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***In Vivo* Time-Lapse Imaging in the Zebrafish Lateral Line: A Flexible, Open-Ended Research Project for an Undergraduate Neurobiology Laboratory Course**Molly H. Marra¹, Zachary J.C. Tobias², Hannah R. Cohen², Greta Glover³, and Tamily A. Weissman²¹*St. Mary's Academy, Portland, OR 97201*; ²*Biology Department, Lewis & Clark College, Portland, OR 97219*; ³*Biology Department, Reed College, Portland, OR 97202*.

The lateral line sensory system in fish detects movements in the water and allows fish to respond to predators, prey, and other stimuli. As the lateral line forms in the first two days of zebrafish development, axons extend caudally along the lateral surface of the fish, eventually forming synapses with hair cells of neuromasts. Growing lateral line axons are located superficially under the skin and can be labeled in living zebrafish using fluorescent protein expression. This system provides a relatively straightforward approach for *in vivo* time-lapse imaging of neuronal development in an undergraduate setting.

Here we describe an upper-level neurobiology laboratory module in which students investigate aspects of axonal development in the zebrafish lateral line system. Students learn to handle and image living fish, collect time-lapse videos of moving mitochondria, and quantitatively measure mitochondrial dynamics by generating and analyzing kymographs of their movements. Energy demands may differ between axons with extending growth cones versus axons that have already reached their targets and are forming synapses. Since relatively little is known

about this process in developing lateral line axons, students generate and test their own hypotheses regarding how mitochondrial dynamics may differ at two different time points in axonal development. Students also learn to incorporate into their analysis a powerful yet accessible quantitative tool, the kymograph, which is used to graph movement over time. After students measure and quantify dynamics in living fish at 1 and 2 days post fertilization, this module extends into independent projects, in which students can expand their studies in a number of different, inquiry-driven directions. The project can also be pared down for courses that wish to focus solely on the quantitative analysis (without fish handling), or vice versa. This research module provides a useful approach for the design of open-ended laboratory research projects that integrate the scientific process into undergraduate Biology courses, as encouraged by the AAAS and NSF Vision and Change Initiative.

Key words: zebrafish; lateral line; mitochondria; time-lapse imaging; in vivo; fluorescence; axon development; growth cone

The lateral line is a mechanosensory organ system in aquatic vertebrates that detects movement and vibration in the water. Crucial for the animal's survival, this sensory system conveys information needed for proper schooling behavior and for locating predators and prey. The lateral line is composed of small clusters of hair cells and support cells, together called neuromasts, which are dotted along the lateral portion of the head and trunk of the fish (Figure 1). The hair cells within each neuromast use stereocilia to convert mechanical signals from the water into electrical potentials. Neurotransmitter is then released via synapses at the base of the hair cell onto peripheral axons that relay information into the central nervous system (reviewed in Dambly-Chaudiere et al., 2003; Harding et al., 2013).

The lateral line system provides an ideal platform for studying mechanisms of axonal development *in vivo*. The axons that innervate hair cells are long, projecting from the lateral line ganglion behind the ear along the length of the fish, and undergo a classic pattern of growth and maturation (Chitnis et al., 2012) that can be followed over a relatively short period of time. Further, the axons can be visualized clearly due to their superficial location along the side of the fish (Dambly-Chaudiere et al., 2003).

Here we focus on axonal development in the lateral line system in zebrafish, an ideal model system for an

undergraduate research project, in part due to the small size of zebrafish, low cost, and transparency during early development (Nüsslein-Volhard and Dahm, 2002). The initial phase of posterior lateral line development in zebrafish occurs between 20-48 hours post fertilization (hpf) (Metcalf, 1985). At 20 hpf, a group of cells called the posterior lateral line primordium begins migrating from behind the embryonic ear toward the tail (Figure 1A-B). Clusters of migrating cells are progressively deposited along the trunk; each cluster will develop into a neuromast. Meanwhile, a group of axonal growth cones, whose cell bodies remain stationary behind the ear, are embedded in and migrate with the primordium (Metcalf, 1985; Gilmour et al., 2004). By 48 hpf, the primordium has reached the tail and deposited a full set of neuromasts (5-7 total) along the length of the trunk. The axons have also fully extended into the tail, innervating newly deposited neuromasts (Figure 1C). The rapid pace of this developmental process allows analysis of multiple stages of axonal development over a short period of time, including growth cone dynamics, axon outgrowth and extension, and target arrival.

As lateral line axons grow, their rapid extension and the highly dynamic remodeling of the actin cytoskeleton at the growth cone likely requires a large amount of ATP, as in

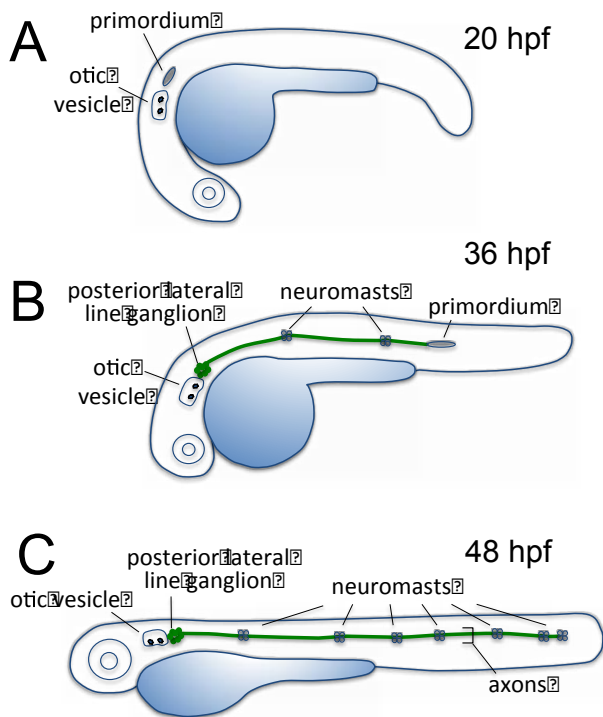


Figure 1. Posterior lateral line development during days 1-2 of zebrafish development. **A.** At around 20 hours post fertilization (hpf), a group of cells called the posterior lateral line primordium begins migrating caudally from behind the embryonic ear (otic vesicle). **B.** The primordium continues to migrate at 36 hpf, depositing clusters of cells along the trunk that will develop into neuromasts. A group of axonal growth cones are embedded in the primordium and extend caudally. **C.** By 48 hpf, the primordium and axons have both reached the tail. A group of fully extended lateral line axons now form synapses to innervate newly deposited neuromasts.

