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Title: Coculture of Axotomized Rat Retinal Ganglion Neurons with Olfactory Ensheathing Glia, as an In Vitro Model of Adult Axonal Regeneration

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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? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

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: 13
Number of Shots: 33

Introduction

1. Introductory Interview Statements

1.1. **Javier Sierra:** Our protocol is a simple and reproducible in vitro model to assess olfactory ensheathing glia capacity to foster adult axonal regeneration.

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

1.2. **M. Teresa Moreno-Flores:** This is a quantitative technique that allows the evaluation of olfactory ensheathing glia regenerative capacity, which can also be extended to other glial cell types.

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

OPTIONAL:

1.3. **M.Teresa Moreno-Flores:** This technique is the first step to study olfactory ensheathing glia as a candidate for cell therapy in central nervous system injuries, before translation to in vivo or clinical studies.

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

Introduction of Demonstrator on Camera

1.4. **Javier Sierra:** Demonstrating the procedure will be María Portela, a PhD student from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. Procedures involving animal subjects have been approved by national and institutional bioethics committees.

Protocol

2. Retinal Tissue Dissection

2.1. To begin, prepare all required media as described in the text manuscript **[1]**. After sacrificing the rat, place its head in a 100-millimeter Petri dish and spray it with 70% ethanol before placing it in a laminar flow hood **[2]**. Cut the rat´s whiskers with scissors so they do not interfere with the eye manipulation **[3]**.

2.1.1. WIDE: Establishing shot of talent at the lab bench preparing solutions.

2.1.2. Talent spraying a rat's head with ethanol.

2.1.3. Talent cutting the whiskers.

2.2. Grip the optic nerve with forceps to pull out the eyeball enough to be able to make an incision across the eye with a scalpel **[1]**. Remove the lens and vitreous humor and pull out the retina, keeping the remaining layers of the eye inside **[2]**. *Videographer: This step is difficult and important!*

2.2.1. Talent pulling out the eyeball.

2.2.2. Talent removing the lens and vitreous humor and pulling out the retina.

2.3. Place the retina in a previously prepared p60 cell culture dish with 5 milliliters of cold EBSS **[1]**. Then, transfer it to a p60 cell culture dish with reconstituted papain plus 50 microliters of APV and 250 microliters of DNase plus 5 microliters of APV **[2-TXT]**. Cut the retina into small pieces with a scalpel **[3-TXT]**. *Videographer: This step is important!*

2.3.1. Talent placing the retina in a p60 cell culture dish.

2.3.2. Talent transferring the retina to the next dish. **TEXT: Use a papain dissociation kit**

2.3.3. Talent cutting the retina into small pieces. **TEXT: < 1 mm**

2.4. Transfer the pieces to a 15-milliliter plastic tube **[1]** and incubate them for 30 minutes in a humidified incubator at 37 degrees Celsius under 5% carbon dioxide **[2]**, agitating every 10 minutes **[3]**.

2.4.1. Talent transferring the tissue to a tube.

2.4.2. Talent putting the tube in the incubator and closing the door.

2.4.3. Talent agitating the tube.

2.5. Dissociate cell clumps by pipetting up and down with a glass Pasteur pipette **[1]**, then centrifuge the cell suspension at 200 x *g* for 5 minutes **[2]**. Discard supernatant **[3]** and resuspend the cell pellet in albumin-ovomucoid protease inhibitor with 150 microliters of DNase and 30 microliters of APV **[4-TXT]**. *Videographer: This step is important!*

2.5.1. Talent pipetting up and down with a Pasteur pipette.

2.5.2. Talent putting the tube in the centrifuge and closing the lid.

2.5.3. Talent discarding the supernatant.

2.5.4. Talent resuspending the cell pellet. **TEXT: 1.5 mL for 2 eyes**

2.6. Carefully add the cell suspension on 5 milliliters of albumin-ovomucoid protease inhibitor **[1]** and repeat the centrifugation **[2]**. While centrifuging, completely remove the ME-10 medium from an OEG 24 well plate and replace it with 500 microliters of NB-B27 medium per well **[3]**. *Videographer: This step is important!*

2.6.1. Talent pipetting the cells suspension into the protease inhibitor.

2.6.2. Talent putting the tube in the centrifuge.

2.6.3. Talent replacing media in the plate.

2.7. Discard the supernatant and resuspend the cells in 2 milliliters of NB-B27 medium **[1]**. Plate 100 microliters of retinal cell suspension into each well of the m24 plate onto PLL-treated or OEG monolayer-coverslips **[2]**. Maintain cultures at 37 degrees Celsius with 5% carbon dioxide for 96 hours in NB-B27 medium **[3]**.

2.7.1. Talent resuspending the cells.

2.7.2. Talent plating cells into a few wells.

2.7.3. Talent putting the plate in the incubator and closing the lid.

3. Immunostaining and Axonal Regeneration Quantification

3.1. After 96 hours, fix the cells for 10 minutes by adding the same volume of 4% PFA in PBS to the culture medium **[1]**.

