FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68384
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=20857168

Title: Whole-Mount Retinal Organoid Visualization with Cellular Resolution

Authors and Affiliations:
Marina Cunquero[1], Helena Isla-Magrané[2], Maria Marsal[1], Maddalen Zufiaurre-Seijo[2], José García-Arumí[2], Miguel Ángel Zapata[2], Anna Duarri[2], Pablo Loza-Alvarez[1]

[1]ICFO-Institut de Ciències Fotòniques, The Barcelona Institute of Science and Technology
[2]Ophthalmology Group, Hospital Universitari Vall d'Hebron, Vall d'Hebron Institut de Recerca (VHIR)

Corresponding Authors:
Pablo Loza-Alvarez pablo.loza@icfo.eu
Email Addresses for All Authors:
Marina Cunquero marina.cunquero@icfo.eu
Helena Isla-Magrané helena.isla@vhir.org
Maria Marsal Maria.Marsal@icfo.eu
Maddalen Zufiaurre-Seijo maddalen.zufiaurre@vhir.org
José García-Arumí josgarci@vhebron.net
Miguel Ángel Zapata miguelangel.zapata@vallhebron.cat
Anna Duarri anna.duarri@vhir.org
Pablo Loza-Alvarez Pablo.Loza@icfo.eu

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. **Filming location:** Will the filming need to take place in multiple locations? **Yes, 1 room apart**

Current Protocol Length
Number of Steps: 20
Number of Shots: 45

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Helena Isla-Magrané:** Our work focuses on developing and study retinal organoids that serve as an *in vitro* human model to study how the human retina forms, how diseases affect, and how we might treat them.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll: 3.1.1*

What technologies are currently used to advance research in your field?

1.2. **Maria Marsal:** To advance the research in the regenerative medicine field, we use 3D retinal organoids combined with optical clearing and immunolabeling to visualize entire structures and study spatial organization of retinal cells with confocal microscopy.

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

What are the current experimental challenges?

1.3. **Marina Cunquero:** Whole-mount imaging faces challenges like uneven antibody penetration in dense tissues and spherical aberrations in organoids, which cause surface-biased labeling and focus loss during deep tissue visualization.

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

What advantage does your protocol offer compared to other techniques?

1.4. **Helena Isla-Magrané:** Our method reveals retinal cell connections, cell types, and their 3D organization, providing crucial insights into the underlying causes of retinal diseases.

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

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Preparation of Custom-Made Fluorescent Secondary Antibodies

Demonstrator: Maria Marsal

2.1. To begin, obtain the fluorophore aliquots dissolved in anhydrous DMSO, dehydrated and frozen **[1].** Add 1 to 10 microliters of DMSO to an aliquot **[2]**.

2.1.1. WIDE: Talent holding 0.02 mg aliquots of the fluorophore.

2.1.2. Talent pipetting and dissolving the fluorophore aliquot into a small Eppendorf tube using dimethyl sulfoxide or ultrapure water for Alexa Fluor 405.

Videographer's Note: Shots 2.1.1-2.1.2 were filmed together

2.2. Now combine 50 microliters of secondary immunoglobulin G or primary antibody, 6 microliters of 1 molar sodium bicarbonate, and 1 to 5 microliters of fluorophore **[1]** and incubate on a rocking platform for 40 minutes at room temperature, protected from light **[2]**.

2.2.1. Talent combining the reagents in a microcentrifuge tube.

2.2.2. Talent placing it on a rocking platform covered with foil.

2.3. While the reaction is progressing, remove the lids of the purification size exclusion columns **[1]** and allow the buffer to pass through **[2]**.
Videographer's Note: Shots 2.3.1-2.3.2 were filmed together

2.3.1. Talent removing column lids.

2.3.2. Shot of the buffer dripping through.

2.4. Equilibrate the column by running 3 rounds of 2 to 3 milliliters of PBS through the column **[1]**. If the last equilibration finishes before incubation ends, put the lids back to avoid them drying and wait for the reaction to finish **[2]**.

