ARTICLE
Teaching Dose-response Relationships Through Aminoglycoside Block of Mechanotransduction Channels in Lateral Line Hair Cells of Larval Zebrafish

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Here we introduce a novel set of laboratory exercises for teaching about hair cell structure and function and dose-response relationships via fluorescence microscopy. Through fluorescent labeling of lateral line hair cells, students assay aminoglycoside block of mechanoelectrical transduction (MET) channels in larval zebrafish. Students acquire and quantify images of hair cells fluorescently labeled with FM 1-43, which enters the hair cell through MET channels. Blocking FM 1-43 uptake with different concentrations of dihydrostreptomycin (DHS) results in dose-dependent reduction in hair-cell fluorescence. This method allows students to generate dose-response curves for the percent fluorescence reduction at different concentrations of DHS, which are then visualized to examine the blocking behavior of DHS using the Hill equation. Finally, students present their findings in lab reports structured as scientific papers. Together these laboratory exercises give students the opportunity to learn about hair cell mechanotransduction, pharmacological block of ion channels, and dose-dependent relationships including the Hill equation, while also exposing students to the zebrafish model organism, fluorescent labeling and microscopy, acquisition and analysis of images, and the presentation of experimental findings. These simple yet comprehensive techniques are appropriate for an undergraduate biology or neuroscience classroom laboratory.

Key words: zebrafish; hair cells; lateral line; MET channel; mechanoelectrical transduction channel; drug competition; dihydrostreptomycin; DHS; FM 1-43; fluorescence microscopy; dose-response curves

Hair cells are mechanosensory receptors found in the auditory, vestibular, and, if present, lateral line systems of vertebrates, where they faithfully transduce mechanical stimuli into trains of action potentials in afferent neurons. Hair cells derive their name from the hair bundle, a collection of specialized stereocilia that project from the cell's apical surface. Mechanical deflection of the hair bundle by movement of the surrounding fluid causes the opening and closing of mechanoelectrical transduction (MET) channels situated at the tip of each stereocilium (Figure 1A) (Fettiplace and Hackney, 2006; Vollrath et al., 2007; Beurg et al., 2009; Gillespie and Müller, 2009). When the MET channel opens, inward cation flow results in depolarization of the hair cell's membrane potential (aka, the receptor potential). This event leads to activation of voltage-gated calcium channels (VGCCs) and the graded release of neurotransmitter from ribbon synapses located along the basal surface of the hair cell (Moser et al., 2006; Gillespie and Müller, 2009; Uthaiah and Hudspeth, 2010). Thus, the features of a stimulus, such as frequency and intensity, are ultimately encoded into the timing of action potentials generated in the innervating afferent neurons (Geisler, 1998; Olt et al., 2016; Troconis et al., 2017).

The ability of hair cells to encode the frequency and intensity of sound waves, vestibular accelerations, or water currents in a graded manner relies on the unique features of the MET channel. While its molecular identity remains under investigation (Kawashima et al., 2011; Pan et al., 2013; Maeda et al., 2014; Kurima et al., 2015), there are decades of excellent studies describing the kinetics of gating and adaptation, as well as the non-specific nature of its permeability. The MET channel is roughly equally permeable to sodium and potassium and also calcium (Farris et al., 2004; Fettiplace, 2009). In addition, the MET channel's large pore width of ~12.5 Å allows large molecules to enter hair cells through the conduction pathway (Farris et al., 2004). These laboratory exercises investigate and take advantage of the non-specific nature of this very large cation channel.

Because of the MET channels' large pore size, aminoglycoside antibiotics are able to enter the hair cell (Alharazneh et al., 2011; Esterberg et al., 2013). Despite being commonly prescribed for the treatment of bacterial infections in humans (Forge and Schacht, 2000), aminoglycosides are associated with ototoxicity (hair cell death) after entry into the hair cell through the MET channel. Overall, up to 25% of patients treated with aminoglycosides have developed irreversible sensorineural hearing loss (Rizzi and Hirose, 2007). Here we take advantage of the ability of aminoglycosides to enter hair cells through the MET channel, in order to learn about the structure and function of both the channel and the hair cell.

Due to their size, aminoglycosides such as dihydrostreptomycin (DHS) have a rate of permeation through the channel that is dramatically slower than smaller cations like sodium and potassium. DHS at sub-saturating concentrations can therefore behave as a permeant blocker to cation flow (Kimitsuki and Ohmori, 1993). This phenomenon can be modeled by considering DHS as "binding" to the channel in a ligand-receptor type interaction

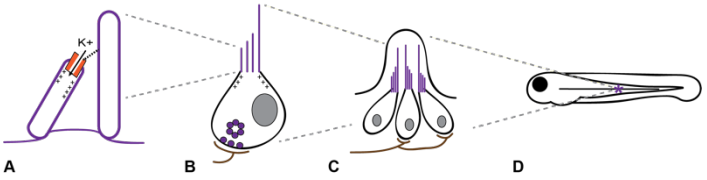

Figure 1. MET channels in lateral line hair cells of zebrafish. (*A*) Two adjacent stereocilia connected by a tip link (dashed line) at the apical end of a hair cell. Deflection of stereocilia opens MET channel (orange) located at their tips, which results in the influx of K^+ and other cations. *(B)* Cation influx through MET channels causes depolarization of the membrane potential resulting in activation of voltage-gated calcium channels, fusion of synaptic vesicles (purple circles), and release of glutamate neurotransmitter onto afferent neurons (brown lines) at the basal end of the hair cell. (C) A collection of hair cells forms a neuromast, which together (D) makeup the lateral line system of zebrafish (purple asterisk = neuromast).

