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Title: ROS Live Cell Imaging During Neuronal Development

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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 MZ 9.5

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No but screen shots when taking images on the microscope.**

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: 20
Number of Shots: 44

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Aslihan Terzi:** Our protocol describes the use of a hydrogen peroxide biosensor in cultured zebrafish neurons and larvae. This method will help researchers to dissect the role of ROS in normal physiology and pathophysiology.

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

1.2. **Sabbir Alam:** The main advantage of this technique is the real-time detection of hydrogen peroxide in living animals and cultured cells.

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

OPTIONAL:

1.3. **Sabbir Alam:** This method can be applied to other species like rodent model systems.

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

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Purdue Institutional Animal Care and Use Committee (IACUC).

Protocol

2. Primary retinal ganglion cell culture derived from zebrafish embryos

2.1. To prepare the coverslips, use acid-cleaned coverslips stored in 100 percent ethanol ~~**[1-TXT]**~~. Use forceps to remove one coverslip from the storage container and flame it to remove residual ethanol **[2]**. Air dry the coverslip completely by placing it at an angle inside a 35-millimeter culture dish **[3]**.

2.1.1. ~~Talent acid-cleaning the coverslip and putting it in ethanol~~

2.1.2. Talent removing and flaming the coverslip using forceps. **TEXT: Coverslip: 22 x 22 mm square; 0.16-0.19 mm thickness**

2.1.3. Talent putting the coverslip at an angle in the culture dish

2.2. Prepare Poly-D-Lysine, or PDL, working solution **[1-TXT].** Apply PDL to the center of each coverslip, avoiding spreading of the solution to the edges, and incubate the PDL for 20 to 30 minutes at room temperature. Make sure the PDL does not dry out **[2-TXT]**. Wash the PDL with 0.5 milliliters of sterile water **[3]** and let the plates dry completely **[4]**.

2.2.1. Talent preparing the solution. **TEXT: Dilute 10x PDL stock (5 mg/mL) in sterile water**

2.2.2. Talent putting PDL at the center of the coverslip. **TEXT: 100 µL of 0.5 mg/mL; 20-30 min at RT**

2.2.3. Talent washing coverslip with sterile water

2.2.4. Talent letting the plates dry.

2.3. Prepare laminin working solution **[1-TXT]** and apply it to the center of each coverslip, making sure to avoid the spread of solution to the edges **[2-TXT]**. Incubate the plates at 37 degrees Celsius in a humidified incubator for 2 to 6 hours. Avoid drying of the laminin solution **[3]**.

2.3.1. Talent preparing the solution. **TEXT: Dilute 50x laminin stock (1 mg/mL) in 1x PBS**

2.3.2. Talent putting laminin at the center of coverslip. **TEXT: 100 µL of 20 µg/mL laminin in PBS**

2.3.3. Talent putting the coverslips in humidified incubator

2.4. To perform embryo dissection and plate retinal ganglion cells, or RGCs, prepare and label four 35-millimeter tissue culture dishes and fill them with 4 milliliters of 70 percent ethanol, E2 media 1, E2 media 2, and E2 media 3 on the day of dissection **[1]**. Keep the dishes in the fridge **[2]**.

2.4.1. Talent pouring 4 mL of solution in a dish.
Author NOTE: We added ethanol to 1 dish, and the same E2 media to other 3 dishes- so there were total of 4 dishes with 4 mL of indicated solutions. This step was in one shot.

2.4.2. Talent putting the dishes in fridge

2.5. When zebrafish embryos are 34 hours post fertilization, take the culture dishes coated with laminin out of the incubator **[1]** and wash the coverslips three times with 0.5 milliliters of 1x PBS **[2]**. After the final wash, add 4 milliliters of ZFCM plus media to each culture dish. Avoid drying the plate **[3-TXT]**.

2.5.1. WIDE: Talent taking the culture dishes out of the incubator.

2.5.2. Talent washing the coverslips

2.5.3. Talent adding media to the dishes. **TEXT: ZFCM (+): Zebrafish culture medium**

2.6. Retrieve the prepared refrigerated culture dishes and let them equilibrate to room temperature **[1]**. Fill 4 to 6 PCR tubes with 15 microliters of ZFCM plus media **[2]**.

