTE: 3.2.1 – 2.2.2 filmed in one shot

3.2.2. Talent placing RPE sheets in 15 milliliter tube.

~~3.2.3. Talent collecting eyes in a tube.~~

3.3. Then, centrifuge the RPE mixture at 340 x *g* for 2 minutes at room temperature **[1]** and discard the supernatant **[2]**.

3.3.1. Talent removing tubes from a centrifuge and closing the lid.

3.3.2. Talent discarding the supernatant.

3.4. Gently resuspend the RPE pellet in a 1-milliliter mixture of 0.25% trypsin-EDTA **[1]** and incubate it for 1 minute in a water bath at 37 degrees Celsius to disaggregate the RPE sheets into single cells **[2]**, then gently pipet up and down 10 times, avoiding bubble formation **[3]**.

3.4.1. Talent resuspending the pellet.

3.4.2. Talent placing mixture on a water bath.

3.4.3. Talent pipetting mixture up and down.

3.5. Add 9 milliliters of freshly prepared RPE medium to dilute and inactivate the trypsin **[1]** and centrifuge the mixture at 340 x *g* for 2 minutes at room temperature **[2]**. Aspirate the supernatant **[3]** and carefully resuspend the cell pellet in 150 microliters of RPE medium with 5% FBS **[4]**.

3.5.1. Talent adding medium to RPE mixture.

3.5.2. Talent placing tubes in the centrifuge and closing the lid.

3.5.3. Talent aspirating the supernatant.

3.5.4. Talent resuspending the pellet.

3.6. Next, remove PBS from the bottom chamber of the previously prepared laminin-coated membrane insert **[1]** and add 700 microliters of RPE medium to it **[2]**. Remove the laminin from the upper chamber of the membrane insert **[3]** and distribute the RPE cell suspension dropwise and uniformly to the center of the chamber **[4-TXT]**.

3.6.1. Talent removing PBS from bottom chamber of membrane inserts. NOTE: 3.6.1 – 3.6.2 filmed in one shot

3.6.2. Talent adding RPE medium to the bottom chamber of membrane inserts.

3.6.3. Talent removing laminin from upper chamber of membrane inserts. NOTE: 3.6.3 – 3.6.4 filmed in one shot

3.6.4. Talent adding RPE cell suspension to membrane inserts. **TEXT: Avoid bubble formation**

3.7. Place the membrane insert undisturbed in a 5% carbon dioxide incubator at 37 degrees Celsius for at least 24 hours **[1]**. Then, check the membrane insert under the microscope to make sure that most RPE cells are attached to the insert **[2]** and the confluence is at least 50%. Do not change the media during first 72 hours **[3]**.

3.7.1. Talent placing membrane insert in an incubator.

3.7.2. Talent observing membrane inserts under the microscope.

3.7.3. LAB MEDIA: Figure 1 A.

Results

4. Results: Morphological and functional validation of RPE cells

4.1. RPE cells isolated using this protocol showed the typical RPE size, morphology, and pigmentation **[1]**. A highly polarized RPE monolayer with two nuclei joined by tight junctions along with the presence of apical microvilli and basal infoldings was observed after one week **[2]**.

4.1.1. LAB MEDIA: Figure 1 A, B.

4.1.2. LAB MEDIA: Figure 1 C.

4.2. Polarization of the RPE monolayer was confirmed by TER values, with the average higher than 200 ohm centimeters squared, which remained stable over time **[1]**.

4.2.1. LAB MEDIA: Figure 2.

4.3. In phagocytosis assays performed on FITC-labeled bovine POS, engulfment and digestion of POS was observed, indicating formation of a functional confluent monolayer of RPE cells **[1]**.

4.3.1. LAB MEDIA: Figure 3.

Conclusion

5. Conclusion Interview Statements

5.1. **Rosario Fernandez-Godino:** Viable cultures require a short dissection time, which is achieved with the experience. Some RPE cells may stay attached to the neural retina, but if several RPE sheets remain attached, the protocol will not succeed. Fresh hyaluronidase must be used every time to prevent this problem.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.2 for viable culture and 2.7.2 for RPE cells.*

5.2. **Rosario Fernandez-Godino:** These functional primary mouse RPE cell cultures are a valuable tool to study the pathobiology of the RPE in many eye diseases. For example, this protocol made it possible to study the pathobiology of the RPE in a mouse model of macular degeneration.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.