(Figure 2B). In this laboratory experiment, students can test this hypothesis by creating a dose-response curve that examines block of the MET channel in the presence of various logarithmic concentrations of the drug. Students can then attempt to fit their data with a Hill equation that describes a one-to-one ligand-receptor interaction (see *Methods* section). Thus, by comparing experimental results to a theoretical curve, students can discuss the dose-response relationship and the mechanism by which DHS blocks MET channels.

Studying the block of MET channels by aminoglycoside antibiotics has traditionally been challenging. In most model organisms, hair cells are difficult to study because they are found within the inner ear and can only be accessed by careful dissection. Prior studies investigating the effects of DHS on the MET channel have been performed on hair cells isolated from bullfrog sacculi and chick and rat cochleae (Kroese et al., 1989; Kimitsuki and Ohmori, 1993; Alharazneh et al., 2011). These organisms require extensive care and maintenance, and experiments on their hair cells necessitate a delicate and time-consuming preparation that is less conducive to an undergraduate laboratory setting. In addition, most studies of MET channel function use patch-clamp electrophysiological techniques to examine mechanotransduction currents, which are beyond the scope of most undergraduate-level teaching laboratories. Here, we take advantage of an alternate method of assaying mechanotransduction block using fluorescence microscopy and the zebrafish lateral line system.

Though classically used to monitor endocytosis and exocytosis of synaptic vesicles (Gaffield and Betz, 2007), the nontoxic fluorescent dye FM 1-43 has also been shown to enter hair cells rapidly through open MET channels (Nishikawa and Sasaki, 1996; Gale et al., 2001; Meyers et al., 2003) (Figure 2). Upon entry into hair cells, the dye's fluorescence intensity dramatically increases as it binds to the plasma membrane (Betz et al., 1996). Furthermore, drugs that block the MET channel are known to limit entry of FM 1-43 into hair cells, and zebrafish mutants that lack functional mechanotransduction channels do not take up FM 1-43 into hair cells (Nishikawa and Sasaki, 1996; Gale et al., 2001; Sidi, 2003; Corey et al., 2004; Kruger et al., 2016). These studies also show that labeling is restricted to neuromast hair cells and not those found in the ear and the labeling is due to MET uptake because the impenetrable skin blocks access to other cellular locations and the short incubation does not allow for endocytosis at the apical surface of the hair cell or other cells. Hence, when hair cells are exposed to both FM 1-43 and DHS simultaneously, the two molecules compete for entry into the MET channel (Figure 2). As a result, the intensity of fluorescently labeled hair cells would be expected to decrease with increasing concentrations of DHS. Thus, we can use fluorescence intensity as a proxy for determining the extent of DHS block of the MET channel.

Zebrafish are an ideal model system for undergraduate students to learn about vertebrate biology and neuroscience (Monesson-Olson et al., 2014; Marra et al., 2015). Zebrafish possess a lateral line system that consists of hair cells arranged in superficially accessible, rosette-like structures termed neuromasts (Figure 1C; Metcalfe et al., 1985; Bleckmann and Zelick, 2009). Each neuromast contains upwards of twenty hair cells that detect and encode hydrodynamic stimuli in a similar manner to hair cells that perceive sound waves and accelerations in the auditory and vestibular systems. Given the accessibility of lateral line neuromasts, larval zebrafish hair cells can be exposed to DHS and FM 1-43 by placing larvae in drug-containing medium. FM 1-43 dye present in the medium is able to access and fluorescently label hair cells directly within seconds (Meyers et al., 2003; Sidi, 2003), and DHS in the medium can competitively block MET channels (Kroese et al., 1989; Alharazneh et al., 2011; Kindt et al., 2012; Kruger et al., 2016). Furthermore, because larval zebrafish are transparent, fluorescently labeled hair cells can be visualized with microscopy in the intact living animal within minutes following brief incubation with the dye (Meyers et al., 2003). Additionally, zebrafish can be obtained at local pet stores and a single female can lay up to 200 eggs per week (Westerfield, 2000). Zebrafish are easy to breed,

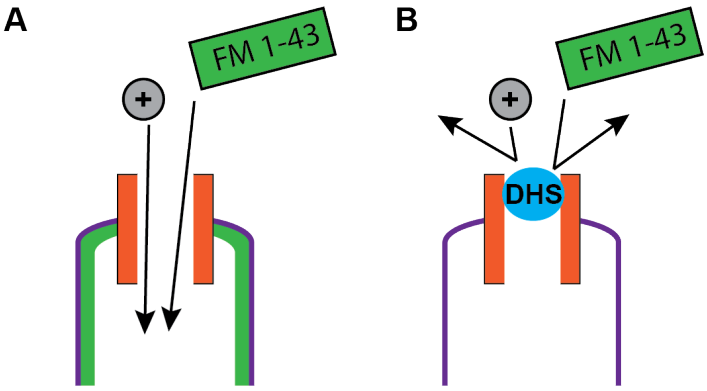

Figure 2. FM 1-43 entry and DHS block of hair cells. (*A*) FM 1-43 (green rectangle) enters hair cells via open MET channels (orange) and fluorescently labels the hair cell membrane. (*B*) DHS (blue circle) permeates slowly through MET channels, blocking ion flow and preventing FM 1-43 entry into hair cells.

maintain, and use in an undergraduate laboratory setting. Below, we outline a laboratory where students are initially encouraged to examine the interaction between the MET channel and DHS by visualizing the concentration-dependent reduction of FM 1-43 labeling of hair cells. Further multi-week experiments can extend the scope of this teaching laboratory to address other aspects of hair-cell mechanotransduction as assayed by FM 1-43 labeling. This laboratory also provides opportunities to teach students about the zebrafish model organism, the lateral line system of fishes, hair cell structure and function, permeation pathways of cation channels, the binding of ligands to receptors, fundamentals of epifluorescence microscopy and imaging, and image analysis methods using ImageJ or similar software. Instructors may emphasize or de-emphasize certain topics according to the needs of their course. In addition, students will strengthen their skills in hypothesis design and testing through experimentation, as well as data collection and analysis of results using curve-fitting and statistics. Moreover, students will improve their scientific writing skills if they are asked to write up their findings as a lab report formatted to model a scientific paper. Altogether, the exercises described here utilize multiple laboratory tools, methods, and techniques to perform *in vivo* hypothesis-based experiments with a vertebrate model organism in order to learn about several important areas relevant to basic biology and neuroscience research.

