Submission ID #: 60966
Scriptwriter Name: Bridget Colvin
Project Page Link: http://www.jove.com/files_upload.php?src=18594678

Title: Confocal Microscope-Based Laser Ablation and Regeneration Assay in Zebrafish Interneuromast Cells

Authors and Affiliations: Bryan A. Volpe[1], Teresa H. Fotino[1], and Aaron B. Steiner[1]

[1]Department of Biology, Pace University

Corresponding Author:
Aaron B. Steiner
asteiner@pace.edu

Co-authors:
bv98841p@pace.edu
tfotino@frontier.com

Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **No**, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars: **Motic SXZ-168-BL Stereomicroscope**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: Screen capture files provided, do not film

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Script Length
Number of Shots: **55**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Aaron Steiner**: Using this protocol, any lab that has a standard confocal microscope equipped with a 405-nanometer laser can perform hair-cell progenitor laser ablations and monitor their regeneration **[1]**.

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

REQUIRED:

1.2. **Bryan Volpe**: Unlike electroablation, this technique limits the damage to surrounding cells and is more accessible than a powerful pulsed UV laser setup. Confocal imaging can also be performed immediately before and after the ablation **[1]**.

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

OPTIONAL:

1.3. **Bryan Volpe**: The technique allows us to better understand the regenerative behavior of sensory progenitors, which may help in the development of therapies for human hearing loss **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

OPTIONAL:

1.4 **Aaron Steiner**: This protocol may be adapted to the ablation of different cell types in zebrafish larvae to facilitate the study of regeneration in other tissues **[1]**.

1.4.1 INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Larva Mounting

2.1. For mounting, first pipette 3-4 anesthetized larvae into a small droplet of E3-tricaine solution **[1-TXT]** into the center of a 35-millimeter dish with a 14-millimeter number 1.5 coverslip bottom **[2]**.

2.1.1. WIDE: Talent loading larvae into pipette, with solution container visible in frame **TEXT: See text for all solution preparation details**

2.1.2. Larvae being added to dish

2.2. Remove the excess solution so that the larvae remain in a small droplet just large enough to contain them **[1]** and place the dish on the stage of a binocular stereo microscope **[2]**.

2.2.1. Solution being removed

2.2.2. Talent placing dish onto microscope

2.3. Manipulate the zoom and focus so that all of the larvae are in the field of view **[1]** and use a transfer pipette to add thin layer of agarose solution onto the coverslip **[2]**.

2.3.1. SCOPE: Larvae coming into view

2.3.2. SCOPE: Agarose being added to coverslip

2.4. Remove the excess agarose until the liquid just fills the well at the bottom of the dish, taking care not to aspirate any larvae **[1]**, and use a hair knife to quickly orient the larvae in the agarose solution with the rostral side facing left **[2]**.

2.4.1. SCOPE: Agarose being removed *Videographer: Important step*
2.4.2. SCOPE: Larvae being arranged *Videographer: Important step*

2.5. Gently press the larvae down against the glass, such that the larvae lie in profile with their right sides facing down **[1-TXT]**.

2.5.1. SCOPE: Larva being pressed *Videographer: Important step* **TEXT: Repeatedly press floating >3 dpf larvae as necessary**

2.6. After about 60 seconds, the agarose will start to solidify and the larvae will not be able to be reoriented **[1]**.

2.6.1. Shot of agarose after 60 seconds

2.7. After 5 minutes, use a transfer pipette to fill the dish halfway with E3 supplemented 1x tricaine **[1]**.

2.7.1. Shot of agarose after 5 minutes, then solution being added to dish

3. Prospective Target Location

3.1. To locate prospective targets, turn on the power to the laser-scanning confocal microscopy system **[1]** and initialize the laser through the integrated imaging software **[2]**.

