**Submission ID #: 68384**

**Scriptwriter Name: Sulakshana Karkala**

**Project Page Link:** [**https://review.jove.com/account/file-uploader?src=20857168**](https://review.jove.com/account/file-uploader?src=20857168)

**Title:** Whole-Mount Retinal Organoid Visualization with Cellular Resolution

**Authors and Affiliations:**

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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?

**Yes**

Ifyour protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

**Stemi 2000-C with a free camera port and Nikon stereo microscope with LV-TV camera adapter with the Digital Sight DS-Fi2.**

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

**Click here to list microscope shots, using the shot numbers from the protocol section of the video script.**

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

If **Yes**, we will need you to record using screen recording software.

We recommend using the screen capture program [OBS](https://obsproject.com/). JoVE’s tutorial for using OBS Studio is provided at this link: <https://review.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k>

As these files are necessary for finalizing your script, please upload all screen-captured video files to your project page as soon as possible: [**https://review.jove.com/account/file-uploader?src=20857168**](https://review.jove.com/account/file-uploader?src=20857168)

**3. Filming location:** Will the filming need to take place in multiple locations?  **Inside the same research institute, but in different rooms/laboratories**

If **Yes**, how far apart are the locations? One room apart

To ensure that your **script can be filmed in one day**, the protocol sections are cumulatively restricted to**55 shots** (shots are the 3-digit numbers like 2.1.1, 2.1.2…etc)

**Current Protocol Length**

Number of Steps: 20

Number of Shots: 45

# Introduction

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

Answers to these questions will become interview statements that you will deliver on camera.

* Answer the **1st REQUIRED** question and **at least 2 other questions (1.2 – 1.10)** below. Up to 5 interview statements will be included in the video.
* Enter the **full name** of the author who will deliver the statement.
* If possible, each author should deliver **no more than two statements**.
* Answer in full sentences, in a style suitable for being spoken aloud.
* Limit the length of each statement to **30 words or fewer**.
* Answers will be edited for length, clarity, and consistency with journal style guidelines.

**REQUIRED:** What is the scope of your research? What questions are you trying to answer?

* 1. Helena Isla: Our work focuses on developing and study retinal organoids—miniature retinas grown from stem cells—that serve as an *in vitro* human model to study how the human retina forms, how diseases affect, and how we might treat them.

What are the most recent developments in your field of research?

* 1. Enter author name**:** Click here to answer question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

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

* 1. 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.

What are the current experimental challenges?

* 1. Marina Cunquero**:** One of the current experimental challenges of whole-mount visualization is achieving uniform antibody penetration in large, dense tissues without surface-biased labeling. Another major challenge of whole-mount imaging of organoids is minimizing spherical aberrations that lead to focus loss during deep imaging

What significant findings have you established in your field?

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

What research gap are you addressing with your protocol?

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

What advantage does your protocol offer compared to other techniques?

* 1. Helena Isla-Magrané**:** Our method allows us to see how retinal cells are connected, what types of cells are present, and how they are organized in 3D. This level of detail is key to understanding the causes of retinal diseases.

How will your findings advance research in your field?

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

What new scientific questions have your results paved the way for?

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

What research questions will your laboratory focus on in the future?

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

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

**Testimonial Questions (OPTIONAL):**

***Videographer: Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.***

Answers to these questions **will not appear in the video** but may be featured in our journal's promotional materials.

* Enter the **full name** of the author who will deliver the statement. **Add your title** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) **as this will included in the promotional materials**.
* Answer in full sentences, in a style suitable for being spoken aloud.
* Answers will be mildly edited for clarity.
* Limit the length of each statement to **50 words or fewer**.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 50 or fewer words.

Can you share a specific success story or benefit you’ve experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

* 1. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 50 or fewer words.