MATERIALS AND METHODS

Animal Ethics and Euthanasia

Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Amherst College under assurance number 3925-1 with the Office of Laboratory Animal Welfare. Adult and larval zebrafish should be euthanized according to AVMA guidelines, generally through hypothermic shock and then storage in a freezer. Larvae mounted on slides are terminated when slide is wiped clean.

Learning Objectives

The objectives of the laboratory exercises presented were as follows:

1) To learn about the structure and function of MET channels
2) To learn about the pharmacology of pore blocking molecules, ligand-receptor interactions, dose-dependent relationships, and the Hill equation
3) To learn about fluorescent dyes and epifluorescence microscopy (excitation and emission filters and dichroic mirrors, etc.)
4) To gain experience capturing and quantifying images using an epifluorescence microscope equipped with a digital camera
5) To gain experience working with the zebrafish model organism including hands-on experimentation with larvae
6) To improve basic research skills including collection and analysis of data, construction of figures, and presentation of results in the form of manuscript-style lab reports
7) To improve writing ability to construct and evaluate hypotheses and contextualize conclusions within the scientific literature

Methods for the Instructor

Background. Before students carried out their experiments, instructors introduced them to the key concepts explored in the lab. This introduction lecture included the structure and function of hair cells and MET channels, the lateral line in zebrafish, FM 1-43 and DHS, fluorescence microscopy, and a review of ligand-receptor interactions and the Hill equation. A key concept in this laboratory is the proposed one-to-one interaction between a molecule of DHS and an individual MET channel, which would be described by

Equation 1. $$L + R \rightarrow LR$$

where L is the ligand (DHS) and R is the receptor (MET channel). Students also learn about the Hill equation, which describes the interaction between the blocker and the channel:

Equation 2. $$I = 100\left(\frac{X^n}{X^n + \mathrm{IC}_{50}{}^n}\right)$$

where for this laboratory, *I* is percent max fluorescence intensity, *X* is the drug concentration, IC_{50} is the DHS concentration that produces half maximal block, and n is the slope of the curve. A.V. Hill first applied this equation in 1910 to characterize the cooperative binding of oxygen to hemoglobin, and it has subsequently been used to analyze other ligand-receptor interactions (Weiss, 1997). Others may wish to examine the dose-response relationship with other pharmacological equations. In this experiment, the Hill equation describes the ability of DHS to block the MET channel as measured by a percent reduction in FM 1-43 fluorescence. The slope (n) is commonly used as an estimate of the number of ligand (drug) molecules that must bind to the receptor (channel) to produce a functional effect (Weiss, 1997). Because we are proposing that DHS interacts with MET channels in a one-to-one manner (see *Equation 2*), this would result in a slope (n) of 1 for the Hill equation. Thus, when the data collected by students are plotted as a percent reduction in fluorescence vs. the concentration of DHS (on a Log_{10} scale), they can test the one-to-one hypothesis by observing whether their data are fit by a Hill equation with a slope of 1 (see *Data Analysis* section). It is important to provide a thorough introduction to the Hill equation and its relationship to the MET channel and DHS interaction, as this part of the laboratory exercise is often challenging for students. In addition, students are encouraged to search PubMed and other sources for learning about DHS interaction with the MET channel (cf., Kroese et al., 1989).

Fish Care

Zebrafish larvae at 4-7 days post fertilization (dpf) are required for this experiment for development and maturation of the lateral line system and its hair cells. The instructor

should obtain and breed adult zebrafish approximately 1 week prior to the teaching lab. Adult zebrafish were kept in tanks under a fixed light cycle (14 hours of light, 10 hours of darkness), separated by sex, and fed twice per day with Gemma Micro 300 (Skretting, Tooele, Utah). Approximately six days before the experiment, four pairs of male and female fish were placed in breeding tanks containing a divider and a plastic plant to encourage egg laying. The following morning, the dividers were removed and the fish were allowed to mate for two hours. After returning the adult fish to their home tanks, eggs were collected by pouring the mating tank water through a tea strainer. The embryos in the strainer were rinsed with E3 embryo medium to transfer them into a petri dish. The eggs were placed into an incubator at 28.5 °C, and the embryo dishes were cleaned daily by removing dead embryos and exchanging ~50-70% of the E3 solution. These methods are detailed in the book *Zebrafish* (Nusslein-Volhard and Dahm, 2002).