3.1.1. Talent adding 4% PFA in PBS to the cells.

3.2. Discard the fixing solution and wash the cells 3 times with PBS for 5 minutes per wash **[1]**, then block with PBS-TS for 30 to 40 minutes **[2-TXT]**. Prepare the primary antibodies in PBS-TS buffer as described in the text manuscript and add them to the cocultures **[3]**. Incubate the cells overnight at 4 degrees Celsius **[4]**.

3.2.1. Talent washing the coverslips with PBS.

3.2.2. Talent adding PBS-TS to the cells. **TEXT: PBS-TS: PBS with 0.1% Triton X-100 and 1% FBS**

3.2.3. Talent adding primary antibodies to the cells.

3.2.4. Talent putting the coverslips in the refrigerator.

3.3. On the next day, discard the antibodies and wash the coverslips 3 times with PBS for 5 minutes per wash **[1]**. Add the corresponding fluorescent secondary antibodies **[2]** and incubate the coverslips for 1 hour at room temperature in the dark **[3]**.

3.3.1. Talent discarding the primary antibody solutions and placing the coverslips in PBS.

3.3.2. Talent adding secondary antibodies.

3.3.3. Talent putting the coverslips in a dark place.

3.4. Then, wash the coverslips 3 times with PBS for 5 minutes per wash in the dark **[1]**. Mount the coverslips with mounting medium and keep them at 4 degrees Celsius **[2]**.

3.4.1. Talent washing coverslips.

3.4.2. Talent adding mounting medium to coverslips.

3.5. Use a 40x objective of an epifluorescence microscope to quantify axonal regeneration. Quantify the percentage of neurons with axons relative to the total population of retinal ganglion neurons **[1]**.

3.5.1. Talent at the microscope imaging the neurons.

3.6. Use the NeuronJ plugin in ImageJ to quantify the axonal regeneration index or mean axonal length, which is the sum of the lengths of all identified axons divided by the total number of counted neurons, whether they presented an axon or not **[1-TXT]**.

3.6.1. SCREEN: 61863_screenshot_1.mp4. 0:03 – 0:23. **TEXT: Image must be 8 bits**

3.7. After opening in the image, click on **Analyze** and **Set Scale** according to microscope settings, working in pixels per micrometer **[1]**. Select **Add Tracings** to begin axon tracking, then track the axons from the soma to the end **[2]**. Click on **Measure Tracings**, **Display Tracing Measurements**, and **Run [3]**.

3.7.1. SCREEN: 61863_screenshot_1.mp4. 0:24 – 0:42.

3.7.2. SCREEN: 61863_screenshot_1.mp4. 0:43 – 1:27. *Video Editor: Speed this up as needed.*

3.7.3. SCREEN: 61863_screenshot_1.mp4. 1:28 – 1:37.

Results

4. Results: Assay for Axonal Regeneration in Cocultures of OEG Lines with Adult Retinal Ganglion Neurons

4.1. Neuroregenerative capacity of olfactory ensheathing glia after neuronal injury was investigated using a reversible immortalized human OEG clonal cell line **[1]**.

4.1.1. LAB MEDIA: Figure 1.

4.2. Ts14 OEG identity was assessed by immunostaining with ensheathing glia markers such as S100 beta **[1]** and vimentin **[2]**. GFAP expression was also analyzed to discard astrocyte contamination **[3]**.

4.2.1. LAB MEDIA: Figure 2 A.
4.2.2. LAB MEDIA: Figure 2 B.
4.2.3. LAB MEDIA: Figure 2 C.

4.3. In the axonal regeneration assay, Ts14 regenerative capacity was compared to Ts12 in RGN-OEG cocultures, using PLL substrate as a negative control **[1]**. Representative images show a lack of capacity of RGN to regenerate their axons over PLL or Ts12 cells **[2]**, while Ts14 stimulates the outgrowth of axons in RGN **[3]**.

4.3.1. LAB MEDIA: Figure 3 A – C.
4.3.2. LAB MEDIA: Figure 3 A – C. *Video Editor: Emphasize A and B.*
4.3.3. LAB MEDIA: Figure 3 A – C. *Video Editor: Emphasize C.*

4.4. Both the percentage of cells with axons as well as the average length of the regenerated axons were significantly higher in neurons cocultured on Ts14 monolayers **[1]**, compared to neurons plated on either Ts12 cells or PLL **[2]**.

4.4.1. LAB MEDIA: Figure 3 D – E. *Video Editor: Emphasize Ts14 bars in both graphs.*
4.4.2. LAB MEDIA: Figure 3 D – E.

Conclusion

5. Conclusion Interview Statements

5.1. **Javier Sierra:** This protocol can be used to elucidate the molecular mechanisms responsible for the neuroregenerative properties of the OEG.

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