# Protocol

**Please review this section to make sure that it accurately describes your protocol. Use Track Changes when making edits or revisions.**

* The two-digit **steps** (e.g., 2.1., 2.2.) are the narration. **JoVE is responsible for the narration of the protocol and results.**
* *Red italics* are pronunciation guides indicating how the word will be spoken.
* Filming should take no more than 10 minutes per step. If a step takes more than 10 minutes, prepare the product for that step in advance.
* The three-digit **shots** (e.g., 2.1.1., 2.2.2.) are the actions that the videographer will capture.

1. **Preparation of Custom-Made Fluorescent Secondary Antibodies**

**Demonstrator:** Maria Marsal

If the same person is the demonstrator throughout, mention them once here and remove the "Demonstrator" field from the other sections; if the demonstrator changes, retain the field in the respective sections.

* 1. To begin, obtain the fluorophore aliquots previously dissolved in anhydrous dimethyl sulfoxide (DMSO), dehydrated and frozen**[1].** Add 1 to 10 microliters of DMSO to an aliquot **[2]**.
     1. WIDE: Talent holding 0.02 mg aliquots of the fluorophore.
     2. Talent pipetting and dissolving the fluorophore aliquot into a small Eppendorf tube using dimethyl sulfoxide or otherwise ultrapure water for Alexa Fluor 405.
  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]**.
     1. Talent combining the reagents in a microcentrifuge tube.
     2. Talent placing it on a rocking platform covered with foil.
  3. While the reaction is progressing, remove the lids of the purification size exclusion columns **[1]** and allow the buffer to pass through **[2]**.
     1. Talent removing column lids.
     2. Shot of the buffer dripping through.
  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 the lids back to avoid them drying up and wait for the reaction to finish **[2]**.
     1. Talent using a pipette to add phosphate-buffered saline to the column for equilibration.
     2. Talent replacing the column lids and setting the columns aside.
  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]**.
     1. Talent adding phosphate-buffered saline to the reaction tube and vortexing.
     2. Talent carefully pipetting the solution into the centre of the column.
  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 **[2]**.
     1. Talent adding 550 µL of PBS.
     2. Talent adding 300 µL of PBS after elution stops.
     3. Talent collecting the elution in a microcentrifuge tube.
  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]**.
     1. Talent places the sample in a spectrophotometer.
     2. Talent placing labelled tubes in a refrigerator.

1. **Antibody Labeling and Confocal Imaging of Organoids**

**Demonstrator:** Marina Cunquero

* 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]**.
     1. Talent adding paraformaldehyde to a dish containing organoids.
     2. Talent adding antigen retrieval solution over the organoids.
     3. Talent placing the organoids in a shaking incubator set to specified conditions.
  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]**.
     1. Talent adding PBST to the organoids.
     2. Talent placing the dish with organoids on a shaker.
  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.
  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]**.
     1. Talent adding diluted primary antibody.
     2. Talent placing the sample in a shaker in the refrigerator.
     3. Talent adding washing solution to the organoids after removing the antibody solution.
  5. Now add the dilution solution secondary antibodies **[1]** and incubate at 4 degrees Celsius for 2 days with mild shaking **[2]**.
     1. Talent adding secondary antibody solution.
     2. Talent placing the dish with secondary antibodies in the refrigerator shaker.
  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].** 
     1. Talent adding fluorescent dyes to the dish.
     2. Talent placing the dish on a shaker.
     3. Talent performing wash.
  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]**. >> all thses shots need to be filmed inside the fume hood and using glass containers
     1. Talent preparing and labelling each propanol solution.
     2. Talent adjusting the pH of the solutions with trimethylamine.
  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]**.
     1. Talent adding 1-propanol solution to the sample. **TXT: Propanol concentrations: 15%, 30%, 45%, 60%, 75%, 90%**
     2. Talent placing the sample on a shaker and setting the temperature.
     3. Talent placing samples in 100 percent 1-propanol.
     4. Shot of the sample on the shaker.
  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]**.
     1. Talent preparing BABB solution.
     2. Talent placing samples in BABB solution and covering the container.
     3. Talent replacing the BABB solution in the sample container.