2.4.1. Talent using a pipette to add phosphate-buffered saline to the column for equilibration.

2.4.2. Talent putting back the column lids and setting the columns aside.

2.5. After incubation, add 140 microliters of PBS to the labelling reaction to bring the volume to approximately 200 microliters and vortex it **[1]**. Add the solution to the centre of the column and let it to enter the column **[2]**.

2.5.1. Talent adding phosphate-buffered saline to the reaction tube and vortexing.

2.5.2. Talent carefully pipetting the solution into the centre of the column.

2.6. After the last drop has eluted, push the solution with 550 microliters of PBS **[1]**. When the liquid stops falling, elute with 300 microliters of PBS **[2]** and collect in a 1.5-milliliter microcentrifuge tube **[3]**.

2.6.1. Talent adding 550 µL of PBS.

2.6.2. Talent adding 300 µL of PBS after elution stops.

2.6.3. Talent collecting the elution in a microcentrifuge tube.
Videographer's Note: Shots 2.6.3-2.6.3 were filmed together

2.7. Measure the absorbance of the sample at 280 nanometers and at the fluorophore-specific wavelengths to calculate the antibody concentration and labelling ratios **[1]**. Store the labelled antibodies at 4 degrees Celsius, protected from light, for up to 6 months **[2]**.

2.7.1. Talent places the sample in a spectrophotometer.

2.7.2. Talent placing labelled tubes in a refrigerator.

3. Antibody Labeling and Confocal Imaging of Organoids

Demonstrator: Marina Cunquero

3.1. Fix the retinal organoids with 4% paraformaldehyde at room temperature for 45 minutes **[1]**. After fixing, add antigen-retrieval solution over the organoids **[2]** and incubate the dish at 60 degrees Celsius with mild shaking at 30 revolutions per minute for 1 hour **[3]**.

3.1.1. Talent adding paraformaldehyde to a dish containing organoids.

3.1.2. Talent adding antigen retrieval solution over the organoids.
Videographer's Note: Shots 3.1.1-3.1.2 were filmed together

3.1.3. Talent placing the organoids in a shaking incubator set to specified conditions.

3.2. Next, permeabilize the organoids with PBS containing 1 percent Triton X-100 **[1].** Incubate the mixture at room temperature with mild shaking for 4 hours **[2]**.

3.2.1. Talent adding PBST to the organoids.

3.2.2. Talent placing the dish with organoids on a shaker.

3.3. Now block the organoids in 2% BSA with 0.1 percent Triton X-100 at room temperature overnight or for over 1 day **[1]**.
2.10.1. Talent adding blocking solution to the dish.

3.4. The next day, add diluted primary antibodies to the organoids **[1].** Incubate at 4 degrees Celsius for 2 days with mild shaking **[2]**. Wash the organoids three times for 15 minutes each in washing solution at room temperature with mild shaking **[3]**.

3.4.1. Talent adding diluted primary antibody.

3.4.2. Talent placing the sample in a shaker in the refrigerator.

3.4.3. Talent adding washing solution to the organoids after removing the antibody solution.

3.5. Now add the dilution solution secondary antibodies **[1]** and incubate at 4 degrees Celsius for 2 days with mild shaking **[2]**.

3.5.1. Talent adding secondary antibody solution.

3.5.2. Talent placing the dish with secondary antibodies in the refrigerator shaker.

3.6. After washing the organoids as demonstrated earlier, incubate the organoids with fluorescent dyes diluted in washing solution **[1]** at room temperature for 1 hour with mild shaking **[2]**. Then wash again **[3].**

3.6.1. Talent adding fluorescent dyes to the dish.

3.6.2. Talent placing the dish on a shaker.

3.6.3. Talent performing wash.

3.7. Next, prepare 1-propanol solutions in ultrapure water at 15, 30, 45, 60, 75, and 90 percent concentrations **[1]**, and adjust each to pH 9.5 with trimethylamine **[2]**.