other examples of growth cone extension (Lin and Sheng, 2015). To meet this high energetic demand, mitochondria are continuously transported from their site of synthesis in the cell body out along the length of the axon (Morris and Hollenbeck, 1995; Ruthel and Hollenbeck, 2003; Maeder et al., 2014). In more mature neurons, a reliable supply of peripheral ATP is still required, to support processes such as synaptic development, synaptic vesicle cycling, and generation of action potentials (reviewed in Sheng, 2014; Lin and Sheng, 2015). Such changing energy demands may necessitate changes in mitochondrial transport patterns and dynamics during development, but such mechanisms are not well understood. Axons in the developing lateral line are well suited to study this question.

In this research module, students test directly whether mitochondrial transport dynamics in the lateral line differ within immature growing axons versus fully extended axons. In zebrafish, as posterior lateral line axons extend, mitochondria are continually transported in both the anterograde and retrograde directions (Drerup and Nechiporuk, 2013). These mitochondria, as well as the

lateral line axons themselves, are easily labeled in zebrafish with fluorescent constructs, and visualized both during and after axon extension. The developing posterior lateral line axons lie just underneath the skin of the embryo, and the body axis of the embryonic fish is relatively flat, making the lateral line in zebrafish particularly amenable to time-lapse imaging with a basic fluorescence microscope. By examining mitochondrial transport (both anterograde and retrograde) in developing zebrafish lateral line axons, students can test predictions about the potentially changing characteristics of mitochondrial transport dynamics and requirements both during and following axon growth *in vivo*. Students measure, quantify, and compare mitochondrial dynamics in lateral line axons at two key stages: ~24 hpf, when the axons are still growing and growth cones are still present; and ~48 hpf, when the axons are fully extended and beginning to form synapses. Students design their own hypotheses regarding the varying requirements for ATP at each of these stages, and quantify mitochondrial dynamics as an assay for ATP synthesis and delivery. In addition to characterizing wild-type transport, students also have a straightforward system in which to address transport mechanisms via the application of drug treatments or genetic manipulations. Furthermore, since additional neuromasts continue to develop after 2 days post fertilization (dpf) (Dambly-Chaudiere et al., 2003), additional time points can be added at later stages. Kymographic analysis is a powerful quantitative tool that undergraduates rarely have an opportunity to generate from their own *in vivo* experiments. This type of movement quantification has broad application to other systems. Furthermore, since little is known about the changes in mitochondrial dynamics during lateral line development, students are pursuing an open-ended research question without simply targeting a right or wrong answer.

STUDENT LEARNING

This module provides students the opportunity to learn a number of key skills that they apply to an open-ended research question. These skills include: 1) handling and anesthetization of living zebrafish embryos; 2) *in vivo* time-lapse fluorescence microscopy that can be done on a basic wide-field epifluorescence microscope (or scaled up for more powerful systems); 3) quantitative analysis of mitochondrial dynamics via generating kymographs to measure moving mitochondria in time-lapse movies (an approach that can be applied broadly to quantifying motion in other types of research projects); 4) quantitative comparison of axons at two different developmental stages; 5) design and completion of an independent research project (optional); and 6) preparation of an oral and/or written presentation. Depending upon the time and resources available at the institution, the overall module can take anywhere from one week to the entire semester. (See Tables 1-2; an incorporation of independent projects can branch off from original research project.) The more lengthy projects allow students to pursue more open-ended and independent research questions.

MATERIALS AND METHODS

We have developed this research project for an advanced undergraduate neurobiology laboratory course with an enrollment of 15-24 junior and senior students. As designed, it is the main focus of the laboratory portion of the class for the semester, taking approximately 10 lab sessions to complete; however, it can be scaled up or down depending upon time and resources available at the institution. The following sections provide background for introducing and conducting this research project with students. Two sample schedules for carrying out the project (one full-length, one abbreviated module) are also included below.

PRE-LABORATORY PREPARATION

Background

To begin the research project, students first require some background in a number of areas, including zebrafish anatomy, lateral line function, axonal development, and mitochondrial trafficking. If some of these topics are already covered in the lecture component of a lecture/laboratory course, the laboratory is an ideal application of what they are learning in the classroom. Students also need a prior lab session on microscopy, with previous practice using the fluorescence microscopes. We provide all of this background in the laboratory, before the project begins. Teaching materials for these subjects are available at weissmanlab.com.

If an active local zebrafish facility is not available for obtaining embryos, wild-type breeding pairs of zebrafish can be acquired from the Zebrafish International Resource Center (ZIRC) or from a local pet store (Westerfield, 2000). The Tg(neuroD:EGFP) line (see below) is also available from ZIRC. A number of useful resources are available that describe zebrafish maintenance, mating, and microinjection techniques (e.g., Westerfield, 2000). Currently, the NIH does not require Institutional Animal Care and Use Committee (IACUC) protocols for zebrafish 3 dpf or younger because they are not considered to be "live, vertebrate animals" until hatching (NIH, 2009; Matthews and Varga, 2012), which occurs around 72 hpf. Regardless, proper euthanasia protocol should be used when discarding embryos; this requires submersion in ice water (0-4°C) for at least 20 minutes, followed by addition of bleach to the water at a concentration of 1:5. Once embryos are exposed to the bleach solution for five minutes, they may be discarded. Institutional restrictions should be followed regarding disposal of organisms containing recombinant DNA. It should be noted that institutional requirements regarding zebrafish embryos may differ from those of the NIH. Additionally, if adults are to be used for breeding, an IACUC protocol is required. For examples, see ZIRC website (<https://zebrafish.org/documents/protocols.php>).

