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Zebrafish in Education

Zebrafish Embryology and Cartilage Staining Protocols for High School Students

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The Life Sciences–Howard Hughes Medical Institute Outreach Program at Harvard University supports high school science education by offering an on-campus program for students and their teachers to participate in investigative, hands-on laboratory sessions. The outreach program has recently designed and launched a successful zebrafish embryology protocol that we present here. The main objectives of this protocol are to introduce students to zebrafish as a model research organism and to provide students with direct experience with current techniques used in embryological research. The content of the lab is designed to generate discussions on embryology, genetics, fertilization, natural selection, and animal adaptation. The protocol produces reliable results in a time-efficient manner using a minimum of reagents. The protocol presented here consists of three sections: observations of live zebrafish larvae at different developmental stages, cartilage staining of zebrafish larvae, and a mutant hunt involving identification of two zebrafish mutants (*nacre* and *chokh*). Here, we describe the protocol, show the results obtained for each section, and suggest possible alternatives for different lab settings.

Introduction

Harvard University conducts a Life Sciences Outreach Program that is hosted by the Department of Molecular and Cellular Biology and supported by a grant from the Howard Hughes Medical Institute (HHMI) Undergraduate Education Program. This program invites local high school biology students and their classroom teachers into Harvard's undergraduate biology teaching laboratories each spring for a single 3 h lab session. MCB graduate students and postdoctoral fellows lead the laboratories in conjunction with the classroom teachers. The goal of this program is to support high school biology education by bringing Harvard scientists together with educators to help enhance and engage the local community. The zebrafish teaching protocol presented here was designed for Life Sciences–HHMI Outreach Program's Laboratory Workshop for High School Classes.

The aim of this zebrafish lab is to provide a meaningful hands-on classroom lesson that incorporates vertebrate embryology and current techniques that yield reliable results. In designing this zebrafish lab teaching protocol, we wanted to introduce students and teachers to a wide range of concepts in vertebrate developmental biology including embryology, heart development, cartilage deposition, genetic influences, fertilization, and animal adaptation. The lab was designed to be modular so that each section could be completed independent of the others and on nonconsecutive days. Section 1 introduces students to zebrafish as a model organism and its utility in developmental research through observations of living fish embryos. Section 2 allows students to perform cartilage stainings while learning about cartilage development. Section 3 introduces students to genetic variations in zebrafish. We have also streamlined the techniques and equipment used in this protocol for greater applicability at other institutions.

We used this teaching protocol for three biology classes of different levels. Students were able to follow the protocol and obtain clear results, which promoted discussions in all three classes on topics such as genetic modifications, human genetics, and environmental adaptation. Feedback from the high school students and teachers was positive, and the zebrafish protocol will be added to the Life Sciences–HHMI Outreach Program's high school laboratory curriculum.

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Results

Our visiting high school classes consisted of 16–22 students. For the three sections, students worked in pairs with each pair sharing a single microscope. An introductory presentation to the students was used to give an overview of the zebrafish and the main concepts of the protocol. The presentation discussed zebrafish as a vertebrate model organism, different embryological stages of the zebrafish, and some currently used techniques and their roles in developmental biology research such as cartilage staining. We also showed a movie of the zebrafish circulatory system (Supplemental Movie S1, available online at www.liebertonline.com). Also included in the presentation was a movie we made giving a tour through our zebrafish facility at Harvard University. After the presentation, the students were given the zebrafish lab protocol (Supplemental Document S1; available online at www.liebertonline.com).

Section 1: observing live embryos

The aim of this section was to get the students acquainted with live zebrafish embryos. The teaching fellows (TFs) distributed wild-type (WT) embryos at two developmental stages, 24 hours postfertilization (hpf) and 3 days postfertilization (dpf) (Fig. 1), in Petri dishes containing fish water to each pair of students. The students examined the embryos under a dissection microscope and were instructed to view the fish using several magnifications. If embryo movement at 3 dpf made observations difficult, students could add 3% methylcellulose to the fish water to increase the fish water viscosity. The students were then asked to find the beating heart and signs of circulation in their 3 dpf embryos. TFs assisted the students in using the dissection microscope and with their observations of the circulatory system.