3.7.1. Talent preparing and labelling each propanol solution.

3.7.2. Talent adjusting the pH of the solutions with trimethylamine.

3.8. Dehydrate the samples sequentially in increasing gradients of 1-propanol solutions **[1-TXT]** for 2 hours each at 30 degrees Celsius with mild shaking **[2]**. Then transfer the sample to 100 % 1-propanol solution **[3]** and incubate overnight at 30 degrees Celsius with mild shaking **[4]**.

3.8.1. Talent adding 1-propanol solution to the sample. **TXT: Propanol concentrations: 15%, 30%, 45%, 60%, 75%, 90%**

3.8.2. Talent placing the sample on a shaker and setting the temperature.

3.8.3. Talent placing samples in 100 percent 1-propanol.

3.8.4. Shot of the sample on the shaker.

3.9. For sample clearing, prepare benzyl alcohol and benzyl benzoate mixture in a 1 to 2 ratio to make BABB *(B-A-B-B)* solution **[1]**. Immerse the samples in BABB at room temperature overnight **[2]**. Refresh the BABB solution before imaging **[3]**.

3.9.1. Talent preparing BABB solution.

3.9.2. Talent placing samples in BABB solution and covering the container.

3.9.3. Talent replacing the BABB solution in the sample container.
Videographer's Note: 3.9.3 in clapboard is 3.9.2
3.9.3 OK in clapboard is 3.9.3

4. Confocal Imaging of Organoids

Demonstrator: Marina Cunquero

4.1. With a glass pipette, position the organoid in a glass-bottom Petri dish with a drop of BABB, ensuring it contacts the surface of the cover glass **[1]**. Now, use an inverted confocal laser-scanning microscope with low and high magnification objectives to acquire Z-stack images for 3D cellular resolution **[2]**.

4.1.1. Talent transferring and adjusting the organoid position carefully in the dish.

4.1.2. Talent positioning the dish containing the samples in the microscope stage while is modifying the imaging settings
Videographer's Note: ID#68384_0049.MXF and ID#68384_0050.MXF are extra shots of step 4.1.2.

4.1.3. SCREEN: 4.1.3_SCREEN.mp4 00:00-00:15

4.2. Recalibrate the step size of the Z-stack acquisition by accounting for the refractive index mismatch between clearing solution and immersion media **[1]**.

4.2.1. SCREEN: 68384_4.2-4.3-4.4.mp4 00:00-00:03.
AND
TEXT ON PLAIN BACKGROUND:

$$Recalibrated\ step\ size\ = \frac{RI_{clearing\ sol.}}{RI_{immersion\ media}} \times step\ size$$

Video Editor: Please play both shots side by side

4.3. Now launch ImageJ *(Image-J)*, update the voxel depth by selecting **Image** and clicking on **Properties** tocreate image projections of the Z-stack **[1]**.

4.3.1. SCREEN 68384_4.2-4.3-4.4.mp4 00:04-00:14

4.4. Inspect the Z-stack depth by selecting **Image**, then **Stacks**, and choosing **Orthogonal views** to display XY*(X-Y)*, XZ*(X-Z)*, and YZ *(Y-Z)* views of the retinal organoid **[1]**. Save the desired regions by clicking on **File** and selecting **Save as [2]**. Lastly, create an animation of the 3D render of the Z-stack using processing software **[3]**.

4.4.1. SCREEN: 68384_4.2-4.3-4.4.mp4 00:18-00:31.

4.4.2. SCREEN: 68384_4.2-4.3-4.4.mp4 00:35-00:49.

4.4.3. SCREEN: 4.4.3_SCREEN.mp4. 00:03-00:08,00:28-01:00
Video Editor: Please speed up the video

Results

5. Results

5.1. Fructose-glycerol-cleared retinal organoids showed the least improvement in transparency, hindering visualization of the organoid core **[1]**. ECi *(E-C-I)* clearing improved visualization of retinal layers but still failed to reveal the organoid core due to persistent light scattering **[2-TXT]**.

