

ARTICLE

Open-Ended Inquiry into Zebrafish Nerve Development Using Image Analysis

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Open-ended laboratory projects increase student success and retention in the sciences. However, developing organismal-based research projects is a challenge for students with restricted laboratory access, such as those attending courses remotely. Here I describe the use of image analysis of zebrafish neural development for authentic research projects in an introductory biology laboratory course. Zebrafish are a vertebrate model that produce large numbers of externally and rapidly developing embryos. Because zebrafish larvae are transparent, fluorescent reporters marking nervous system structures can be imaged over time and analyzed by undergraduate scientists. In the pilot of this project, remote first-year college students independently developed biological questions based on an image collection comparing zebrafish mutants and wild-type siblings. Students created and mastered techniques to analyze position, organization,

and other morphological features of developing neurons and glia in the images to directly test their biological questions. At the end of the course, students communicated their project results in journal article format and oral presentations. Students were able to hone skills in organismal observation and data collection while studying remotely, and they reported excitement at applying lecture-based knowledge to their own independent questions. This module can be adapted by other instructors for both students on- and off-campus to teach principles of neural development, data collection, data analysis, and scientific communication.

Key words: neurodevelopment, neuroanatomy, zebrafish, sensory nerves, lateral line, motor nerves, authentic inquiry, open-ended laboratory, first-year research, image analysis, Fiji, ImageJ, remote learning

Authentic inquiry experiences early in collegiate careers are associated with improved outcomes for undergraduate science students, with increases in learning gains, persistence in the field, and sense of belonging (Bangera and Brownell, 2014; Indorf et al., 2019; Buffalari et al., 2020). The COVID-19 outbreak necessitated a shift to online, remote learning for many introductory labs. Although studies suggest that students can perform research projects as well remotely as in-person (Kirkpatrick et al., 2019; Doctor et al., 2021), lab course content had to be significantly adjusted. One challenge for all labs, but particularly at the introductory level, was providing authentic, open-ended inquiry involving data collection and analysis for students studying remotely.

Image analysis is frequently employed in research studies but is taught less often than traditional wet-lab techniques in introductory laboratories. Because image analysis can be done entirely on a computer after collecting data at the microscope, it is well-suited to remote learning. Furthermore, undergraduate students can derive suitable questions for open-ended inquiry when provided with an image collection that contains multiple parameters to analyze (Pemberton et al., 2018; Nahmani, 2019).

Zebrafish larvae are particularly amenable to live imaging and analysis, even for novices and at the introductory level (Pike et al., 1992; Marra et al., 2015; Peterson et al., 2018; Wilk et al., 2018; Prakash and Toro, 2019). Zebrafish are small, freshwater teleosts that produce many larvae from a single cross of a fertile male and female. External fertilization permits phenotypic analysis of nervous system patterning across time in a single living animal, while rapid embryonic development results in identifiable spinal cord

and peripheral nerve patterning within 3 days post-fertilization. Transgenic fluorescent reporters can be used to label neurons and glia in different genetic backgrounds and are readily observed in transparent larvae. Importantly, the major genetic regulators of nervous system development are conserved from zebrafish to humans, allowing students to draw connections to disease mechanisms.

Here, I present a laboratory module using image analysis of the larval zebrafish nervous system for remote open-ended inquiry in an introductory laboratory course. In the pilot of this module, remote first-year biology students used an instructor-created collection of images of novel zebrafish mutants with mispatterned nervous systems and compared them to age-matched wild-type siblings. This allowed students to develop their own testable questions about the development of neurons, glial cells, and related structures and tissue types in a vertebrate model system. Students then designed appropriate image analysis techniques using Fiji (ImageJ with Bio-Format Importer) to test their predictions, analyzed their data using R programming via RStudio, and presented their findings in written and oral format. In all, students were able to collect and handle novel data and develop analytic skills for independent inquiry while in a remote laboratory course setting.