Laboratory Consumables

- Polystyrene disposable 100x15 mm petri dishes
- Polystyrene disposable 50x9 mm petri dishes
- Disposable 1 mL transfer pipettes
- 50 mL polypropylene conical tubes
- Depression slides and coverslips
- Colored labeling tape

Solutions

- E3 embryo medium (5 mM NaCl, 0.33 mM $MgCl_2$, 0.33 mM $CaCl_2$, 0.17 mM KCl, 10 % methylene blue, pH 7.2)
- 0.016% Tricaine (MS-222; Sigma-Aldrich A5040; St. Louis, MO) in E3 (protect from light). Make stock concentration at 0.4% in distilled water
- 2-3% low gelling temperature agarose (Sigma A9414) in E3 medium with 0.016% tricaine (kept in 44°C water bath)
- 0.03, 0.1, 0.3, 1, 3 and 10 mM Dihydrostreptomycin (DHS; CAS Number: 5490-27-7; Sigma-Aldrich D1954000; St. Louis, MO) in E3 via serial dilutions of stock quantities of DHS and 100x E3
- FM 1-43 (Thermo-Fisher T35356) at stock concentration of 1 mg/L (1,000x) in molecular grade water. Protect from light and store in small aliquots at -20°C

Solution Hazards

Stocks of DHS and FM 1-43 are handled by the instructor using gloves and proper technique. FM 1-43 is listed as not hazardous on its Material Safety Data Sheet (MSDS). DHS has health warnings and is considered harmful if swallowed. As with all chemicals in the lab, care should be taken when working with solutions.

Equipment

- Dissection microscope to observe gross motor behaviors of zebrafish larvae, which can include startle responses by lightly tapping on the side of the petri dish with a pen (ideally one per laboratory group)
- Nikon Eclipse 50i epifluorescence microscope or similar with 10x and 40x objectives (air or water objectives will work) and GFP/FITC fluorescence
- Digital microscope camera
- Computer for image and data analysis
- Laboratory timers (one per group)

Imaging and Analysis Software

- Igor Pro (Special Coursework License; Wavemetrics; Lake Oswego, Oregon), Microsoft Excel (Microsoft Corporation; Redmond, WA) or other comparable data analysis and spreadsheet software
- Proprietary imaging software or MicroManager open access software for controlling digital camera
- FIJI, an open-source image-processing package of ImageJ that includes useful tools for microscopy (National Institutes of Health; Bethesda, MD)

Methods in the Classroom

We have offered this lab in multiple separate semesters and found that it works best to teach it over two laboratory sessions. In the first session, students practice handling individual larvae, moving them quickly between dishes and with a minimal amount of liquid transfer to reduce contamination of rinse dishes with the FM 1-43 dye. Practice is also needed for mounting larvae on sides. During short lectures at the onset of the laboratories, students learn about the information necessary to understand their experiments, which includes hair cell structure and function, the zebrafish lateral line system, and fundamentals of epifluorescence microscopy and fluorescent molecules. A brief introduction to the use of FM 1-43 as a measure for open mechanotransduction channels is also provided (Gale et al., 2001). Thus the first laboratory session is primarily a run-through of the entire method without the use of DHS, and the second session requires data collection from control and experimental fish, which students complete with more efficiency due to the experience gained in the first lab session. Students typically work in groups of 2-3, with each group labeling and collecting data from both control larvae and experimental larvae with a single DHS concentration.

Fluorescent labeling of control larvae

Each group of 2-3 students was given ~10 larval zebrafish in a petri dish filled with E3-embryo medium at room temperature. Using a 1 mL transfer pipette, students transferred a single larva into a 50 x 9 mm petri dish containing 2 µl of the 1,000x fluorescent dye FM 1-43 diluted in 2 mL of E3 medium (Figure 3B). The larva was labeled for 30 seconds and then transferred and rinsed subsequently in two separate 100 mm petri dishes containing E3 medium only (Figure 3C, top). To avoid cross-contamination of the rinse dishes, laboratory tape of different colors was used to match separate pipettes to each petri dish. Finally, students placed the larva into a petri dish containing the anesthetic tricaine (0.016% in E3) for ~2 min and verified the absence of a startle response by tapping on the side of the dish (Figure 3D). The anesthetized larva was transferred with a new pipette into a 50-mL conical tube containing low gelling temperature agarose with tricaine. Finally, students rapidly removed a small aliquot of low-melt agarose containing the larva and deposited both onto a

depression slide that was immediately covered with a glass coverslip (Figure 3E). The ideal position of the larva is on its left or right side, however head neuromasts can still be imaged if the larva is not in this position. After waiting ~5 min for the agarose to solidify, the mounted larva was ready for fluorescent imaging (see below). Performing the last step expediently was important to guarantee appropriate mounting of the larva onto the microscope slide. FM 1-43 solutions and dishes should be protected from light when not in use, perhaps using tin foil over the petri dish lids.

Fluorescent labeling of experimental larvae

To assay the dose-dependent DHS block of the MET channel, the experimental larvae were fluorescently labeled with FM 1-43 in the presence of a range of DHS concentrations. Each group of students placed a larva into a pre-labeling petri dish containing a specific concentration of DHS in E3 (Figure 3A, bottom). Each was responsible for one concentration of DHS, with ideally two or more groups overlapping on a given concentration. Because ligand binding typically follows the Law of Mass Action, the following log and half-log concentrations were chosen for DHS: 0.03, 0.1, 0.3, 1, 3 and 10 mM. Students were shown that for a Hill equation with slope n = 1, a reduction from 10% to 90% of fluorescence will fall within a span of one log unit to either side of the IC_{50}. This relationship is shown with *Equation 2* using hypothetical values of 3 mM IC_{50} (50% block) and 0.3 (~10%) and 30 mM (~90%) DHS concentrations. Next, the larva was transferred to the labeling dish, which contained 2 µL of FM 1-43 and 2 mL of E3 plus DHS at the experimental concentration (Figure 3B, bottom). It was crucial that all groups worked with a fixed volume of E3 and a fixed concentration of FM 1-43. As with control larvae, each larva was labeled for exactly 30 seconds. Rapid transferring of the larva took practice as did transferring the larva with a minimum amount of E3+FM 1-43. After labeling, each larva was rinsed in E3 solutions that also contained the experimental concentration of DHS to avoid labeling of unblocked hair cells by contaminating FM 1-43 (Figure 3C, bottom). The anesthesia and mounting steps followed the same procedure as described for control larvae (see Figs. 3D and E).