5.1.1. LAB MEDIA: Figure 1B. *Video editor: Please highlight the CLSM image*

5.1.2. LAB MEDIA: Figure 1C. **TXT: Eci: Ethyl Cinnamate** *Video editor: Please highlight the CLSM image*

5.2. FluoClear BABB-cleared organoids exhibited the highest transparency, clearly revealing both the cortex and core with consistent fluorescence **[1]**. Both ECi and FluoClear BABB-cleared organoids exhibited visible shrinkage in brightfield images due to dehydration steps **[2]**.

5.2.1. LAB MEDIA: Figure 1D. *Video editor: Please highlight the CLSM image*

5.2.2. LAB MEDIA: Figure 1C and D. *Video editor: Please highlight the BF images in both 1C and 1D*

5.3. BABB-induced shrinkage increased sample compactness, enabling the use of high magnification objectives to image deeper structures **[1]**.

5.3.1. LAB MEDIA: Figure 2. *Video editor: If possible, please show figure 2A first then zoom out to 2B*

5.4. TUJ1 *(T-U-J-One)* immunolabelling at 40 days *in vitro* showed a single, thin neuronal layer without visible stratification **[1]**. By 90 days, the cells densely populated the apical region and at 170 days, the organoid exhibited clear signs of layered organization with a defined apical zone **[2]**.

5.4.1. LAB MEDIA: Figure 3. *Video editor: Please highlight the 40 DIV panel on the left and show the "neuroretina" image*

5.4.2. LAB MEDIA: Figure 3. *Video editor: Sequentially highlight the 90 DIV image and then the170 DIV image .*

5.5. At 200 days, elongated cell projections extended inward from the surface into the core and the organoid developed into three distinct retinal layers at 250 days **[1]**.

5.5.1. LAB MEDIA: Figure 3. *Video editor: Highlight the 200 DIV panel and 250 DIV panel. For 250 DIV, please show zoomed out image labeled "Stratified Neuroretina".*

5.6. Neuronal fibers extended from the organoid center toward the periphery across maturation, becoming thicker and more complex by 250 days *in vitro* **[1]**.

5.6.1. LAB MEDIA: Figure 4. *Video editor: Sequentially show the images from 40 DIV to 250 DIV (with the zoomed in top images)*

5.7. Cone photoreceptors expressing blue and green-red opsins appeared at late stages and showed elongated morphologies with bright tips **[1]**. Rod photoreceptors expressing rhodopsin were identified by their central nuclei and peripheral opsin distribution **[2]**.

5.7.1. LAB MEDIA: Figure 5. *Video editor: Highlight the top image row*

5.7.2. LAB MEDIA: Figure 5. *Video editor: Highlight the bottom image row*

5.8. Chx10 *(C-H-X-Ten)*-positive cells were initially distributed widely but later localized specifically to the inner nuclear layer during organoid maturation **[1]**. Endogenous GCaMP6s *(G-Camp-Six-s)* expression was preserved following BABB clearing, with GFP *(G-F-P)* signal detectable in the neuroretina after long-term fixation **[2]**.

5.8.1. LAB MEDIA: Figure 6. *Video editor: Please sequentially highlight the images from 40 DIV to 250 DIV (along with top zoomed in images)*

5.8.2. LAB MEDIA: Figure 7.

Pronunciation Guide:

1. Organoid

- **Pronunciation link**: https://www.merriam-webster.com/medical/organoid
- **IPA**: /ˈɔːr.gə.nɔɪd/
- **Phonetic Spelling**: OR-guh-noydmerriam-webster.com+7merriam-webster.com+7merriam-webster.com+7merriam-webster.commerriam-webster.com+1merriam-webster.com+1

---

2. Confocal

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/confocal
- **IPA**: /kənˈfoʊ.kəl/
- **Phonetic Spelling**: kuhn-FOH-kuhlmerriam-webster.com+1merriam-webster.com+1

---

3. Paraformaldehyde

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/paraformaldehyde
- **IPA**: /ˌpær.ə.fɔːrˈmæl.dəˌhaɪd/
- **Phonetic Spelling**: PAR-uh-for-MAL-duh-hidemerriam-webster.com+8merriam-webster.com+8merriam-webster.com+8