3.1.1. WIDE: Talent turning on microscope
3.1.2. Talent initializing laser, with monitor visible in frame

3.2. Select the 63x Plan-Apochromat oil immersion objective **[1]** and apply immersion oil to the lens **[2]**.

3.2.1. Talent selecting objective
3.2.2. Talent applying oil

3.3. Secure the dish in a circular stage insert with the rostral aspect of the larvae facing to the left **[1]**.

3.3.1. Talent securing dish in place

3.4. Using bright-field or differential interference contrast illumination, select one of the mounted larvae for imaging **[1]** and use the focus knob to bring the skin on the side of the fish closest to the coverslip into focus **[2]**.

3.4.1. Talent selecting brightfield or DIC
3.4.2. SCOPE: Fish coming into focus *Videographer: Difficult step*

3.5. Switch to epifluorescent illumination in the GFP channel **[1]** and locate the posterior lateral line by GFP expression along the horizontal myoseptum **[2]**.

3.5.1. Talent switching to GFP, with monitor visible in frame
3.5.2. SCOPE: Lateral line being located *Videographer: Difficult step*

3.6. Rings of fluorescent cells are indicative of the neuromast mantle cells **[1]** and elongated strands of cells are the interneuromast cells **[2]**.

3.6.1. LAB MEDIA: Figure 1B *JoVE Video Editor: please emphasize green rounded cell sections*
3.6.2. LAB MEDIA: Figure 1B *JoVE Video Editor: please emphasize elongated cell sections*

3.7. Beginning with the first migrating primordium neuromast, use the stage control joystick to visually scan caudally along the horizontal myoseptum, following the string of interneuromast cells until the region between the third and fourth migrating primordium neuromasts is reached **[1-TXT]**.

3.7.1. Talent using joystick to scan myoseptum, with monitor visible in frame **TEXT: Prim1L3-Prim1L4 most amenable to ablation**

3.8. If several larvae are to be imaged, select **Position** to set the first stage position **[1-TXT]**.

3.8.1. SCREEN: screenshot_1: 00:50-00:55 **TEXT: Repeat for each larva**

4. Pre-Ablation Imaging

Vid NOTE: For sections 4,5,6 all screen captures were filmed in one continuous take and I had the talent describe what they were doing while showcasing the software

4.1. After cell bodies in the L3-L4 region have been identified, switch to the acquisition mode **[1]** and use an appropriate laser to activate the GFP imaging track **[2]**.

4.1.1. WIDE: Talent switching to acquisition mode
4.1.2. SCREEN: screenshot_1: 01:12-01:20

4.2. To add a transmitted light channel to the activated laser track, click the T-PMT (T-P-M-T) box in the **Imaging Setup** dropdown menu **[1]**.

4.2.1. SCREEN: screenshot_1: 01:21-01:25

4.3. To image ET20 (E-T-twenty) larvae, select the 488-nanometer laser, set the **laser power** to 6%, the **pinhole size** to 1 Airy unit equivalent, and the **digital gain** to 750 **[1]**.

4.3.1. SCREEN: screenshot_1: 01:28-01:48 *Videographer: please speed up*

4.4. Adjust the gain such that cell bodies are saturated to capture otherwise dim projections and filipodia and set the **frame size** to 1024- x 1024-pixels, the **averaging** to 2, and the **digital zoom** to 0.7 **[1]**.

4.4.1. SCREEN: screenshot_1: 01:55-02:20 *Videographer: please speed up*

4.5. Check the z-stack box to bring up the z-position dropdown menu **[1]**.

4.5.1. SCREEN: screenshot_1: 02:25-02:28

4.6. While fast scanning, focus out until the interneuromast cells are just out of focus and set the first slice **[1]**.

4.6.1. SCREEN: screenshot_1: 02:39-03:23 *Videographer: please speed up*

4.7. Focus through the sample until the interneuromast cells are once again out of focus and set the last slice **[1-TXT]**.

4.7.1. SCREEN: screenshot_1: 03:23-03:37 *Videographer: can speed up* **TEXT: Ensure stack encompasses approximately 25 micrometers of tissue**

4.8. Then click **Stop** and click **Start experiment** to capture a pre-ablation z-stack **[1]**.

4.8.1. SCREEN: screenshot_1: 03:38-03:45

4.9. If the stage positions have been added, inactivate the positions option so that only the current position is imaged and save the file once it has been captured **[1]**.