Labeling the zebrafish lateral line *in vivo*

To study mitochondrial dynamics *in vivo*, neurons in the lateral line, as well as elsewhere in the fish, are labeled by green fluorescent protein (GFP) driven by the neuroD

promoter (neuroD:EGFP) (Figure 2A-B) (Mo & Nicolson, 2011). NeuroD is an early transcription factor expressed in many differentiating neurons including the lateral line ganglia (Thomas et al., 2012). GFP fills the cytoplasm in labeled neurons, making them clearly distinguishable from background. The neuroD:EGFP DNA can be microinjected in plasmid form into one cell-stage embryos, in which case labeling is mosaic, or alternatively we can provide the stable transgenic line Tg(neuroD:EGFP) upon request (Obholzer et al., 2008; also available from ZIRC). Mitochondria are labeled via a separate DNA construct, neuroD:mito-TagRFP (Figure 2A-B). This plasmid uses the neuroD promoter to drive expression of a fusion protein composed of a mitochondrial targeting sequence (Clontech) linked to a red fluorescent protein (TagRFP, available for purchase from Evrogen). The neuroD:mito-TagRFP plasmid was assembled using Tol2 Multisite Gateway cloning from Invitrogen (Kwan et al. 2007; Obholzer et al. 2008; Drerup and Nechiporuk, unpublished observations). This plasmid is introduced via microinjection into one-cell stage embryos. If using the Tg(neuroD:EGFP) stable line, only the neuroD:mito-TagRFP plasmid needs to be injected; if using wild-type zebrafish, both the neuroD:mito-TagRFP and neuroD:EGFP plasmids can be injected together.

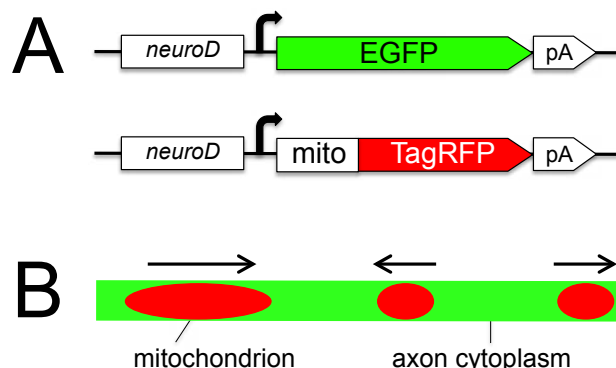


Figure 2. Labeling the lateral line in living zebrafish. **A.** Two DNA constructs are available that label the lateral line neurons with a cytoplasmic enhanced green fluorescent protein (EGFP; upper construct), and label mitochondria within lateral line neurons with a tagged form of TagRFP that localizes to the mitochondrial membrane (lower construct). Both constructs are driven by the neuroD promoter. **B.** Expression in a lateral line axon appears as red punctate mitochondria in the context of GFP-labeled cytoplasm (green). Trafficking of mitochondria is imaged in living fish.

Before the lab session, fish should be screened for expression of GFP and mitoTagRFP in the lateral line. Screening is most easily done in the GFP channel (green filter), either using a fluorescence dissection microscope (if available), or wide-field epifluorescence system. While the GFP expression should be visible at low magnification, mito-TagRFP expression in mitochondria is punctate (Figure 2B) and only visible at higher magnification (e.g., 20X). If both constructs have been co-injected together, GFP expression can be used as an indicator of both constructs, since both likely express in the same cells.

During screening, it is useful to categorize fish into those that express on the left side versus the right (not necessary for Tg(neuroD:EGFP) fish, which will express on both sides). This can aid students when mounting; however, if large numbers of fish are mounted there are good odds that expression will be visible without this added L/R screening step. Using the Tg(neuroD:EGFP) fish will help facilitate screening and lower the number of injected plasmids from two to one. In-crosses are advisable with this heterozygous line (i.e., heterozygous crossed to heterozygous) to generate expression in roughly 75% of embryos (25% homozygous + 50% heterozygous offspring). For efficiency purposes, microinjection and screening are typically done 'behind the scenes' by our instructional staff; however, students are invited to our zebrafish facility outside of the lab session if they wish to learn the microinjection technique. Microinjection is a powerful technique that can be learned over several weeks by students or technicians if desired (described in detail in Westerfield, 2000; Rosen et al., 2009). Instructors and support staff are encouraged to inject a large excess of embryos to ensure attainment of an adequate number of expressing embryos.

EQUIPMENT NEEDED

- Microinjection equipment (e.g., pipette puller, micromanipulator, pressure source, etc.) if constructs to be injected in-house
- Dissection microscopes with transillumination (1-2 per student group)
- Digital heat block (for low-melt agarose if used for mounting fish)
- Upright or inverted fluorescence compound microscope, equipped with standard green (FITC/Alexa488/GFP) and red (TRITC/Alexa555/mCherry) filter sets, and monochromatic digital camera (color camera also suitable but not needed), for imaging of embryos.
- Basic image acquisition software that allows for time-lapse capture (e.g., AxioVision, SPOT)
- Computers (Mac or PC) equipped with ImageJ or FIJI (students can use personal laptops as software is open access)
- Optional - fluorescence dissection microscope (for embryo screening)

REAGENTS NEEDED

- E3 Embryo Medium (Recipe from Nüsslein-Volhard and Dahm, 2002): 5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33mM MgSO₄, 10⁻⁵ % Methylene Blue. For 1L of 60x stock, dissolve 17.2g NaCl, 0.76g KCl, 2.9g CaCl₂•2H₂O, and 4.9g MgSO₄•7H₂O in 1L of DI or RO H₂O. Add Methylene Blue when making 1X solution.)
- Low-melt agarose for mounting live embryos (Amresco; J234)
- Anesthetic: Ethyl 3-aminobenzoate methanesulfonate salt (MESAB/Tricaine/MS-222; Fluka A5040) – store on ice. Stock of 4 mg/mL; add approx. 0.25-0.5 mL (~5-10 drops) to 3 mL E3 medium (~half full 35 mm Petri dish); ~(0.33-0.66mg/mL final concentration).

- N-Phenylthiourea [PTU] (Alfa-Aesar: CAS 103-85-5); Starting at 24 hpf, this may be added to E3 medium at 0.003% if desired to inhibit pigmentation. (Note: PTU is considered hazardous by the Occupational Safety and Health Administration, OSHA.)

