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Title: Cone-enriched cultures from the retina of chicken embryos to study rod to cone cellular interactions

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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.

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: 19
Number of Shots: 38

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Thierry Léveillard:** This method is used to study cone photoreceptors that are essential for daylight vision. An important advantage of this method is the use of fertilized chicken eggs, which are easily accessible and one of the only sources of primary cone cultures.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.1.2, 2.2.1*

OPTIONAL:

1.2. **Thierry Léveillard:** The protocol was used to identify the rod-derived cone viability factor, a very promising future treatment of inherited retinal degenerations. Furthermore, the protocol was used to successfully to study cone photoreceptor metabolism.

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

1.3. **Géraldine Millet Puel:** By carefully following the described protocol, an individual with expertise in primary cell cultures should be able to obtain cone-enriched cultures after only a few attempts.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 4.1.1*

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Committee on the Ethics of Animal Experiments of the University Pierre and Marie Curie and the French ministry of research.

Protocol

2. Recovery of the chicken embryos

2.1. Begin by collecting weekly fertilized eggs from an industrial hatchery **[1-TXT]**. Maintain the fertilized eggs at 17 degrees Celsius in the laboratory **[2]**.

2.1.1. WIDE: Establishing shot of talent walking to the incubator with the eggs. **TEXT: strain I 657, red label** NOTE: take 1

2.1.2. Talent putting the eggs in the incubator and closing the door. NOTE: as 2.1.1 take 2

2.2. For each culture, incubate seven fertilized eggs for 24 hours at 20 degrees Celsius and then 136 hours at 37 degrees Celsius in a humidified chamber with intermittent reversion of inclination **[1]**.

2.2.1. Eggs incubating.

2.3. To recover the chicken embryos, wash the surface of the eggs with disinfectant **[1]**. Break the eggshell by making a hole at the top of the shell with large straight pliers **[2]**, then cut the shell to remove the hat from the egg **[3]**.

2.3.1. Talent washing the surface of the egg.

2.3.2. Talent breaking the eggshell. NOTE: take 2

2.3.3. Talent cutting the shell and removing the hat.

2.4. Gently extract each embryo from the eggshell with curved forceps **[1]** and transfer it to a Petri dish containing sterile PBS previously heated to 37 degrees Celsius **[2]**. Carefully remove the envelope that surrounds the embryos **[3]**. *Videographer: This step is important!*

2.4.1. Talent extracting an embryo.

2.4.2. Talent placing the embryo in a Petri dish with PBS.

2.4.3. Talent removing the envelope.

2.5. Verify the stage of development of each embryo by visual comparison to Hamburger and Hamilton **[1]** and select two embryos at the 29^{th} stage of development **[2]**. Enucleate the eyes of these selected embryos **[3]** and transfer them into carbon dioxide-independent medium **[4]**. *Videographer: This step is difficult and important!*

2.5.1. Talent looking at the embryos under the microscope.

2.5.2. SCOPE: Stage 29 embryo OR LAB MEDIA: Figure 1, stage 29 image.

2.5.3. SCOPE: Talent enucleating the eyes.

2.5.4. Talent placing the eyes into medium.

3. Dissection of the retinas

3.1. Working in carbon dioxide-independent medium, position four eyes with the cornea facing down and the optic nerve facing the experimenter **[1]**. Drill a hole in the optic nerve using two straight forceps **[2]**. *Videographer: This step is important!*

3.1.1. SCOPE: Talent positioning the eye.

3.1.2. SCOPE: Talent drilling a hole in the optic nerve.

3.2. Insert a branch of each forceps between the retina and the pigment epithelium, then pull and rotate the eye to detach the epithelium from the retina **[1]**. Remove the cornea, followed by the lens and the vitreous **[2]**. *Videographer: This step is difficult and important!*

3.2.1. SCOPE: Talent detaching the epithelium from the retina.

3.2.2. SCOPE: Talent removing the cornea, lens, and vitreus. NOTE: with 3.2.1

3.3. Transfer the four retinas into a Petri dish containing Ringer's medium at pH 7.2 **[1]**.

3.3.1. Talent placing the retinas in the Ringer's medium.

4. Preparation of retinal cell suspension

4.1. Cut the four retinas into very small pieces using two straight pliers **[1]** and wash the pieces twice with Ringer's medium **[2]**.

