Confocal imaging of live larval zebrafish for assessing peripheral neuropathy

Version: 1
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Summary:

This protocol describes a method to mount live 8 day-old zebrafish larvae in agarose for capturing fluorescent transgene-expressing peripheral somatosensory axons with confocal imaging. We have previously described a similar method in the Journal of Visualized Experiments (*1*).

Reagents and Materials:

Reagent/Material	Required concentration/ volume	Vendor	Order Number
Tricaine (3-amino benzoic acid ethyl ester) pH 7.0	0.4%	Sigma-Aldrich	A-5040
Glass bottom culture dish as imaging chamber		MatTek Corporation, Ashland, USA	P35GCol-1.5-14-C
Dow Corning High Vacuum Grease	1 ml	Dow Corning, USA	
Agarose, Low gelling temperature	1.2 %	Sigma-Aldrich, USA	A9414
Sea salt	0.3 g/L	Instant Ocean (Pet store)	
phenyl-2-thiourea (PTU)	0.003 %	Sigma-Aldrich	P7629
CREST3:Gal4VP16_14xUAS-GFPand isl2b:GFP transgenic zebrafish		Our lab	
Fluorescent stereomicroscope		Discovery II, Zeiss Germany	
Fluoview 1000 inverted confocal microscope		Olympus, USA	

Reagent Preparation:

Reagent 1: Embryo medium
Preparation: Add 0.03 % Instant Ocean salt (Pet store) into double distilled water.

Reagent 2: Embryo medium with 1-phenyl-2-thiourea (PTU) to prevent pigment formation.
Preparation: To embryo medium in a small beaker add a final of 0.003% PTU and dissolve overnight using a magnetic stir bar. Keep PTU solution in the dark.

Reagent 3: Tricaine (according to the Zebrafish Book Recipes)
Preparation: Tricaine (3-amino benzoic acid ethyl ester also called ethyl 3-aminobenzoate) comes in a powdered form from Sigma (A-5040). First, make a tricaine stock solution by combining the following in a glass bottle with a screw cap:

400 mg tricaine powder
97.9 ml DD water
~2.1 ml 1 M Tris (pH 9). Adjust pH to ~7. Store this solution at 4ºC until use. To make a tricaine working solution for anesthesia, combine the following:

4.2 ml tricaine stock solution
~100 ml embryo medium containing 0.003 % PTU. Use immediately and keep unused solution refrigerated.

Reagent 4: Low-melt agarose for mounting of larval zebrafish.
Preparation: Mix 1.2 % of low-melt agarose (0.6 g) with 50 ml of embryo medium (no PTU) and bring to a boil in the microwave (about 1 minute). Boil until agarose is dissolved completely and add 1 ml into 42ºC pre-warmed 1.5 ml tubes. Place tubes into 42ºC heat block until use.

Protocol:

1. Mounting of larvae for imaging

Before mounting, select fluorescent larvae under a fluorescent stereomicroscope. For this, add a few drops of tricaine stock to the dish in which the larvae are raised and wait until larvae are anesthetized. Assess anesthesia by touching larvae with a pipette tip. If no response is observed, transfer fluorescent larvae into small petri dish containing the tricaine working solution. With a glass Pasteur pipette, transfer anesthetized larvae (one at a time) from the petri dish into a 1.5 ml tube containing 1.2 % of the low-melt agarose (keep at 42ºC in a heat block prior to use to maintain as a liquid). Transfer a drop of the agarose containing the larva into an imaging chamber. Immediately position the larva on its side for imaging from the lateral view and on its back to image from the dorsal view. Immobilized embryos are then immersed in tricaine working solution and the chamber is covered with a lid (if using the glass bottom dish) or sealed with a frosted slide following the application of silicon grease to the top of the Teflon ring (when using the Teflon chamber apparatus). Do not leave air bubbles in the imaging chamber after it is sealed, as they will interfere with imaging in the bright field mode.

2. Confocal imaging

Following positioning of the mounted larvae on the inverted microscope, utilize a PlanNeofluar 20x/0.50 (Zeiss) air objective and a 1.6x digital zoom to capture the GFP-expressing axons with a 473 nm diode laser. To record stacks, capture 3 µm sections according to the owner's manual. Project each stack into a single image. The projected images can be exported as tiff files and processed in Adobe Photoshop for publication. Alternatively, load stacks into Imaris imaging software and process with Filament tracer module to quantify features of axon degeneration such as axon debris and axon density.

Potential Pitfalls:

1. Larvae die during the mounting or imaging procedure: a) If larvae die, the agarose may be too warm, which is typically the case if the agarose is prepared immediately prior to mounting of the larvae. In this case, let agarose cool down for sufficient amount of time (at least 30 minutes). b) The tricaine concentration may be too high, which negatively affects larval health. Also, re-test the pH of the tricaine, which should be pH 7. c) Larvae are unhealthy due to growth in the medium or lack of food. The larvae typically need to be fed with larval diet after 5 dpf due to the depletion of the yolk.

2. Images on the confocal microscope are blurry. This could be due to a dirty objective or excessive opening of the pinhole.

References:

1. G. S. O'Brien, S. Rieger, S. M. Martin, A. M. Cavanaugh, C. Portera-Cailliau, A. Sagasti, Two-photon axotomy and time-lapse confocal imaging in live zebrafish embryos. *J Vis Exp*, (2009).
2. S. Rieger, A. Sagasti, Hydrogen peroxide promotes injury-induced peripheral sensory axon regeneration in the zebrafish skin. *PLoS Biol* **9**, e1000621 (2011); published online EpubMay (10-PLBI-RA-9260R3 [pii] 10.1371/journal.pbio.1000621).
3. A. J. Pittman, M. Y. Law, C. B. Chien, Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points. D*evelopment* 1**35,** 2865-2871 (2008); published online EpubSep