ADDITIONAL MATERIALS FOR EACH LAB GROUP

- For Step 1: Embryo manipulation tool (numerous design options)
- For Step 1: Needle syringes (optional, for dechoriation; ~0.45 mm gauge)
- For Steps 1-2: Petri dishes (100mm x 15mm for embryo storage; 60mm x 15mm for dechoriation, anatomy; 35mm x10mm for anesthetization)
- For Steps 1-2: 1-2 disposable transfer pipettes, or Pasteur pipettes with bulb or pump
- For Step 2: Fine-tipped transfer pipettes may be desired for agarose mounting
- For Steps 2-4: Glass slides (for upright microscopes) or small petri dishes with coverslip bottoms (for inverted microscopes)
- For Step 2: No 1.5 coverslips (66x22mm to coverslip fish for air objective; 22x22mm for bridge mounting method; or similar sizes – e.g., see Figure 3).

RESULTS

LABORATORY PROCEDURES

Step 1. Fish handling & anatomy

Students begin by inspecting fish at different ages using a dissection microscope with transillumination. We provide fish at ~1, 2, and 5 dpf for comparison. Although these recommended ages are flexible, it is useful for students to become accustomed to the ages they will use for their experiments (1-2 dpf), and it is also useful to compare these young developmental stages with a slightly older fish. They will become familiar with anatomical landmarks (e.g., ear, heart) and should note various age-related differences, such as the lack of yolk and presence of swim bladder at later stages. Online zebrafish atlases or published resources can be used to aid students in this activity (e.g., Kimmel et al., 1995). We have developed a worksheet that requires the students to make various measurements, including heart rate (available at weissmanlab.com). Our laboratory clusters students into lab groups of three.

Students then practice manually removing the chorion from the fish after viewing online videos of the procedure (e.g., JoVE; <https://www.youtube.com/watch?v=sDUuMwnHRFM>). We have the students use two needle syringes as tools for this process. Some students struggle with dechoriation, either in terms of their fine motor control and/or because they have psychological issues with harming the fish. While this step can be skipped (i.e., if embryos are provided to students dechorionated), teaching the students to dechorionate their own fish allows them to be more independent later in the semester. Students then learn to anesthetize their dechorionated fish by adding Tricaine (MESAB; see Reagents) directly to the dish and

waiting for the startle response to disappear upon light touch to embryo with manipulation tool.

We spend one three-hour lab session on fish handling and anatomy, allowing students to maximize time in the subsequent lab session on the microscopes. Alternatively, however, some students could begin shifts at the fluorescence microscope during the first session (perhaps with pre-prepared slides) while other students are focusing on anatomy and handling.

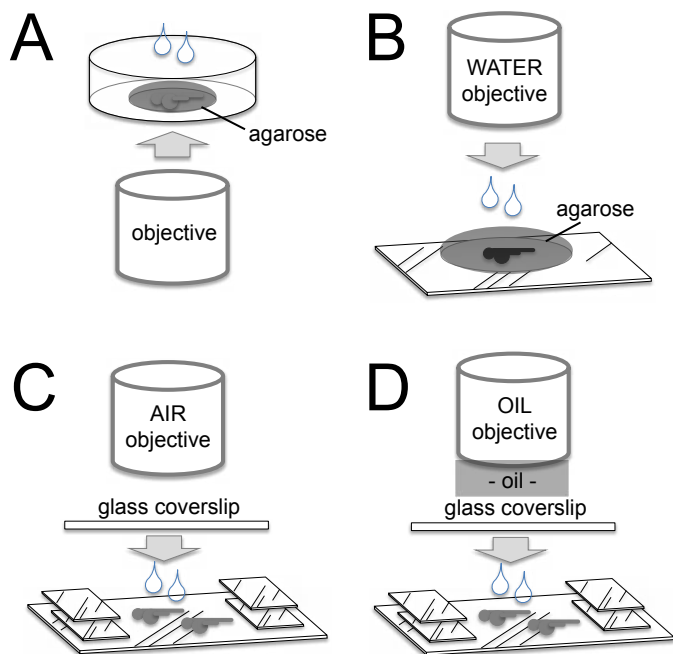


Figure 3. Mounting options. Living fish must be mounted in a water-soluble environment on the slide. **A.** For inverted microscopes, place fish onto a coverslip (e.g., bottom of coverslipped petri dish), and cover with low-melt agarose followed by E3 medium. **B.** With upright water objective, fish can be mounted in low-melt agarose with no coverslip. Place fish as close to the surface of agarose as possible. **C.** With upright air objective, fish should be mounted in E3 medium (or fish system water) and cover-slipped using some type of 'bridge' method to protect fish from being squashed. **D.** Oil objectives are not recommended due to their short working distance, but they may work if the lateral surface of the fish abuts the coverslip. More detailed mounting instructions are useful for students, and are available at weissmanlab.com.

Step 2. Mount fish onto slides

Mounting fish onto glass slides for imaging is one of the more difficult tasks for students in this project. There are many different options for mounting, and the ideal method depends upon which objectives are available at the microscope. Since the fish are alive during the imaging session, they must be in a water-soluble environment on the slide (in either E3 embryo medium or agarose made with E3). Both upright and inverted microscope systems are appropriate. Since the lateral line is close to the surface of the skin, one need not focus far into the organism to capture images. An inverted microscope is thus appropriate (Figure 3A) if the fish is placed onto a coverslip (e.g., bottom of coverslipped petri dish), and covered with low-melt agarose for stability, followed by E3

medium or fish system water for hydration (see Reagents). If an upright microscope with a water objective is being used, fish can be mounted directly onto slides in agarose with no coverslip (Figure 3B). The fish should be placed close to the surface of the agarose, to minimize optical aberrations, and a water column should be present between the agarose bed and the objective. (Use either E3 or de-chlorinated water; distilled water typically contains chlorine.) If an upright microscope with a dry air objective is being used, fish should be placed in E3 embryo medium on the slide, then cover-slipped (Figure 3C). It is crucial to build a supportive structure on the slide so that the coverslip does not flatten the fish. This can be made from a number of readily available items, such as small coverslips stacked on either end of the slide, or vacuum grease dotted on the four corners of slide surface. Depression slides may be used and cover-slipped directly, but the fish may sit too low in the well to be captured within the working distance of the objective. Oil objectives are not recommended due to their typically short working distances, but could potentially work in either upright or inverted systems if the side of the fish abuts the coverslip (e.g., Figure 3D). If desired, fish may also be mounted between two large coverslips in order to image from both top and bottom. Additional detailed mounting instructions and handouts are available at weissmanlab.com.