Visualization: Hair Cells and Fluorescent Imaging

Students placed the depression slide with the mounted larva on an upright, epifluorescence microscope equipped with a GFP/FITC filter set. Students first observed the larva under transmitted light with the 10x and 40x objectives. Initially, they noted the major organs of the larva, including the eyes, ears, olfactory pits, heart and circulatory system and lateral line system. After a neuromast of the anterior or posterior lateral line system was identified under transmitted light (see Ghysen and Dambly-Chaudière, 2004; Chitnis et al., 2012 for review of lateral line system in zebrafish larvae), students observed the hair cells under fluorescence illumination. Bright field and fluorescence images of three different neuromasts at different focal planes were taken using the SPOT software and an 8-bit digital camera (Figure 4). The students were instructed to select the exposure time and gain manually with settings that resulted in the clearest image and to hold these settings constant for all subsequent images for both their control and experimental conditions. It is critical that pixel intensity in captured images is not saturated as linear changes in fluorescence intensity would be lost. Similarly, it is very important that camera settings are not changed throughout the experiment. Students should be shown how to check for saturation using the camera software and perhaps also within the ImageJ software. Also, control labeling and imaging should be performed first because these images will be brighter and allow the camera settings to be optimized to the brightest images. These points can be highlighted on the laboratory handout as well as during the instructor's pre-lab lecture. We also note that depending on the number of available microscopes, lab sessions can be staggered to have students arrive in groups to minimize students waiting to use

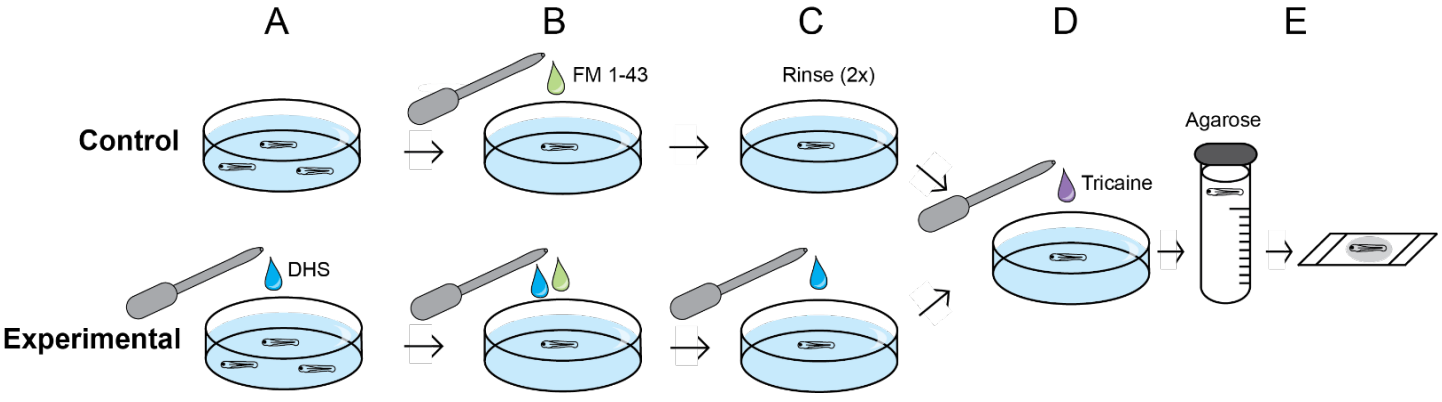

Figure 3. Experimental method for labeling larvae with FM 1-43. (*A*) Pre-labeling dishes for control and experimental larvae. DHS (blue drop) is added at the test concentration to all experimental dishes. (*B*) For control larva, the labeling dish contains FM 1-43 (green drop) and the experimental dish contains both FM 1-43 and DHS. (*C*) Rinse dishes for experimental larvae contain DHS. Multiple rinses (2 or 3x) with several different dishes is recommended for both control and experimental larvae. (*D, E*) The larva is moved to an anesthesia dish and then an agarose conical for immobilization of larvae prior to mounting on a microscope slide for fluorescence microscopy.

