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# Confocal imaging of live larval zebrafish for assessing peripheral neuropathy V.2

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We use this protocol and it's working

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## Abstract

### 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).

### Diabetic Complication:



Neuropathy

### References:

1. G. S. O'Brien, S. Rieger, S. M. Martin, A. M. Cavanaugh, C. Portera-Cailliau, A. Sagasti, Twophoton 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. Development 135, 2865-2871 (2008); published online EpubSep

## Materials

### MATERIALS

☒ Tricaine (3-amino benzoic acid ethyl ester) pH 7.0 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A-5040**

☒ Glass bottom culture dish as imaging chamber **MatTek Corporation Catalog #P35GCol-1.5-14-C**

☒ Dow Corning High Vacuum Grease **Dow Corning**

☒ Agarose Low gelling temperature **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9414**

☒ Sea salt **Instant Ocean (Pet store)**

☒ phenyl-2-thiourea (PTU) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P7629**

☒ CREST3:Gal4VP16\_14xUASGFPand isl2b:GFP transgenic zebrafish **Catalog #lab**

☒ Fluorescent stereomicroscope **Zeiss**

☒ Fluoview 1000 inverted confocal microscope **Olympus Catalog #Fluoview 1000**

### Reagent: Required Concentration / volume

Reagent/Material	Required concentration/ volume
Tricaine (3-amino benzoic acid ethyl ester) pH 7.0	0.4%
Dow Corning High Vacuum Grease	1 ml
Agarose, Low gelling temperature	1.2 %
Sea salt	0.3 g/L
phenyl-2-thiourea (PTU)	0.003 %

### 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 4°C 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 42°C pre-warmed 1.5 ml tubes. Place tubes into 42°C heat block until use.

**Note:**

**Sigma-Aldrich ([RRID:SCR\\_008988](#))**

## 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 42°C 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.

## 3 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.