4.9.1. SCREEN: screenshot_1: 07:04-07:13

5. Laser Ablation

5.1. For laser ablation of the targeted cell bodies, click **Show all Tools** in the acquisition interface **[1]** and, in the **Imaging Setup** menu, select **Add a new track [2]**.

5.1.1. WIDE: Talent clicking show all tool, with monitor visible in frame
5.1.2. SCREEN: screenshot_2: 00:09-00:16

5.2. Click **Dye** and select DAPI **[1]**.

5.2.1. SCREEN: screenshot_2: 00:17-00:24

5.3. Under **Channels**, select 405 for the **Laser setting** and increase the laser power to 75% **[1]**.

5.3.1. SCREEN: screenshot_2: 00:29-00:41

5.4. Unclick the DAPI channel to turn off the laser while scanning for candidate cell bodies for ablation **[1]**.

5.4.1. SCREEN: screenshot_2: 00:45-00:48

5.5. Click **Live** and, with the body of an interneuromast cell centered in the field of view **[1]**, zoom into the scanning frame to 20-22x **[2-TXT]**.

5.5.1. SCREEN: screenshot_2: 00:51-00:55
5.5.2. SCREEN: screenshot_2: 01:28-02:10 **TEXT: Adjust frame position as necessary to keep cell body in center**

5.6. Stop the live scanning as soon as the cell body fills the field of view **[1]**.

5.6.1. SCREEN: screenshot_2: 02:11-02:15

5.7. Check the 405-nanometer laser shutter box to activate the track **[1]** and set a timer for 45 seconds **[2]**.

5.7.1. SCREEN: screenshot_2: 02:16-02:20
5.7.2. Talent setting timer

5.8. Then activate continuous scanning and start the timer, immediately stopping the scanning at 45 seconds **[1]**.

5.8.1. SCREEN: screenshot_2: screenshot_2: 02:22-03:10 *Videographer: please speed up*

6. Post-Ablation Imaging and Regeneration Time-Lapse Microscopy

6.1. For imaging of the cell bodies post-ablation, under the **Channels** menu **[1]**, unclick the **DAPI** track to inactivate the ablation laser and open the **Acquisition mode** menu **[2]**.

6.1.1. WIDE: Talent opening Channels menu, with monitor visible in frame
6.1.2. SCREEN: screenshot_3: 00:11-00:18

6.2. Click **Zoom** and decrease the zoom to 0.7 **[1]**.

6.2.1. SCREEN: screenshot_3: 00:15-00:26

6.3. To assess the success of the cell ablation, fast scan the field of view **[1]**.

6.3.1. SCREEN: screenshot_3: 00:27-00:53 *Videographer: please speed up*

6.4. Using the same settings as those for the pre-ablation imaging, capture and save a post-ablation image **[1-TXT]**.

6.4.1. LAB MEDIA: Figure 1C **TEXT: Repeat ablation if fluorescent cell remnants or cell bodies are observed**

6.5. Inspect the transmitted light photomultiplier tube channel image to further confirm the cellular damage **[1]**.

6.5.1. SCREEN: screenshot_4: 00:16-00:22

6.6. Damaged cells will demonstrate a granular appearance and the nuclei will frequently swell or appear irregular in shape **[1]**.

6.6.1. LAB MEDIA: Figure 2

6.7. To assess the post-ablation cell body recovery, activate both the stage position and time options for time-lapse image capture **[1]** and set the time parameters to the appropriate experimental time point and 15-minute intervals **[2]**.

6.7.1. Talent activating stage position and/or time options
6.7.2. SCREEN: screenshot_4: 01:00-01:24 *Videographer: please speed up*

6.8. The start the experiment to acquire images and save the resulting file when complete **[1]**.

6.8.1. LAB MEDIA: Movie 1

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.4., 2.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.5.-3.7., 6.3.-6.5.