Regardless of the mounting approach selected, several anesthetized fish should be mounted together onto one slide in order to increase the likelihood of finding at least one unobstructed, well-labeled lateral line axon in a fish that is sitting at the appropriate angle. At 1-2 dpf, the yolk protrudes ventrally from the fish trunk, conveniently and naturally turning the fish onto its side when placed on the glass. This is the ideal angle for viewing the lateral line. Great care should be taken when manipulating the fish, however, as to not disturb/rupture the yolk, which is sticky and pervasive, and can contribute significantly to optical aberrations during imaging. Fish will remain healthy during the imaging session for up to several hours. (If the yolk is ruptured, fish will not survive.) Following the imaging session, fish should be removed and euthanized/discarded appropriately (see Background section of Materials and Methods above).

Step 3. Live imaging of zebrafish at 1 dpf

Students should first use a low-powered objective (e.g., 5X) to locate a fish on the slide. This step can be somewhat tricky for students because zebrafish are transparent; it may be helpful for them to use the GFP channel as opposed to transmitted white light to locate the fish. Next, center on the trunk of one fish (Figure 4A) by moving the slide/microscope stage in X and Y directions until the trunk region is in the very center of the viewable field. Then, switching to a higher magnification (e.g. 20X), locate the lateral line axon/s in the GFP channel (Figure 4B-C). These axons will appear long, smooth, and relatively thick, projecting along the side of fish, quite superficially (just under the skin). It should be relatively easy to distinguish the lateral line axons from the deeper spinal cord neurons, which are narrower in caliber and

slightly more dorsal than the lateral line (Figure 4B). Once the lateral line is identified, scroll in the rostral/caudal direction until a region is found that is contained in one focal plane (filling most of the field). Although searching may take some time for students, they should be encouraged to work relatively quickly, since overexposure may bleach the fluorophores. Multiple axons may be labeled with neuroD:EGFP, especially if the transgenic line is used.

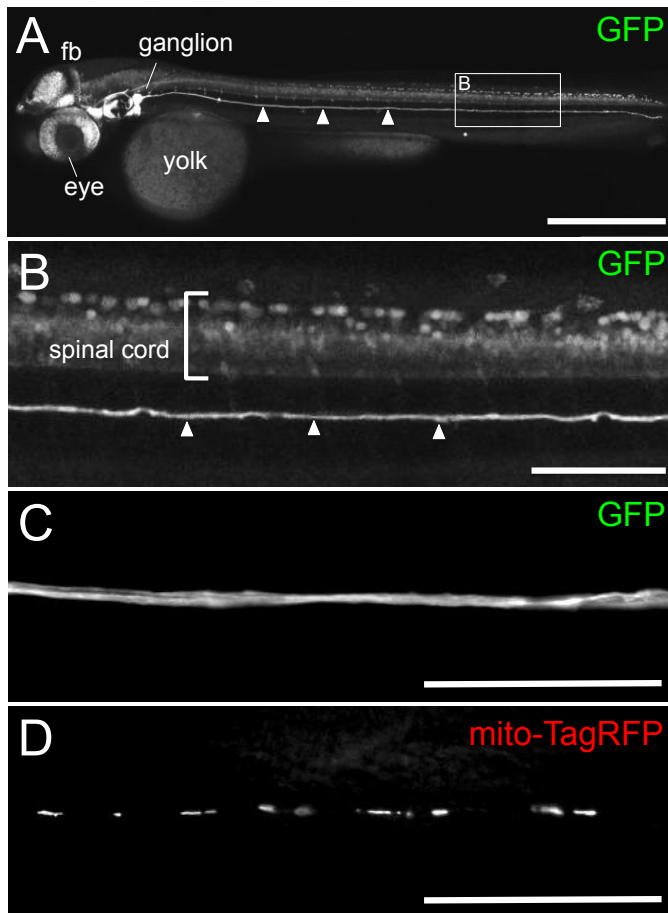


Figure 4. Lateral line labeling in a living 2 dpf zebrafish. **A.** Here, the neuroD-EGFP construct labels the lateral line ganglion behind the fish ear and at least one lateral line axon (arrowheads), as well as neurons in the developing eye and forebrain (fb). Fish imaged from the lateral view using a 10X air objective (montage of six fields). **B.** Box from **A** is shown magnified. Neurons in the spinal cord (upper part of image) are deeper into the tissue than the superficial lateral line axon, but both depths are shown for demonstration purposes in this confocal 3-D projection. The lateral line axon is thicker in caliber, smooth, and more superficial, making it distinct from axons in the spinal cord. **C.** Lateral line axons viewed using a wide-field epifluorescence microscope (40X water objective). **D.** Mitochondria are shown within an axon from **(C)**. While multiple lateral line axons may be visible in the GFP channel, especially if the Tg(neuroD:EGFP) transgenic line is used, mito-TagRFP ideally expresses in only a subset of axons. Scale bars: 500 μ m in **A**; 100 μ m in **B**; 50 μ m in **C** & **D**.

Once a relatively flat region (e.g., Figure 4C) is identified, the experiment is almost complete. Take a quick image of the axon/s in the green channel if desired (not

necessary for analysis, but useful for presentation purposes). Now switch to the red channel to visualize mitochondria, which should appear as tiny spots along the axon (Figure 4D). Ideally the mitochondria within only one axon have been labeled, even if multiple axons were visible in the green channel. After focusing on the punctate mitochondria and adjusting exposure appropriately, set up the acquisition software to collect a short time-lapse movie in the red channel only. Length of the movie should be approximately 1-2 minutes long, with a time interval of approximately 1-2 seconds between frames. (Time intervals longer than 1-2 seconds will be too slow to capture quick mitochondrial movements.) Save the time-lapse data in a format that will be recognized by ImageJ (e.g., tiff series, .zvi, .oib, .lif, etc.), and be sure to record the time interval between frames. A quick preview of the movie should reveal mitochondria moving along the axon, likely in both directions. Ideally one axon is visually distinct from neighboring axons within the lateral line (e.g., Figure 4B,D); imaging labeled mitochondria in two neighboring axons may complicate the analysis. Students can complete a thorough analysis even if they are able to acquire only one or two movies, as long as some mobile mitochondria can be identified. The kymographic analysis can be done on poorly resolved (e.g., blurry) mitochondria, because the center of the object can be followed over distances. Furthermore, since the acquired time-lapse series can be quite short (~60 seconds), even microscopes *without* an automated shutter can be used. (Simply leave the shutter open for the duration of the acquisition.) Slight decreases in mitochondrial intensity (e.g., due to bleaching) will not drastically affect the kymographic analysis.