2.6.1. Talent taking the dishes out of the refrigerator.

2.6.2. Talent putting media in PCR tubes

2.7. Retrieve zebrafish embryos from the incubator and immerse embryos in a 35-millimeter tissue culture dish containing 70 percent ethanol for 5 to 10 seconds to sterilize **[1]**.

2.7.1. Talent putting the zebrafish embryos in dish containing ethanol
NOTE: 2.7.1 and 2.8.1 were taken in the same shot as it was a continuous process.

2.8. Using a transfer pipette, transfer embryos to the E2 Media 1 dish containing sterile E2 media to wash off excess ethanol **[1]**. Then, transfer the embryos to the E2 Media 2 dish **[2]** and remove their chorions with sharp forceps **[2]**. Finally, transfer embryos to the E2 Media 3 dish to perform dissections **[3]**.

2.8.1. Talent transferring the embryos to dish 1.

2.8.2 Added step: Talent transferring the embryos in dish 2.

NOTE: 2.7.1, 2.8.1, 2.8.2 were taken in the same shot as it was a continuous process.

2.8.2. Talent removing the chorion

2.8.3. Talent transferring the embryos to dish 3.

2.9. Using a pair of fine forceps, position and hold embryos anterior to their yolk with one of the forceps and remove the tail posterior to the yolk sac with the other forceps **[1]**. Grab the neck with forceps and take off the head to expose the brain and eyes to the E2 media **[2]**.

2.9.1. SCOPE: Talent removing the tail

2.9.2. SCOPE: Talent grabbing the neck and exposing the brain and eye to the media *Videographer: This step is difficult and iimportant!*

NOTE: 2.9.1, 2.9.2, 2.10.1 were taken in the same shot as it was a continuous process.

2.10. With the tip of fine forceps, gently roll the eyes from the head while holding the cranial tissue down with the second forceps **[1-TXT]**. Transfer four eyes to one of the previously prepared tubes containing ZFCM plus **[2]**.

2.10.1. SCOPE: Talent rolling the eyes off the head **TEXT: Avoid cutting the yolk sac** *Videographer: This step is difficult and important!*

NOTE: 2.9.1, 2.9.2, 2.10.1 were taken in the same shot as it was a continuous process.

2.10.2. Talent transferring the eyes to the media containing tubes

2.11. Gently triturate up and down with the P20 pipette about 45 times to dissociate cells **[1]**. Transfer the ZFCM plus with the dissociated cells to the center of the coverslips **[2].** Maintain cultures on the benchtop at 22 degrees Celsius on a polystyrene foam rack to absorb vibrations **[3]**.

2.11.1. Talent dissociating the cells. **TEXT: Avoid any air bubbles** *Videographer: This step is important!*

2.11.2. Talent putting the dissociated cells on coverslips

NOTE: 2.11.1 and 2.11.2 were taken in the same shot as it was a continuous process.

2.11.3. Shot of culture dish on a polystyrene foam rack

3. In vitro ROS imaging of cultured RGC neurons

3.1. On the day of imaging, check cells under the microscope to validate growth of RGC axons **[1]**. For live-cell imaging, transfer the coverslips from the culture dish to a live cell imaging chamber **[2]**.

3.1.1. Talent checking cells under the microscope.

3.1.2. Talent transferring coverslip to a live cell imaging chamber

3.2. Use an inverted microscope equipped with a DIC objective, OG590 long-pass red filter, and an EM-CCD camera **[1]**. Before imaging, replace the ZFCM plus medium with ZFCM minus **[2]**. After positioning the cells with the 10x objective, acquire images at 60x magnification using an oil immersion objective. Use an additional 1.5x magnification **[3]**.

3.2.1. Talent setting the microscope.

3.2.2. Talent replacing the medium

3.2.3. Talent positioning the cells, then changing the objective. *Videographer: This step is important!*

3.3. First, acquire DIC images **[1]**, then image roGFP2-Orp1 (Pronounce: "ro G F P two orp one") using an appropriate filter set **[2-TXT]**.

3.3.1. LAB MEDIA: Example DIC image of RGC neuron at 90x

3.3.2. Talent setting the filter range and acquiring the image **TEXT: 405/20 and 480/30 nm excitation and 535/30 nm emission** *Videographer: This step is important!*

Author NOTE: We could not take real-time image acquisition due to the experimental failure. We used previous images to look like acquiring real-time. Please edit video accordingly to obscure any cursor movements- if they are visible.