4.1.1. Talent cutting the retinas.

4.1.2. Talent washing the pieces with Ringer's medium. NOTE: May be 4.1.1B or 4.2.1B

4.2. After the second wash, let the pieces of retina fall to the bottom of the tube and remove the media **[1]**. Treat the retinal pieces with a solution of trypsin for 20 minutes at 37 degrees Celsius **[2]**.

4.2.1. Talent removing the media. NOTE: use 4.1.2

4.2.2. Talent adding trypsin solution to the pieces.

4.3. After ~~10~~ 20 minutes, ~~disperse the trypsin solution by successive suction **[1].** Discharge using a pipette and check for dissociation of the retinal pieces~~ **[2]**. Stop the reaction by adding culture media supplemented with 10% inactivated foetal calf serum **[3]**.

~~4.3.1. Talent dispersing the trypsin.~~

4.3.2. Talent checking for dissociation.

4.3.3. Talent adding culture media to the reaction. NOTE: take 2

4.4. Incubate the cell suspension with 0.05 milligrams of DNase I *('one')* **[1]**, then immediately dissociate the cell clusters and the DNA by pipetting up and down with a ~~Pasteur~~ pipette **[2]**. Wash the retinal cell suspension twice with chemically defined culture medium, or CDM **[3]**.

4.4.1. Talent adding DNase I to the cells.

4.4.2. Talent pipetting up and down.

4.4.3. Talent washing the cells with CDM. NOTE: take 1 remove medium, take 2 add new medium)

5. Retinal cell seeding

5.1. Treat two black 96-well culture plates with a transparent bottom with poly-L-lysine for 2 hours at 37 degrees Celsius **[1-TXT]**. When finished, rinse the plates twice with M199 culture medium **[2]**. ~~Resuspend the cell pellet in 1 milliliter of CDM **[3]**.~~

5.1.1. Talent adding the poly-L-lysine to a few wells. **TEXT: 32.25 µg/cm²** NOTE: take 2

5.1.2. Talent rinsing the plate. NOTE: take 1 remove medium , take 3 add new medium

~~5.1.3. Talent resuspending the cells.~~

5.2. Add trypan blue to an aliquot of 10 microliters of the cell suspension to stain the living cells **[1]**, then add the cells suspension specimen to a hemocytometer **[2]**.

5.2.1. Talent adding trypan blue to the cell suspension aliquot.

5.2.2. Talent adding the cells to the hemocytometer.

5.3. After counting the cells, bring the cell suspension to the appropriate concentrations using CDM **[1-TXT]**. Add 50 microliters of the library of molecules to be screened using a predefined pattern **[2].** NOTE: Moved from 5.5 to 5.3

5.3.1. Talent diluting the cells. **TEXT: 5.6 x 10^4 cells/mL and 1.12 x 10^5 cells/mL** (take 2)

5.3.2. Talent adding the library molecules. NOTE: take 2, moved from 5.5

5.4. Seed 50 microliters of the two cell suspensions into the two pretreated black 96-well culture plates. Distribute the cells in the plates with a multichannel pipette from the right of the plate to the left, homogenizing between each column **[1]**.

5.4.1. Talent seeding the cells. NOTE: CU at the end

5.5. Then incubate the plates for seven days at 37 degrees Celsius under 5% carbon dioxide with no change of media **[2]**.