---

4. Antigen

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/antigen
- **IPA**: /ˈæn.tə.dʒən/
- **Phonetic Spelling**: AN-tuh-jenmerriam-webster.com+3merriam-webster.com+3merriam-webster.com+3merriam-webster.com+1merriam-webster.com+1

---

5. Triton

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/triton
- **IPA**: /ˈtraɪ.tən/
- **Phonetic Spelling**: TRY-tuhnmerriam-webster.com+4merriam-webster.com+4merriam-webster.com+4merriam-webster.com+2merriam-webster.com+2merriam-webster.com+2

---

6. BSA (Bovine Serum Albumin)

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/BSA
- **IPA**: /ˌbiː.esˈeɪ/
- **Phonetic Spelling**: BEE-ess-AYmerriam-webster.com+8merriam-webster.com+8merriam-webster.com+8

---

7. Retinal

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/retinal
- **IPA**: /ˈrɛt.n.əl/

- **Phonetic Spelling**: RET-nuhlmerriam-webster.com+39merriam-webster.com+39merriam-webster.com+39

---

8. Microscopy
- **Pronunciation link**: https://www.merriam-webster.com/dictionary/microscopy
- **IPA**: /maɪˈkrɒs.kə.pi/
- **Phonetic Spelling**: my-KROS-kuh-peemerriam-webster.com+1merriam-webster.com+1

---

9. Immunolabeling
- **Pronunciation link**: https://www.howtopronounce.com/immunolabeling
- **IPA**: /ɪˌmjuː.noʊˈleɪ.bəl.ɪŋ/
- **Phonetic Spelling**: ih-MYOO-noh-LAY-buh-ling

---

10. Alexa Fluor
- **Pronunciation link**: https://www.howtopronounce.com/alexa-fluor
- **IPA**: /əˈlɛk.sə ˈflʊər/
- **Phonetic Spelling**: uh-LEK-suh FLOOR

---

11. Benzyl Alcohol
- **Pronunciation link**: https://www.howtopronounce.com/benzyl-alcohol
- **IPA**: /ˈbɛn.zɪl ˈæl.kə.hɒl/
- **Phonetic Spelling**: BEN-zil AL-kuh-hol

---

12. Benzyl Benzoate
- **Pronunciation link**: https://www.howtopronounce.com/benzyl-benzoate
- **IPA**: /ˈbɛn.zɪl ˈbɛn.zoʊ.eɪt/
- **Phonetic Spelling**: BEN-zil BEN-zoh-ate

---

13. BABB (Benzyl Alcohol and Benzyl Benzoate)
- **Pronunciation link**: https://www.howtopronounce.com/babb
- **IPA**: /bæb/
- **Phonetic Spelling**: BAB

---

14. ImageJ
- **Pronunciation link**: https://www.howtopronounce.com/imagej
- **IPA**: /ˈɪ.mɪdʒ ˈdʒeɪ/
- **Phonetic Spelling**: IM-ij JAY

---

15. GCaMP6s
- **Pronunciation link**: https://www.howtopronounce.com/gcamp6s
- **IPA**: /ˈdʒiː kæmp sɪks ɛs/
- **Phonetic Spelling**: GEE-kamp-six-ess

---

16. Opsin

- **Pronunciation link**: https://www.howtopronounce.com/opsin
- **IPA**: /ˈɒp.sɪn/
- **Phonetic Spelling**: OP-sin

---

17. Rhodopsin

- **Pronunciation link**: https://www.howtopronounce.com/rhodopsin
- **IPA**: /roʊˈdɒp.sɪn/
- **Phonetic Spelling**: roh-DOP-sin

---

18. Chx10

- **Pronunciation link**: https://www.howtopronounce.com/chx10
- **IPA**: /siː eɪtʃ ɛks tɛn/
- **Phonetic Spelling**: C-H-X tenmerriam-webster.com+7merriam-webster.com+7merriam-webster.com+7

---

19. TUJ1

- **Pronunciation link**: https://www.howtopronounce.com/tuj1
- **IPA**: /ti juː dʒeɪ wʌn/
- **Phonetic Spelling**: T-U-J one