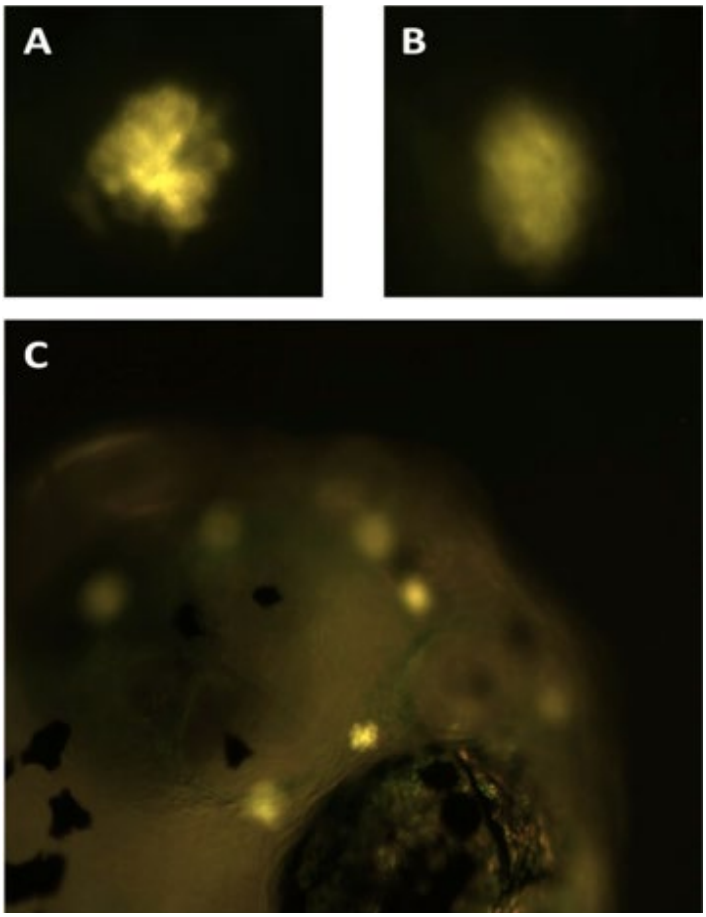

Figure 4. Figure of fluorescent neuromasts taken from a student lab report. (*A*) FM1-43-labeled neuromast from a control larva at 40x magnification. The circular region of interest (ROI) was drawn in FIJI. (*B*) Fluorescent image of a neuromast from an experimental larva (0.3 mM DHS). (C) Image showing fluorescently labeled neuromasts surrounding the head of a zebrafish larva at 10x magnification (overlay of DIC light and GFP fluorescent images). Figure by Alex Ordoobadi, Amherst College class of 2015.

the microscope(s). The process from labeling through to imaging can be completed within an hour if necessary, with analysis and discussion happening offline or during another class or lab session.

The collected images were analyzed using ImageJ. A region of interest (ROI) was drawn around each neuromast using the circle outline tool. It was important to minimize the amount of background surrounding the neuromast included in the ROI, which would otherwise bias the mean pixel intensity. Under Analyze>Tools>ROI Manager, the students clicked on "Add [t]" and used the "Measure" option to obtain the mean pixel intensity for the ROI as computed by the program. Students carried out this process for neuromasts from both control and experimental larvae. In future iterations of this lab, it would be beneficial to subtract background fluorescence by measuring an ROI in a non-hair cell region of the image, which would reduce the potential confounding effect of certain ROIs containing more background area than others.

Data Analysis

Each group used IgorPro or another comparable data analysis and spreadsheet program to average the mean intensities of the three or more neuromasts imaged from each control and each experimental larva. The average fluorescence intensity reduction was determined from the following equation:

Equation 3. $$\% \text{ reduction} = 100 \times (I_0 - I_x)/I_0$$

where I_0 is the value of the control fluorescence intensity and I_X is the experimental fluorescence intensity for each concentration of DHS tested. Normalizing the data obtained from each group accounted for differences in microscopes, camera settings, and individual technique. The students then entered their individual data in a Google Docs sheet so that every student had access to fluorescence measurements taken under every concentration of DHS. The mean percent reduction in fluorescence for each DHS concentration was plotted against the concentration of DHS with the X-axis set to a log scale (Figure 5). Students were asked to fit the Hill equation (*Equation 2*) to their plot of the data and calculate the IC_{50} value. Finally, the students were given the freedom to choose their own approach to

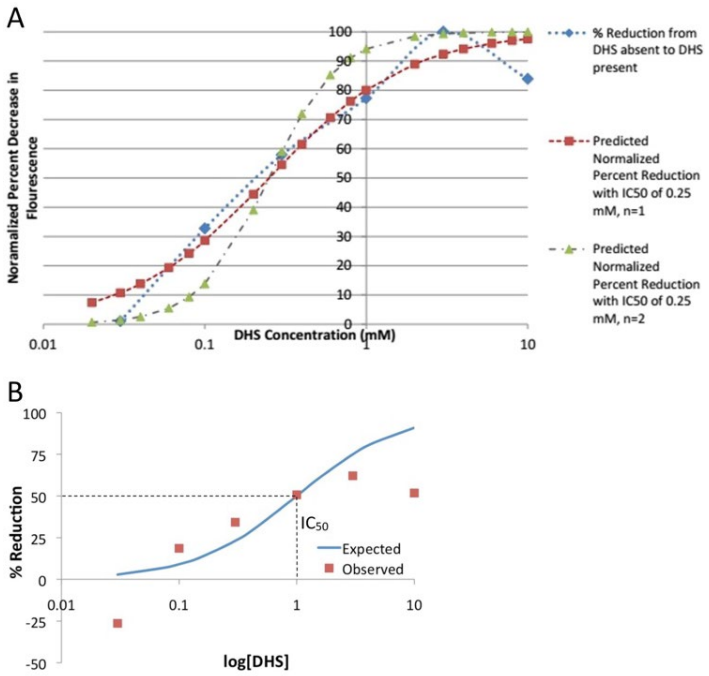

Figure 5. Two different plots created by students using data collected in the laboratory. Both graphs plot the percent reduction in fluorescence versus the concentration of DHS (note X-axis on a log scale). (*A*) Shown are two hypothetical Hill equations with slopes of n = 1 (red) and n = 2 (green) and the student's data connected by a line (blue). Plot by Kyra Shapiro, Amherst College class of 2015. (*B*) A plot where the student did not normalize the percent-reduction to 100% block. The Hill equation with slope of 1 did not fit to the data as well, but still allowed the student to visualize the IC_{50} value, a concentration that would produce a half-maximal decrease in fluorescence. Plot by Alex Ordoobadi, Amherst College class of 2015.

manipulating the curve and presenting their hypothesis and results in the context of a biologically relevant research question. The format of the lab report was chosen to be a scientific paper as described in the *Background* section.