5.5.1. Talent putting the plates in the incubator and closing the door.

5.6. To count the viable cells, add 2.7 micromolar calcein AM and 0.3 millimolar ethidium homodimer to each well of the plate **[1]**. Incubate the plates for 1 hour at room temperature in the absence of light **[2]**.

5.6.1. Talent adding calcein AM and ethidium homodimer to a few wells.

5.6.2. Talent covering the plate from light.

5.7. Read the fluorescence on an automated plate reader composed of an inverted microscope equipped with a mercury lamp with two excitation filters at 485 and 520 nanometers, two emission filters at 520 and 635 nanometers, and a charge-coupled device camera **[1]**.

5.7.1. Talent at the microscope, imaging the cells. NOTE: 2sd part + CU at the end

Results

6. Results: Epithelium-derived cone viability factor (EdCVF), clone 0073-09-37

6.1. This protocol was used to screen a normalized cDNA library made of choroid and retinal pigmented epithelium from 400 eyes of 8-week-old Long-Evans rats. A total of 2,112 sets of 100 clones corresponding to 211,200 individual clones were evaluated **[1]**.

6.1.1. LAB MEDIA: Table 1.

6.2. Among the 42 pools of clones with a ratio greater than 2, pools 0080 and 0073 had a viability ratio 16 and 14 times higher than the negative control after 7 days of culture **[1]**.

6.2.1. LAB MEDIA: Supplementary Figure 1. *Video Editor: Emphasize the 0080 and 0073 bars.*

6.3. Each selected pool of 100 clones was subdivided into 16 sets of 10 clones from their glycerol stock **[1]**. The sub-pool 0073-09 gave the strongest viability ratio and was subdivided to produce 16 individual clones that were tested in a third round of screening on cone-enriched cultures **[2]**.

6.3.1. LAB MEDIA: Supplementary Figure 2A.

6.3.2. LAB MEDIA: Supplementary Figure 2A. *Video Editor: Emphasize the 09 bar.*

6.4. The clone 0073-09-37 stood out with a viability ratio of 2.5 **[1]**. Further analysis confirmed that this clone has a robust and reproducible effect on cone survival. The test was repeated independently **[2]**, and the insert of 1.8 kilo bases was sequenced **[3]**.

6.4.1. LAB MEDIA: Supplementary Figure 2B. *Video Editor: Emphasize the 37 bar.*

6.4.2. LAB MEDIA: Figure 3A.

6.4.3. LAB MEDIA: Figure 3B.

6.5. A bioinformatic analysis revealed that the clone 0073-09-37, which was named epithelium-derived cone viability factor, or EdCVF, contains three open reading frames **[1]**. When tested independently, only ORF1 exerted a protective effect on the cones **[2]**.

6.5.1. LAB MEDIA: Supplementary Figure 3.

6.5.2. LAB MEDIA: Supplementary Figure 3. *Video Editor: Emphasize the ORF1 bar.*

6.6. ORF1 was produced as a glutathione S-transferase fusion protein **[1]**. The epithelium-derived cone viability factor was purified and the GST tag was removed **[2]**. Analysis of

trophic activity demonstrated that EdCVF is able to prevent cone degeneration in the cone-enriched culture system **[3]**.

6.6.1. LAB MEDIA: Figure 4A.

6.6.2. LAB MEDIA: Figure 4B.

6.6.3. LAB MEDIA: Figure 4C. *Video Editor: Emphasize the EdCVF bar.*

Conclusion

7. Conclusion Interview Statements

7.1. **Geraldine Millet-Puel:** When attempting this procedure, the stage of development of the embryos should be carefully checked in order to get the cone-enriched cultures.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.5.1 – 2.5.2*

7.2. **Geraldine Millet-Puel:** Following this protocol, the cells can be electroporated with plasmid DNA to study the molecular mechanisms of any survival factor, as such RdCVF.

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

7.3. **Thierry Léveillard:** This technique paves the way for metabolic research, including the development of mathematical models of cone survival.

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