PROJECT DEVELOPMENT AND RESOURCES

Learning Objectives and Skills Objectives

Following this project, students should be able to:

- Distinguish neuroanatomical features of the zebrafish nervous system, including central and peripheral

nervous systems, sensory and motor nerves, and neuron soma and axons

- Apply neuroanatomical terminology for orientation (dorsal vs. ventral, anterior vs. posterior) and cellular features (soma vs. axons) of zebrafish nervous systems
- Identify, compare, and contrast normal (wild-type) nervous system patterning with aberrant (mutant) neural development

Additionally, students develop skills in the following areas:

- Use primary literature to gain content knowledge and as models for writing scientific papers
- Collaborate with peers in experimental design
- Use Fiji to measure neuroanatomical features in fluorescent micrograph images
- Analyze data and produce graphs using RStudio

Course Structure

The introductory biology laboratory at Kenyon College, BIOL 109-110Y, is a year-long course typically taken by first- and second-year students. This course is required for students majoring in neuroscience, biology, and molecular biology and serves as a prerequisite for upper-level lab courses in those fields. The goals of the course are to develop experimental design, data collection and management, analysis, and writing skills. By the end of the first semester (prior to the project period), students are familiar with reading literature, setting up datasheets for data collection, descriptive and inferential statistics, and creating graphs in RStudio. Thus, this module can be incorporated into the end of an introductory course, or as part of a course with more senior students that are proficient with these skills.

In the second semester of this year-long course, students develop an independent project in consultation with a faculty mentor and write a research project proposal. Project development is carried out in parallel to “traditional” inquiry-based labs in the first seven weeks of the semester; that is, students strategize with prospective mentors outside of, and in addition to, weekly lab course meetings (Table 1). In the final seven weeks of the course, students carry out their proposed project with a partner. Students independently perform data collection and analysis, with their work culminating in a final oral presentation and complete project

Week	Activity
1-4	Identification of project mentor, reading literature, meeting with faculty
5	Submit annotated bibliography of five papers related to project
6-7	Draft and submit independent project proposal
8-13	Data collection, analysis, and writing. Weekly reports submitted to course instructors detailing progress, goals, and literature read
14	Oral presentation in-class
15	Final project paper in style of journal manuscript

Table 1. Independent project structure by course week.

paper in the style of a journal article manuscript. In Spring 2021, students enrolled in this course were mixed in-person and remote; on-campus students (46 of 82) generally conducted research projects in person, while remote students (36 of 82) were limited to remote research projects. Six remote first-year students participated in this independent project of zebrafish image analysis.

Zebrafish Larvae Rearing and Imaging

Early in the second semester (~8 weeks before projects commenced), zebrafish larvae were crossed, collected, and imaged by the instructor to create the database for student analysis. This project used the novel *stl159* mutant that was previously identified in a forward genetic screen with multiple developmental defects in peripheral nervous system patterning (manuscript in preparation). Additional suitable mutants are also available, as are pharmacological manipulations; these would be generally useful for this module (see “*Opportunities for modification or extension*”).

Presently, the *stl159* lesion is not fully characterized genetically; therefore, progeny were considered siblings or mutants based strictly on their phenotype. For this study, heterozygous adults for the novel *stl159* lesion were crossed to produce homozygous wild-type and heterozygous *stl159/+* siblings, which have a normal patterning phenotype called “wild-type”, and *stl159* homozygous mutants, which have broad mispatterning of the peripheral nervous system referred to as “mutant” (Figure 1).

Larval progeny of heterozygous crosses were sorted for the presence of two transgenes. All imaged larvae contained a transgene that expresses red fluorescent protein in neurons under the control of the *neural b-tubulin* promoter, *nbt:dsRed* (Peri and Nüsslein-Volhard, 2008). Fluorescent dsRed protein can be strongly observed in neuron cell bodies in the brain and spinal cord, but also in axons that extend throughout the peripheral nervous system. Additionally, larvae expressed *sox10:megfp*, which is predominantly expressed in neurons and glia within the spinal cord as well as neural crest derivatives, including Schwann cells in the peripheral nervous system (Smith et al., 2014). These transgenic strains are available for distribution from the original study authors and other groups.

Individual dsRed+; mEGFP+ larvae from two different clutches of *stl159/+*; *nbt:dsRed*; *sox10:megfp* parents were sorted and placed individually in numbered 24-well dishes at 24 hours post-fertilization (hpf), before peripheral nerves are patterned. In total, 34 larvae were sampled (22 from first clutch, 12 from second) with 30 used for the image collection. At 36, 48, and 72 hpf, larvae were removed from their individual wells, anesthetized with 0.02% tricaine, and imaged in 9-well glass dishes. Brightfield, dsRed (dsRed filter cube), and mEGFP (GFP filter cube) was imaged using a Zeiss Discovery V.8 fluorescent dissecting stereoscope with Axiocam 506 color camera. Care was taken to take images consistently; for instance, exposure was held constant (500 ms), larvae were oriented in a lateral view horizontally across the image field, magnification (32x, 50x, and 80x) was chosen that showed the same wide-field and detailed features for every larva, and the yolk extension was included in high-magnification images of the midbody to