An additional option at this stage, while the fish is still on the microscope (especially if mitochondria are not labeled or visualized well), is to locate and image the tips of the GFP-labeled lateral line axon growth cone, which is still growing until ~36 hpf. Switching now to the GFP channel, a longer time-lapse movie can be acquired (approximately 5-10 minutes total (or more if desired/able), with time interval of approximately 10-20 seconds between frames). Even short movies should reveal growth cone dynamics for visualization by students.

The major bottleneck with this research module comes at the microscope, since resources are limited and there are typically five to eight different groups (based on a class size of 15-24) who need to rotate through imaging stations. This bottleneck can be improved by increasing the number of lab sessions/weeks dedicated to imaging. We schedule four 3-hour imaging sessions (on 3-4 usable fluorescence microscopes), with 1.5 hour shifts at each imaging station. During each lab session, students may have a number of goals, including anatomical study of live fish (see Step 1), mastery of kymograph preparation (on sample movies), and subsequent analysis of their own data (see Step 5).

Step 4. (Optional) Live imaging of zebrafish at 2 dpf

To expand the inquiry-based aspect of this project, we ask students to compare mitochondrial dynamics between younger and older fish. At ~24 hours, the lateral line axons

are still growing/extending along the rostro-caudal axis. At 36-48 hours, the period of extension is complete. By including additional imaging sessions at the later developmental stage, students can put together a more independent and creative analysis of their results, comparing a still-growing axon with one that has already reached its target and is undergoing synapse formation. Methods are identical to Step 3, but using older embryos (which can be slightly easier to mount). Student groups will differ in their efficiency at imaging. In imaging sessions 2-4, we provide both ages of fish so that students can focus where needed.

Step 5. Analysis

Once the students have collected time-lapse images of mitochondria in the lateral line at 1dpf (and/or 2dpf), they can analyze the velocity and movements of individual mitochondria using kymograph analysis in ImageJ or FIJI. This software, as well as the plugins required for the kymographic analysis, are freely available online for instant download, and can be used on either Mac or PC platforms. In addition to installing ImageJ, students will need to download two additional plugins: Bio-formats Package (<http://downloads.openmicroscopy.org/bio-formats/5.1.0/>) and the Kymograph Plugin (http://www.embl.de/eamnet/html/body_kymograph.html). Step-by-step instructions for this analysis, in the form of a lab handout, are available at weissmanlab.com.

Using the Bio-formats importer in ImageJ, students will first open a time-lapse image series that is in a recognizable file format (e.g., tiff series, .zvi, .oib, .lif, etc.). In an abbreviated module, or if students are not successful in obtaining their own images, students can perform kymographic analyses on image series provided by the instructor or other lab groups. (We will be providing some of these resources on our lab webpage: weissmanlab.com.) Upon opening an image file, the options for "Standard ImageJ" and "Autoscale" should be selected. Once the image series is opened, students should be able to move through the time-lapse series with a scroll bar at the bottom of the image file. The size of the image file should be noted (in microns or pixels; often located in the top left corner of image) for later calculating distance and velocity. (If the image size is unknown, it can be calculated by imaging a micrometer with the same objective.) It is useful to then duplicate the image in order to run multiple analyses (Image>Duplicate, select "Duplicate Stack").

Students should select a region of the axon that is in focus throughout the time series and that shows at least one mitochondrion moving (Figure 5A). To simplify the view, they may select a small portion of the image using the rotate (Image>Transform>Rotate) and crop (Image>Crop) tools. Students should keep track of the orientation of the selected axon with respect to the anterior/posterior axis of the fish; this will be important for comparing retrograde versus anterograde mitochondrial transport.

Students can then draw a segmented line along the axon by right clicking on the line tool (Figure 5A). The use

of the segmented line tool allows students to trace axons that may bend slightly over their length. From this line, they will generate a kymograph using the kymograph plugin (Plugin>Multiple Kymograph), which will prompt to select a line width. The students can experiment with which line width works best to create the cleanest kymograph, but we suggest starting with width = 1 first and working up by odd numbers. If movement has occurred during the time-lapse collection, it can be corrected using a freely available ImageJ plugin called "StackReg".