Student Evaluation

To evaluate whether the laboratory was successful in achieving the above stated learning objectives, the following survey was given to the class of students in the fall of 2016:

Please evaluate from 1 to 5 how effective the zebrafish MET channel lab was in accomplishing each of the following statements:

(1 = not at all effective and 5 = very effective).

1) Teaching me about the structure and function of MET channels
2) Teaching me about the pharmacology of pore blocking molecules, ligand-receptor interactions, dose-dependent relationships, and the Hill equation
3) Teaching me about fluorescent dyes and epifluorescence microscopy (excitation and emission filters and dichroic mirrors, etc.)
4) Teaching me how to capture and quantify microscopic images using an epifluorescence microscope with a digital camera
5) Teaching me about the zebrafish model organism including hands-on experimentation with larvae
6) Improving my research abilities including collection and analysis of data, constructing figures and presenting results in the form of manuscript-style lab reports
7) Improving my writing ability to construct and evaluate hypotheses and contextualize my conclusions within the scientific literature

RESULTS

Classroom Results

Students successfully labeled hair cells from lateral line neuromasts with FM 1-43 in both control (without DHS) and experimental (in the presence of a fixed concentration of DHS) larvae. Images captured by students demonstrated that control neuromasts (Figure 4A) appeared to have brighter fluorescence than those treated with DHS (Figure 4B). After photographing and analyzing control and experimental neuromasts, students calculated the percent reduction in fluorescence between experimental and control neuromasts. They then plotted these data against the concentrations of DHS and generated dose-response curves. Some students chose to fit their data to the Hill equation by hand using a large column of incrementing X values and solving for intensity (I) values based on estimated parameter values for IC_{50} and Hill slope (n), while others used a curve fitting program. This decision was based mostly on the level of math and computer science courses that students have taken.

Figure 5 shows example graphs generated by students, both of which include the recorded data and an attempt at fitting the data with the Hill equation. Both of the curves have a sigmoidal shape, and the bulk of the fluorescence reduction (~10-90%) takes place within two log units surrounding the predicted IC_{50} value that each student calculated. As mentioned in the Methods section, this finding indicates that the Hill slope is likely to be around n = 1. For these data, the students found that saturating DHS decreased fluorescence by a maximum of 60%, and the top plot shows normalized percent reduction values using this percentage as the "maximum" reduction. The result of a 60% max reduction rather than a complete 100% loss of fluorescence may be due to multiple sources of experimental error, including transfer of FM dye into the rinse steps. Using a normalized maximal fluorescence intensity allows the students to fit to the Hill equation, which assumes 100% effect. Students were encouraged to discuss these sources of error in their lab reports. The data shown in Figure 5A were fit to both a Hill equation with n = 1 (shown in red) and n = 2 (shown in green). The quality of fit of the Hill equation with a slope of 1 allowed the student to conclude that DHS appeared to bind to MET channels in a one to one manner. In contrast, the student that created the plot shown in Figure 5B chose to not normalize the percent reduction to 100% and to fit with only n=1. Ultimately, students were given the freedom to interpret their data and draw their own conclusions in their individual lab reports.

Student Evaluation Results

Sixteen students out of the twenty that participated in the Fall 2016 laboratory exercises responded to the survey described in the above *Student Evaluation* section. In the survey, students ranked the extent that the laboratory had been successful in their achievement of each learning objective (these results are summarized in Figure 6). For all of the learning objectives, a majority of students reported either a 4 or 5, and no student reported a score below 3 for any objective. Objective 4, "to gain experience capturing and quantifying microscopic images using an epifluorescent while objective 2, "to learn about the pharmacology of pore blocking molecules, ligand-receptor interactions, dose-dependent relationships, and the Hill equation," had the

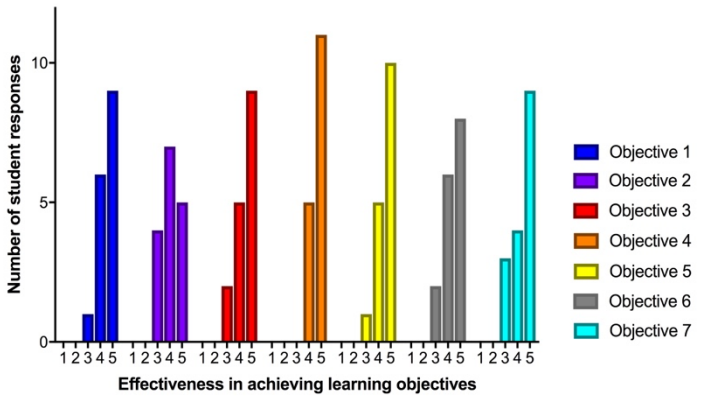

Figure 6. The results of the evaluation filled out by students from the fall 2016 laboratory. There were 16 out of 20 student responses to the survey. Students rated the effectiveness of the teaching lab from 1 to 5 (one was not at all effective and 5 was very effective) in achieving each of the learning objectives, as outlined in the *Methods* section.

lowest average rating. In future iterations of the course, we could imagine adding a category on learning experimental design and the importance of controls, replicates, et cetera.

DISCUSSION

Summary

This laboratory provided students with an opportunity to develop many important skills needed for biological research. Through hands-on experimentation, students gained experience working with the zebrafish model organism, labeling cells with fluorescent molecules, and using epifluorescence microscopes, digital cameras, and image analysis. Through lectures and their own independent research for lab reports, students learned about hair cells, MET channels, pore-blocking molecules, dose-dependent interactions, and the Hill equation. Writing their lab reports as structured scientific papers, students furthered their ability to collect, analyze, and present scientific data, construct high-quality figures, and clearly express their hypotheses and conclusions.