Section 2: cartilage stain

This procedure allowed students to experience a fairly common technique that scientists use to stain cartilage in larval zebrafish.[1,2] We opted to use *nacre* (*nac*) mutant larvae for this procedure as this mutant lacks melanophores throughout embryonic and larval development.[3] The *nac* mutant larvae are completely transparent and only express pigment in the pigment epithelium layer of the retina. We decided to use this mutant to facilitate the visualization of the cartilage staining. However, it is not necessary to use this mutant, and WT larvae will yield similar results. We chose two developmental stages: 3 dpf, an early stage that would not produce cartilage staining, and 9 dpf, a later stage that would yield good cartilage staining particularly around the jaw. Larvae at the two developmental stages were prepared in advance by fixation in acid/ethanol and kept at 4°C. Before the arrival of the students, the TFs aliquoted the embryos into Petri dishes containing the fixative. Students were required to wear latex gloves and safety goggles throughout this procedure. Before the staining, the students compared the two

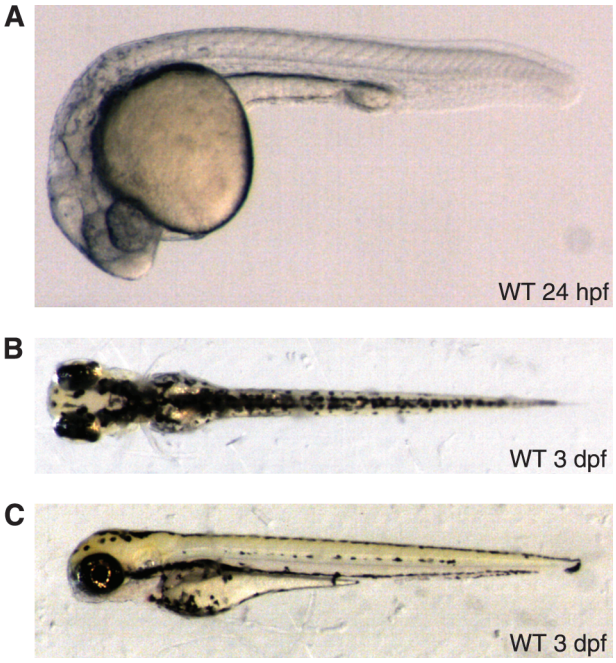

FIG. 1. Different stages of WT embryos. (**A**) Embryo at 24 hpf. (**B**) Dorsal view of embryo at 3 dpf. (**C**) Lateral view of embryo at 3 dpf.

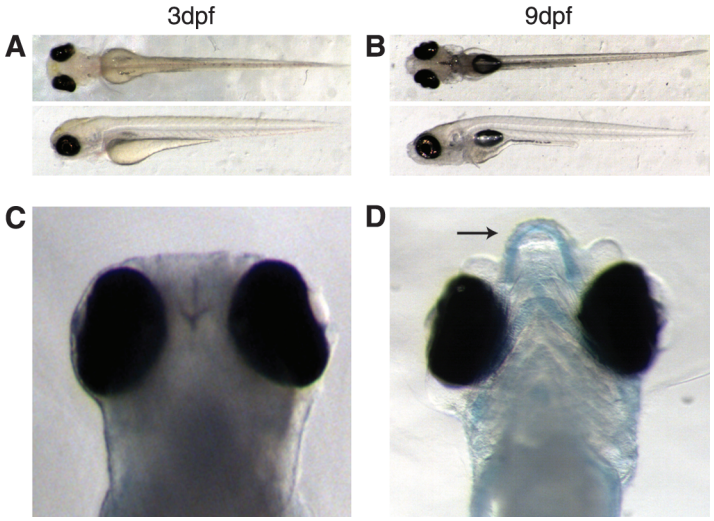

FIG. 2. Results of cartilage staining of *nacre* embryos. (**A**) Embryo at 3 dpf and (**B**) 9 dpf before staining. Upper and lower panels are dorsal and lateral views, respectively. (**C**) Close up of head in ventral view of alcian blue cartilage staining at 3 dpf. At this stage the cartilage staining is diffuse and nonspecific. (**D**) At 9 dpf the cartilage staining is visible and the jaw becomes prominent (arrow).

developmental stages (Fig. 2A, B) and noted the differences, such as size and morphology.

The students removed the fixative from the dishes using small transfer pipettes while avoiding damage to the samples. The embryos were then submerged in 0.2% alcian blue and stained for at least 30 min. During this incubation time, examples of other vertebrate stainings using alcian blue were passed around, such as a mouse embryo.

To wash the samples, the students removed the stain from the dishes with a small transfer pipette and added acid/ethanol. The embryos were left submerged for at least 10 min to remove any stain not bound to cartilage. After the washing step was completed, the acid/ethanol was removed from the dishes and KOH/glycerol was added to the embryos to clear the samples. The purpose of the KOH/glycerol in making the embryos transparent for visualization of the cartilage stain was explained to the students. After the embryos incubated in KOH/glycerol, the students examined the results using microscopes. An example of the cartilage stain is shown in Figure 2C and D.