Results

7. Results: Representative Interneuromast Cell (INMC) Ablation Imaging and Gap Measurement

7.1. In this representative experiment, the region of the lateral line located between the third and fourth migrating primordium neuromasts was identified **[1]** and pre-ablation images were captured **[2]**.

7.1.1. LAB MEDIA: Fig-1A *oVE Video Editor: please emphasize region in schematic that is outlined with small rectangle in original Figure 1A*

7.1.2. LAB MEDIA: Figure 1B

7.2. Post-ablation scanning confirmed that no cell bodies remained in the ablated region **[1]**, leaving a gap between the elongated projections of the adjacent interneuromast cells **[2]**.

7.2.1. LAB MEDIA: Figure 1C

7.2.2. LAB MEDIA: Figure 1C *JoVE Video Editor: please emphasize black gaps in center of image*

7.3. Analysis of the transmitted light photomultiplier tube channel after ablation **[1]** reveals damaged and dying cells marked by swollen and irregularly shaped nuclei and a granular appearance **[2]**.

7.3.1. LAB MEDIA: Fig-2

7.3.2. LAB MEDIA: Fig-2 *JoVE Video Editor: please add outline and/or emphasize cells within dotted outline as in original Figure 2 T-PMT image*

7.4. The recruitment of large amoeboid cells that are likely macrophages may also be observed **[1]**.

7.4.1. LAB MEDIA: Fig-2 *JoVE Video Editor: please add asterisk and/or emphasize cell indicated by asterisk in original Figure 2 T-PMT image*

7.5. In this experiment, the ablation of several cells in double-transgenic larvae **[1]** created sizeable gaps in the interneuromast cell string **[2]** but had little or no effect on the lateral line nerve **[3]**.

7.5.1. LAB MEDIA: Figure 3

7.5.2. LAB MEDIA: Figure 3 *JoVE Video Editor: please emphasize gap in green signal in bottom right image*

7.5.3. LAB MEDIA: Figure 3 *JoVE Video Editor: please emphasize solid red line across center of bottom right image*

7.6. Following laser ablation, gap sizes can be measured **[1]**, ranging from just a few microns **[2]** up to 100 microns, depending upon the width of the individual interneuromast cells and how many cells are selected for ablation **[3]**.

7.6.1. LAB MEDIA: Figure 5

7.6.2. LAB MEDIA: Figure 5 *JoVE Video Editor: please emphasize black dots from about 0-40 micrometers*

7.6.3. LAB MEDIA: Figure 5 *JoVE Video Editor: please emphasize black dots from about 80-100 micrometers*

7.7. After ablation, some interneuromast cells recover within the first several hours of imaging **[1]**, with the probability of gap closure positively correlating with the gap size **[2]**.

7.7.1. LAB MEDIA: Movie 1

7.7.2. LAB MEDIA: Figure 5 *JoVE Video Editor: please emphasize blue data line*

7.8. Even in interneuromast cells that are unable to recover, however, the formation of long projections from neighboring interneuromast cells, which can resemble extending neuronal growth cones, can be observed **[1]**.

7.8.1. LAB MEDIA: Movie 2 *JoVE Video Editor: please emphasize ends of green signal that reach out into but do not close gap*

Conclusion

8. Conclusion Interview Statements

8.1. **Bryan Volpe**: It's important to thoroughly inspect the T-PMT channel for damaged cells exhibiting irregularly shaped nuclei and granularity and to allow sufficient time for these indicators of cell death to become visible **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (6.5., 6.6.)

8.2. **Aaron Steiner**: Further analysis of the resulting time-lapse microscopy data can potentially reveal novel cellular behaviors induced by laser ablation and may guide the development of experiments for identifying regulators of regeneration **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

8.3. **Bryan Volpe**: This technique has enabled us to study the molecular regulators of interneuromast cell regeneration by providing a rapid and cost-effective method for selectively damaging these cells **[1]**.

8.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*