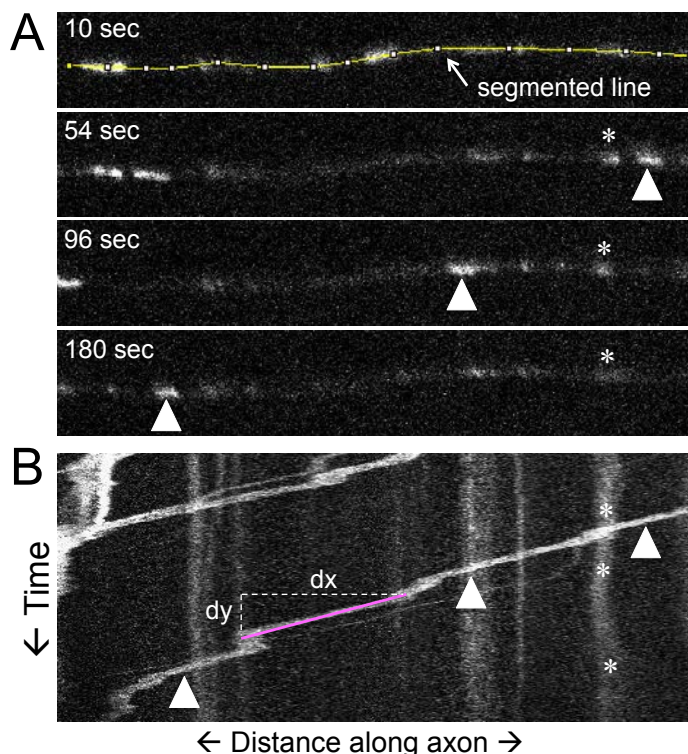


Figure 5. Sample time-lapse images with kymograph. **A.** Four single time points are shown at 10, 54, 96, and 180 seconds. (In ImageJ, sequential images appear as an image stack.) Arrowhead tracks a single mitochondrion, which moves toward left over time. Using the ImageJ tools, a segmented line is drawn along the axon of interest (shown at 10 sec). ImageJ measures the fluorescence intensity at each pixel along that line. **B.** A kymograph is generated that displays the fluorescence intensity present along the segmented line (x-axis) over time (downward on y-axis). The first time frame of the time-lapse movie is shown as the very top horizontal row of pixels; the last frame is shown in the bottom pixel row (see text). Arrowheads in (B) correspond to same object moving toward left in movie above. Asterisks in (A) and (B) follow a stationary object. Diagonal lines in kymograph demonstrate moving objects over time; vertical lines illustrate stationary objects. The ImageJ plugin measures velocity (distance/time) from straight lines drawn on kymograph.

At this point, students should have generated a kymograph (e.g., Figure 5B). It is crucial that the students understand how to interpret these graphs. In a kymograph, the top horizontal row of pixels displays the intensity values along the length of the segmented line at the first time point. If a bright mitochondrion is present at the first time point of the movie (e.g., Figure 5A, top), then in the

kymograph, bright pixels should appear at that same x location in the very top row (Figure 5B). The x-axis simply maps distance along the segmented line (i.e., the length of the axon). The next row of pixels down in the kymograph shows the intensity values along the same segmented line but now at the next time point, and so on down the y-axis, which displays time progressing downward. The kymograph thus shows imaged fluorescence along the length of the segmented line (x-axis) over time (y-axis downward). Fluorescent objects (e.g., mitochondria) that are present throughout a portion of the movie should appear along the vertical dimension of the kymograph that corresponds to those time points. The location along the x-axis will represent an object's specific location at each point in time. Since an object is typically present over a period of time, it will appear as a white line going downward on the graph. While an object is stationary, its location along the x-axis should not change over time, thus it should appear as a vertical line (asterisks in Figure 5A-B). While an object is in motion, its x location changes over time, thus it can be tracked as a diagonal line going downward on the kymograph (arrowheads in Figure 5A-B). This type of graphic display is extremely useful for directly calculating various aspects of object movement, including velocity, distance traveled, direction traveled, number of starts and stops, length of bout, etc. If students save their kymograph image, it can be included in their manuscript or presentation later.

The velocity of moving objects can be calculated from the kymograph using the 'tsp' tool (Plugins>Macros>Install>tsp050607). Using the straight line tool, students will draw a straight line over a diagonal region on the kymograph, starting at the earlier time points (toward top) and ending with the later time points toward the bottom of the image, as shown in Figure 5. Each bout of movement (diagonal section, possibly interrupted by a brief still period, which would appear vertical) should be analyzed separately. Note that even blurry mitochondria can be analyzed; students will simply track the centroid of the fuzzy line. To generate a velocity value for each segment, select Plugins>Macros>"read velocities from tsp."

Students can copy and paste each reading into a spreadsheet program such as Microsoft Excel to keep track of velocity values for multiple mitochondria. Anterograde and retrograde direction of movement (either down toward left or down toward right) should be recorded at this step. The value "dy sum" corresponds to change in y (time), and "dx sum" corresponds to change in x (distance). Note that these values are reported as number of frames (time) and pixels (distance), respectively. Before calculating the actual velocity (dx/dy), however, values need to be converted into seconds and microns. To convert the reported time values from number of frames to seconds, simply divide by the number frames per second. (This value was set by user during image acquisition.) Note that if students used a line width greater than one, dy will have been multiplied by that factor. Next, to convert distance values from pixels to microns, students must multiply each value by the appropriate number of microns

per pixel. These pixel dimensions can be viewed under Image>Show Info (may be reported as "Resolution"). If such metadata is not available, students will at least be able to read the number of pixels across the width of the image. They can then capture a new image of a micrometer using the same objective on the same microscope. Knowing the distance between ticks on the micrometer, students can calculate the approximate number of microns across their image, then divide this by the pixel width. If the instructors wish to simplify this step for students, they can pre-calculate the pixel dimensions for each objective; however, this tedious calculation is effective in helping students understand how they actually calculate velocity from their images.

There are multiple ways for students to analyze the measurements they acquire from the kymograph analysis, depending upon the questions they wish to answer. For example, they could compare average mitochondrial velocities across different ages of fish, compare retrograde and anterograde mitochondrial transport (Figure 6A), or calculate the number of bouts per minute and/or the length of each bout (which reflects attachment to or detachment from motor proteins.)

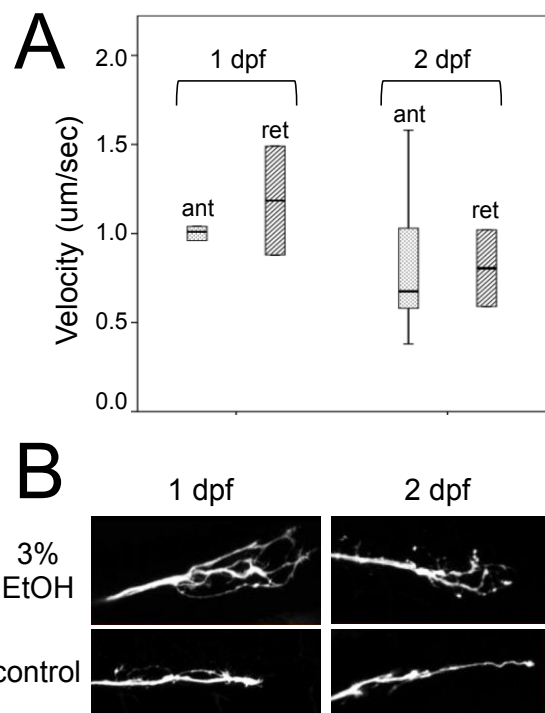


Figure 6. Examples of student work. **A.** Boxplots compare mitochondrial transport velocity in the retrograde (ret) and anterograde (ant) directions, at 1 and 2 dpf. **B.** In this independent project, students compared growth cone morphology and dynamics in the developing lateral line of living zebrafish embryos at 1 and 2 dpf that had been exposed to 3% ethanol or control. These students also tested the escape response in the same fish (data not shown).