3.4. After taking the first set of images, exchange media with media containing different treatment solutions. Media should be changed every 30 minutes of imaging to avoid pH and osmolarity changes **[1-TXT]**

3.4.1. Talent exchanging the media.

4. In vivo ROS imaging of developing embryos

4.1. For in vivo imaging, keep 22 to 24-hour post fertilization embryos in E3 media containing 0.003 percent Phenylthiourea without methylene blue **[1]**. Exchange media and remove dead embryos daily **[2]**.

4.1.1. Embryos in media.

4.1.2. Talent removing the dead embryos

4.2. At the desired age, anesthetize and mount embryos in 1 percent agarose on 35-millimeter glass bottom culture dishes. Embryos can be oriented dorsally, ventrally, or laterally, depending on the region of interest for imaging. **[1-TXT]**.

4.2.1. Talent mounting the embryos *Videographer: This step is important!*

4.3. After the agarose solidifies, fill the dishes with E3 media without methylene blue or 0.016 percent tricaine **[1]**. Set up the microscope for imaging. Use an inverted laser scanning confocal microscope to image embryos mounted on top of an agarose drop. **[2-TEXT].**

4.3.1. Talent transferring media to solidified agarose containing dish *Videographer: This step is important!*

4.3.2. Talent setting the microscope.

4.4. Excite roGFP2-Orp1 and acquire corresponding images with the desired emission filters **[1-TXT]**. Acquire z-stacks with 5-micrometer section thickness through the desired part of the embryos **[2]**.

4.4.1. Talent taking the images through microscope **TEXT: 405/20 and 480/30 nm excitation and 535/30 nm emission**

4.4.2. Talent Z-stacking the images in the region of interest

Author NOTE: We could not take real-time image acquisition due to the experimental failure. We used previous images to look like acquiring real-time. Please edit video accordingly to obscure any cursor movements- if they are visible.

4.5. After imaging, remove embryos from agarose with fine forceps **[1]** and keep them in the incubator in methylene blue-free media with Phenylthiourea until the desired age **[2]**.

4.5.1. Talent removing the embryos from agarose

4.5.2. Talent keeping embryos in the incubator

Results

5. Results: H_2O_2 imaging in cultured and in vivo zebrafish RGCs with roGFP2-Orp1.

5.1. A representative image of the hydrogen peroxide -biosensor in cultured zebrafish RGC extend axons is shown here **[1]**. The cell body, axon, and growth cones are clearly visible in individual neurons. The addition of hydrogen peroxide to the culture media increases the ratio values, showing that real-time changes can be detected with this system **[2].** Quantification of hydrogen peroxide levels is shown in panel B **[3].**

5.1.1. LAB MEDIA: Figure 4 *Video editor: Emphasize Figure 4A*

5.1.2. LAB MEDIA: Figure 4A *Video editor: Emphasize the right panel image in Figure 4A*

5.1.3. LAB MEDIA: Figure 4B *Video editor: Emphasize Figure 4B*

5.2. To determine hydrogen peroxide levels in whole zebrafish embryos, the mRNA was injected during the one-cell stage, causing all tissues to express the roGFP2-Orp1 biosensor **[1]**. The head region of zebrafish larvae is shown at two different time points, focusing on the hydrogen peroxide levels in the retina **[2]**.

5.2.1. LAB MEDIA: Figure 5

5.2.2. LAB MEDIA: Figure 5A *Video editor: Emphasize the highlighted regions of Figure 5A*

5.3. The basal levels of hydrogen peroxide in the zebrafish embryos at 2 days post fertilization and 5 days post fertilization were measured **[1]**. At 2 days post fertilization, the ratio values were significantly lower than at 5 days post fertilization **[2]**.

5.3.1. LAB MEDIA: Figure 5B and Figure 5C

5.3.2. LAB MEDIA: Figure 5B

5.4. Furthermore, each animal showed a different level of increase in their retinal hydrogen peroxide content **[1]**.

5.4.1. LAB MEDIA: Figure 5C

Conclusion

6. Conclusion Interview Statements

6.1. **Aslihan Terzi:** Using fine forceps for removing the eyes and gentle dissociation of the eyes with the pipet are the most critical steps in this procedure.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.10.1 and 2.11.1.*

6.2. **Sabbir Alam:** This protocol can be followed by drug treatments to investigate the factors involved in ROS signaling.

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