1. **Confocal Imaging of Organoids**

**Demonstrator:** Marina Cunquero

* 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]**.  
     Authors: Please create screen capture videos of the shots labeled as SCREEN, create a screenshot summary, and upload the files to your project page as soon as possible: [**https://review.jove.com/account/file-uploader?src=20857168**](https://review.jove.com/account/file-uploader?src=20857168)
     1. Talent transferring and adjusting the organoid position carefully in the dish.
     2. Talent positioning the dish containing the samples in the microscope stage while is modifying the imaging settings
     3. SCREEN: The sample is being scanned and images are being acquired.
  2. Recalibrate the step size of the Z-stack acquisition by accounting for the refractive index mismatch between clearing solution and immersion media **[1]**.
     1. SCREEN: The step size is being recalibrated.   
        **AND**  
        TEXT ON PLAIN BACKGROUND:

*Video Editor: Please play both shots side by side*

* 1. Now launch ImageJ *(Image-J)*, update the voxel depth by selecting **Image** and clicking on **Properties** **[1]**. Then create image projections of the Z-stack **[2]**.
     1. SCREEN: ImageJ is being launched and **Image | Properties** is being clicked
     2. SCREEN: Z-stack image projections is being created.
  2. 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]**.
     1. SCREEN: Image, Stacks and Orthogonal views are being clicked and the XY. XZ, YZ views are being seen.
     2. SCREEN: File and Save As is being used to save selected regions of interest.
     3. SCREEN: Show 3D rendering and animation process in the chosen software.

# Results

**Please review this section to make sure that it accurately reflects your findings.**

* This section **will not be recorded** by the videographer. It only includes the figures/tables from your manuscript (called LAB MEDIA).
* Use Track Changes when making edits or revisions. Ensure the voiceover length is below 200 words. Current word count: 210
* Please note that the video **cannot** include voiceover without an accompanying visual.

1. **Results   
   AUTHORS: Please confirm that the results have been accurately summarized. If you wish to add any specific results, please substitute the existing content since the results section is limited to 200 words**
   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]**.
      1. LAB MEDIA: Figure 1B. *Video editor: Please highlight the CLSM image*
      2. LAB MEDIA: Figure 1C. **TXT: Eci: Ethyl Cinnamate** *Video editor: Please highlight the CLSM image*
   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]**.
      1. LAB MEDIA: Figure 1D. *Video editor: Please highlight the CLSM image*
      2. LAB MEDIA: Figure 1C and D. *Video editor: Please highlight the BF images in both 1C and 1D*
   3. BABB-induced shrinkage increased sample compactness, enabling the use of high magnification objectives to image deeper structures **[1]**.
      1. LAB MEDIA: Figure 2. *Video editor: If possible, please show figure 2A first then zoom out to 2B*
   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]**.
      1. LAB MEDIA: Figure 3. *Video editor: Please highlight the 40 DIV panel on the left and show the “neuroretina” image*
      2. LAB MEDIA: Figure 3*. Video editor: Sequentially highlight the 90 DIV image and then the170 DIV image .*
   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]**.
      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”.*
   6. Neuronal fibers extended from the organoid center toward the periphery across maturation, becoming thicker and more complex by 250 days *in vitro* **[1]**.
      1. LAB MEDIA: Figure 4. *Video editor: Sequentially show the images from 40 DIV to 250 DIV (with the zoomed in top images)*
   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]**.
      1. LAB MEDIA: Figure 5. *Video editor: Highlight the top image row*
      2. LAB MEDIA: Figure 5. *Video editor: Highlight the bottom image row*
   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-Sixe-S)* expression was preserved following BABB clearing, with GFP *(G-F-P)* signal detectable in the neuroretina after long-term fixation **[2]**.
      1. LAB MEDIA: Figure 6. *Video editor: Please sequentially highlight the images from 40 DIV to 250 DIV (along with top zoomed in images)*
      2. LAB MEDIA: Figure 7.