Step 6. Independent projects

Once they have completed the basic module (Steps 1-5), students are capable of repeating these experiments on

their own. They are typically eager to select any number of manipulations or ideas for expanding their research question. For example, they may wish to look at the same two time points in the presence of a pharmacological agent, in a mutant zebrafish line, or in fish housed within a different environment. They may wish to analyze a new time point, or simply repeat the experiment to increase sample size and power in their analysis. Alternatively, they may wish to focus on growth cone dynamics (Figure 6B). Depending upon the preferences of the instructional staff, students may be given a great deal of freedom on the design of their project, or they may be asked to design new experiments that focus on a particular manipulation (e.g., calcium dynamics).

In our course, after submitting a structured proposal for their independent work and receiving feedback/approval from the instructor, students are encouraged to work independently (both inside and outside of class time) on their projects. Assistance is typically required in obtaining (and diluting) necessary reagents, timing injections of constructs, and microscopy. While students are consistently able to generate satisfying data during the independent project portion of the semester, they often report that they could use more time for their independent work, even when they are given a full five weeks.

Step 7. Presenting their work

There are numerous options for presentation and assessment. In our course, for Steps 3-5, student groups write a scientific manuscript with Introduction, Methods, Results, Discussion, and References Sections (worth 40 points out of 150 total for lab). They are requested to design and include at least three different figures (not tables, and not simply raw data) that summarize and visually display various aspects of their data (e.g., Figure 6A). For the independent projects, a written project proposal (15 points) is required that includes a statement of the specific question to be addressed, hypothesis to be tested, description of approach/techniques, proposed schedule, and list of required supplies and animals/ages. Following completion of the independent projects, students present their work in an oral presentation (Powerpoint style), and must include background, methods, detailed results, analysis, future ideas, and literature cited. All presentations are given on a final lab day, and are worth 40 points (out of 150 total for lab). A scaled-down version of this lab could require a basic worksheet from the kymographic analysis that asks students to report mitochondrial velocity in anterograde vs. retrograde directions, number of bouts per minute, etc.

Steps 1-7 can be modular in that they need not all be done. We provide two sample schedules in Table 1 (full length version) and Table 2 (abbreviated version).

DISCUSSION

We present here an open-ended research project appropriate for advanced undergraduates who wish to investigate axonal development in the zebrafish lateral line system. Students learn multiple useful skills, including working with living fish, *in vivo* time-lapse fluorescence

Week 1	Zebrafish development & anatomy	Project overview. Practice dechorionating, anesthetizing, basic fish anatomy, mounting, etc.
Week 2	Fluorescence microscopy	Overview of fluorescence microscopy. Practice mounting fish & using fluorescence microscope.
Week 3	<i>In vivo</i> time-lapse session 1	Introduction should be complete by now (lateral line, axonal transport, etc.). Groups screen & mount fish, begin time-lapse at first time point (~24 hpf)
Week 4	<i>In vivo</i> time-lapse session 2 + analysis	Continue imaging. Fish also available at ~36 hpf for some groups. Data analysis can begin.
Week 5	<i>In vivo</i> time-lapse session 3 + analysis	Continue imaging. Each group should have 3-4 good quality time-lapse series at each time point. Data analysis ongoing.
Week 6	<i>In vivo</i> time-lapse session 4 + analysis	Some groups finish up imaging. Most groups focus on data analysis. Results summary & proposal for independent projects due in Week 7.
Weeks 7-10 (+)	Independent projects (At least four dedicated weeks – more is better for students)	Fish available at various time points throughout week, depending upon student needs. Students work largely independently and often meet outside of lab time, but may utilize instructor help during regular lab sessions.
Week 11	Final presentations	

Table 1. Sample schedule I (full length version)

Weeks 1-2	Zebrafish overview & <i>in vivo</i> time-lapse imaging sessions	Skip anatomy & dechorionation. Students simply mount fish & collect time-lapse for one age only (24 or 36 hpf). Imaging is heavily supervised. Time needed for all groups to image will depend on microscope bottleneck/equipment available. While waiting for imaging stations, students can work on sample movie analysis.
Weeks 2-3	Data analysis	Work on generating and analyzing kymographs

Table 2. Sample schedule II (abbreviated version)

microscopy, and, importantly, experimental design and quantitative analysis. The project is modular in that it works either in full-length form, or with only a subset of steps (Tables 1-2). Although the imaging portion of the project benefits from multiple fluorescence microscopes on hand, it can be done with only one basic wide-field epifluorescence microscope, or could even be done by analyzing movies acquired at a different facility.

We have incorporated into this research project an advanced quantitative analysis of movement known as the kymograph. Kymographic analysis is a quantitative approach that graphs movement over time. It is rare that students have an opportunity to perform this type of analysis on data that they have generated through their own *in vivo* experiments. Here, students are surprised to learn how accessible the approach is. They often report feeling empowered by this concrete mathematical approach, which helps them envision new ways to visualize and communicate their scientific results.

Importantly, since little is known about the specific changes that occur in mitochondrial trafficking dynamics during lateral line development, students learn to design experiments and interpret data that investigate an open-ended research question that does not have a clear answer in the lab manual. The 2011 report on the Initiative for Vision and Change in Undergraduate Biology Education (AAAS, 2011) highlighted the critical need for an integration of the scientific process into undergraduate Biology courses. Real research experiences that practice inquiry-based learning are foundational for providing students with an understanding of how to do science and how to understand the scientific process (Sadler and McKinney, 2010), and such experiences can be provided within the context of regular laboratory courses. We have worked to develop this open-ended research project for an undergraduate neurobiology laboratory course that allows students to design and ask their own research question in developmental neurobiology, learn cutting-edge imaging techniques in an *in vivo* zebrafish model system, and master a broadly applicable quantitative approach for analyzing and presenting their data. This project has the added advantage of investigating a relatively undiscovered area of neuroscience research. Because of this, students interpret open-ended data sets and think like true researchers.

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