The two classes that carried out this laboratory were successful in collecting data and benefitted from their experiences as shown in a survey of the class that completed the lab in 2016. The results indicate that the students considered the lab effective in helping them achieve the stated learning goals. The survey also helped identify the strongest aspects of the lab as well as those that have the potential to cause students difficulty and thus merit more careful discussion by instructors. As noted above, most students gave the highest rating to learning objective number 4, “to gain experience capturing and quantifying microscopic images using an epifluorescence microscope with a digital camera”, which indicates that the lab did a good job of exposing students to the use of epifluorescence microscopes and the acquisition of digital images. In contrast, also noted above, objective 2, “to learn about the pharmacology of pore blocking molecules, ligand-receptor interactions, dose-dependent relationships, and the Hill equation,” had the lowest average rating. Curve fitting and applying the Hill equation is one of the more challenging areas of the lab, and the relatively lower rating for this objective suggests that students will benefit from more in-depth presentation of background information before beginning their own data analysis and lab reports.

We note that many students mentioned in their end of semester evaluations how much they enjoyed this laboratory. They noted that labs that are based on testing hypotheses and performing experiments where the outcome was not known, or at least not well established, was far more enjoyable than following a recipe-like laboratory. Students also mentioned how variability of their results was challenging to consider and that it gave them a stronger idea of how experiments in the “real world” don’t always go as planned. A large number also remarked on how much they learned through the process of plotting and analyzing their data and crafting a formal lab report in the style of a journal article with writing that is motivated by hypothesis testing.

Another positive aspect of this laboratory is that it follows a relatively simple and inexpensive protocol. Zebrafish are easy to maintain and breed and many institutions already possess a zebrafish facility or have access to a zebrafish lab at a nearby school. The hair cells of the lateral line system are exposed to the external environment, which makes fluorescent labeling straightforward, while the transparency of larvae makes visualizing fluorescently labeled neuromasts uncomplicated, without requiring additional dissection or sectioning. Altogether, the laboratory is accessible and informative for undergraduate biology and neuroscience students.

Adapting the laboratory to course goals and alternative experiments

There are multiple ways to teach this lab and instructors may adapt their teaching in order to fulfill a variety of different course goals. The background information presented to students may vary depending on what questions instructors wish them to answer in their lab reports. One option is to have students focus on specific information about only one of the drugs (either FM 1-43 or DHS) and ask them to predict and model the behavior of the other drug (e.g., if DHS blocks the MET channel in a 1-1 fashion, what is the expected shape of a dose response curve?) What does it mean if the curve shows a 10-90% reduction in fluorescence between a log unit on either side of the DHS IC_{50}? Another possibility is to give students information about both drugs and how they interact and ask them to determine that the interaction occurs as predicted. Are the relative sizes of FM 1-43, DHS, and the MET channel consistent with their observed results? Students can also be asked to characterize the binding of DHS to MET channels and determine the best fit slope for the Hill equation (*Equation 2*). Instructors may choose an approach that best fits with the focus of the lecture portion of their class, including forgoing the DHS component and simply labeling hair cells with the FM dye.

There are many ways in which the experiments carried out in the laboratory exercise can be modified or extended based on specific course needs. For example, other aminoglycoside antibiotics or other types of drugs/compounds/small molecules can be used with this experimental protocol (e.g. Kruger et al., 2016, for example). These experiments would allow for calculation of respective IC_{50} values and maximal fluorescence reduction or even potentiation of fluorescence. Results between multiple compounds could be compared to characterize their relative effects on hair cell mechanotransduction. For instance, the relationship between a compound’s molecular structure and its ability to block the channel (or enhance conductance) could be used to estimate the size of the channel pore (Farris et al., 2004). Each lab group could also perform multiple DHS concentrations themselves using the same camera exposure settings and compare these results to the entire classes normalized results. Finally, if a confocal microscope is available, capture of higher resolution images and more accurate quantification of results could be obtained. This method would also present an opportunity to teach students about the principles of confocal microscopy.

Limitations

There are several limitations in the experimental design that may influence results obtained. Students should be

encouraged to identify these sources of error and discuss them and their impact on the results in their lab reports. One possible limitation is that results may be skewed by the small sample size due to the constraints of carrying out such an experiment in a classroom setting over a short period of time. A second possible limitation of this experiment is that the control and experimental neuromasts are examined from different larvae and each larva is labeled and imaged by different groups. Both sources of variability may result in differences in the measured fluorescence values. Additionally, using ImageJ to analyze circular ROIs drawn around neuromasts can result in non-hair-cell "background" regions that may influence the measurement of each ROI's mean fluorescence. There may also be a variability in the size of the ROI from student group to group. Instructors may wish to explore the freehand tool in ImageJ for improved ROI creation. Another possible source of error in the experiment is variation of labeling time between larvae during FM 1-43 incubation (Gale et al., 2001). The amount of time larvae spend in the labeling dish affects the amount of FM 1-43 that enters hair cells and thus the amount of fluorescence measured in neuromasts. Longer labeling times are also associated with endocytosis at the apical surface and this would also affect percent block calculations. Contamination of FM 1-43 in the transfer pipettes and rinse dishes can also be a source of error, which is why we recommend including DHS (at the experimental concentration) in all rinse dishes. Students should also be able to transfer larvae quickly between dishes and with minimal liquid in the transfer pipette. The shelf life of FM 1-43 is limited once resuspended in water and we suggest using a fresh aliquot for every lab section and a fresh stock for every course iteration. Finally, photobleaching may also skew fluorescence measurements (Iwabuchi et al., 2014). For this reason, students should take care not to expose FM 1-43 labeled larvae to light for an extended period of time prior to and during imaging, as it may artificially reduce the measured fluorescence.

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