Section 3: examining WT and mutant zebrafish embryology

This section introduced the effects of genetic mutations in zebrafish embryology. Two mutants with distinct and easily identifiable phenotypes were used. The *nac* mutant, as mentioned above, lacks melanophores throughout development; the eyeless *chokh* (*chk*) mutant has a mutation in the Rx3 homeodomain–containing transcription factor and completely lacks eyes from the earliest stages of development.[4] Mutant and WT embryos were fixed in advance at two developmental stages: 24 hpf and 5 dpf (Fig. 3). Students were required to wear latex gloves and goggles for this section of the protocol.

Each pair of students received one of the two mutants and WT embryos at the same two developmental stages. WT and mutant embryos were distributed in separate dishes so that the students could compare the genotypes. First, the students were instructed to compare the two developmental stages of only one set of the unidentified embryos. Then, they were asked to examine the other set of embryos to identify any differences between the two sets at the two developmental stages. The students transferred the fixed embryos into a Petri dish containing a viscous solution of 3% methylcellulose so that they could easily view the samples from all angles using a toothpick to align the samples. Comparing the two embryonic stages reinforced the differences in development and also provided an example for discussion that phenotypes of different mutations are not all identifiable at the same stage of development. The *nac* mutant at 24 hpf does not look significantly different from the WT embryos at 24 hpf (compare Fig. 3A and B). However, by 5 dpf the *nac* mutant larvae are easily distinguishable from the WT larvae due to a lack of body pigmentation (compare Fig. 3D and E). In contrast, the lack of eye development of the *chk* mutant at 24 hpf was obvious to students when compared to the WT embryos at the same developmental stage. The students drew the embryos and compared their finding with their classmates to discuss what the two mutant phenotypes might be. The TFs then discussed how the different phenotypes arise from mutations in specific genes.

Discussion

The Harvard University Life Sciences–HHMI Outreach classes that we taught consisted of a small but diverse sampling of the Boston-area population with students enrolled in remedial science through Advanced Placement biology. For all classes, we had enthusiastic responses to the protocol from the students and educators. However, we found that student interactions and scientific discussions were highest when students were less absorbed in didactic note taking. The students especially enjoyed the sketching portion of the laboratory and spent time taking careful observations and recording them. The visualization of the live embryo's movements, heart beating, and circulation was very exciting to them. We have included a movie (Supplemental Movie S1; available online at www.liebertonline.com) of the embryo's heartbeat and circulation that we used in our introduction. It depicts what students were typically able to observe. Another video that we used during our lab introduction that students particularly enjoyed is a time-lapse of embryonic development. This video is available on YouTube.com under the title "Fish Embryonic Development flat-beat."

Throughout the teaching lab, students were kept constantly engaged in the steps of the procedure and through additional satellite workstations and activities. Because the students used fixed embryos for many of the procedures, we used several satellite stations that had live mutant larvae at 5 dpf in Petri dishes for students to observe under a light microscope.

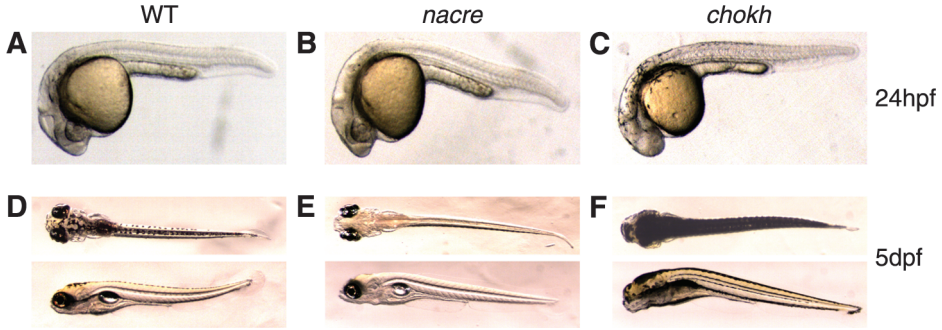

FIG. 3. Embryos used for the mutant-hunt. Twenty-four hours postfertilization embryos of (**A**) WT, (**B**) *nacre*, and (**C**) *chokh* zebrafish. Embryos at 5 dpf of (**D**) WT, (**E**) *nacre*, and (**F**) *chokh* zebrafish. Note lack of body pigmentation in *nacre* mutants, and lack of eye development in *chokh* mutants. Upper and lower panels are dorsal and lateral views, respectively.

We also had satellite stations with examples of adult fish of different genetic backgrounds such as WT AB, WT TL, *nacre*, and other mutants. To demonstrate zebrafish mating, fertilization, and egg-laying behavior, and how we obtain eggs, a small tank containing a male and female adult zebrafish and their eggs was shown to the students at another workstation. Although all of the WT embryos we used in the protocol were of a separate background (AB strain) than the mutants, our mutant hunt protocol can be adapted to add a section on classical Mendelian genetics. For example, after students have properly identified mutant phenotypes they can be given dishes of mixed WT and mutant embryos at specific ratios, and recessive versus dominant traits can be discussed.

Sections 1 and 2 of our protocol can also be used at institutions without a zebrafish facility, and instructions for breeding zebrafish from a pet store to obtain embryos are also included (Supplemental Document S2; available online at www.liebertonline.com).

Materials and Methods

Animals

Zebrafish were obtained from the Harvard University Fish Facility. Zebrafish were maintained on a 10-h dark and 14-h light cycle at 28°C.[5] All WT embryos used in the protocol were of AB strain. The *chk* mutation is maintained in a heterozygous line in a TL background. To obtain homozygous *chk* mutant embryos, crosses between heterozygous adult fish were set up at night and eggs were collected the following day. The *chk* mutation is recessive, and the eyeless phenotype is fully penetrant.[4] Mutant embryos were screened at both 22 hpf and 5 dpf using the lack of eye development and darker body pigmentation as morphological markers. The *nac* mutation is also recessive, and embryos were obtained from a cross between homozygous adult *nac* mutants. This mutation specifically affects pigment cell fate in the neural crest. Homozygous *nac* mutants lack melanophores throughout development resulting in the loss of the zebra stripes along the body. The *nac* mutants do have normal dark eye pigments, however, as the retinal pigment epithelium is nonneural crest derived.[3]

The different developmental stages and genotypes used for each part of the protocol are shown in Table 1. The number of fish given to each pair of students was 5–7 for each genotype.

Staging of embryos

All embryos were collected 2 h after the beginning of the light cycle, and staging of embryos was performed under a light microscope according to Kimmel *et al.*[6]

Table 1. Genotypes and Embryonic Stages Used in the 3 Sections

Procedure	*Type of embryo*	*Developmental stage*	*Fixation*
Section 1	WT	24 hpf and 3 dpf	No
Section 2	*nac* mutant	3 and 9 dpf	Yes
Section 3	WT	24 hpf and 5 dpf	Yes
	nac mutant	24 hpf and 5 dpf	Yes
	chk mutant	24 hpf and 5 dpf	Yes

Fixation of embryos

For Sections 2 and 3, students used fixed embryos. Before the lab, TFs prepared the samples to minimize the students' exposure to hazardous chemicals. Staged embryos were anesthetized in a dish containing a solution of 0.001% w/v MS-222 (Tricaine, Sigma A-5040; Sigma-Aldrich, St. Louis, MO). Samples were immersion fixed with acid/ethanol (0.37% HCl and 70% ethanol) and transferred to 2 mL tubes for storage at 4°C. For optimal results, embryos were fixed for at least 10 h at 4°C before stainings.

Cartilage staining

Fixed *nac* mutant embryos at 3 and 9 dpf were gently transferred into small 35×10 mm Petri dishes with lids using a small plastic transfer pipette and given to students. During the cartilage staining procedure, students wore gloves and goggles and disposed of liquid waste in a designated waste bottle. Without disturbing the embryos in the dish, the acid/ethanol fixative was removed and replaced with the alcian blue cartilage stain consisting of 0.2% w/v alcian blue (Sigma A-3157) diluted in the acid/ethanol fixation solution. The embryos were submerged in the stain for 30 min at room temperature. The alcian blue stain was removed and replaced with the original acid/ethanol fixation solution. The embryos were washed with gentle intermittent plate swirling for at least 10 min to remove any stain not bound to cartilage. After this washing step the acid/ethanol was removed and the embryos were submerged in KOH/glycerol (1% KOH; 50% glycerol) for at least 20 min.

Manipulation of embryos

To visualize embryos each pair of students used a dissection stereomicroscope. For immobilization of live embryos or to position cartilage stained embryos, 3% methyl-cellulose (Sigma M-0387) was added to the Petri dishes containing the embryos. Toothpicks were used to gently position the embryos for visualization.

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Disclosure Statement

No competing financial interests exist.

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