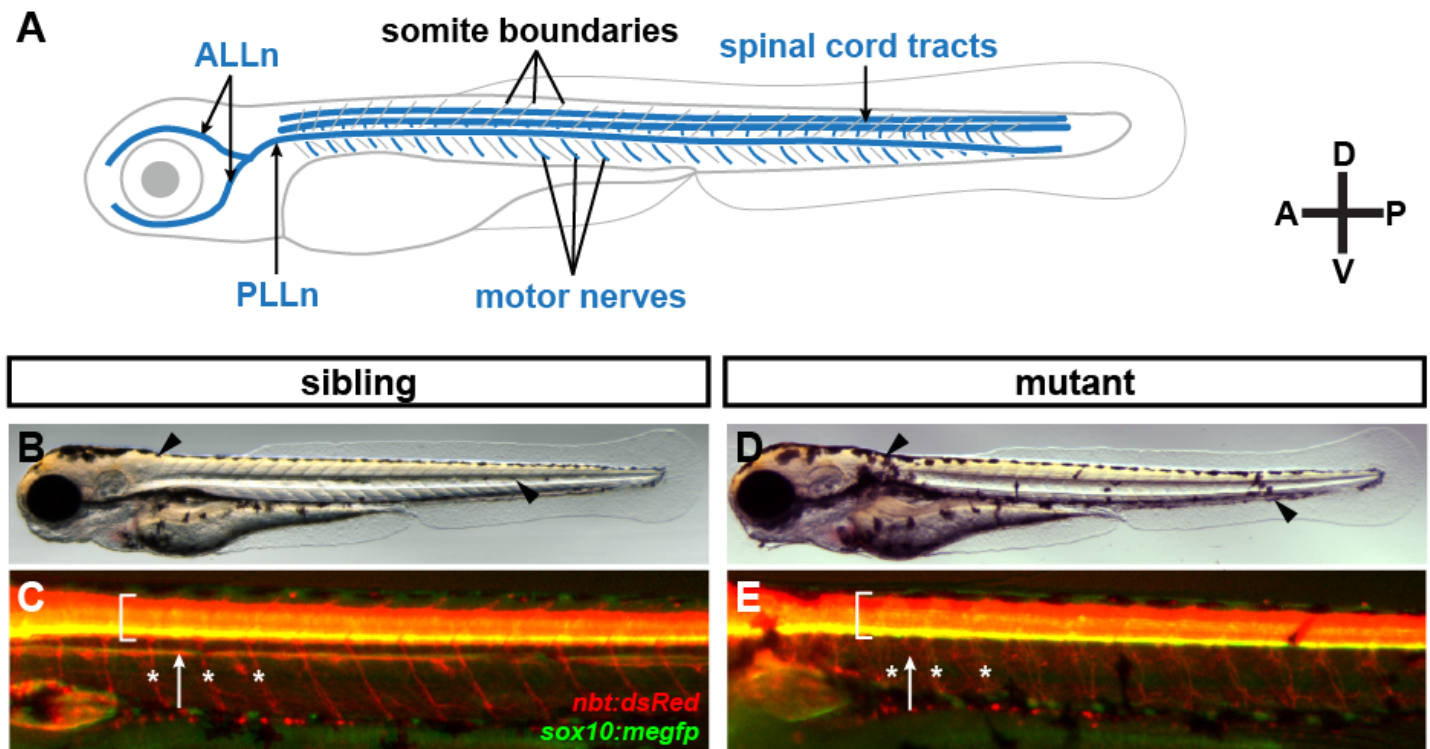


Figure 1. Lateral schematic of key nervous system features in a zebrafish larva. The anterior lateral line nerve (ALLn) and posterior lateral line nerve (PLLn) are sensory nerves. Motor nerves are shown emanating from spinal cord. Boundaries of body wall muscle segments (somites) are shown in gray. Compass denotes dorsal (D)-ventral (V) and anterior (A)-posterior (P) axes. (B-E) Patterning of wild-type sibling (B-C) and *stl159* mutant (D-E) larvae at 72 hours post-fertilization (hpf). (B, D) Brightfield lateral view shows normal melanocyte patterning in sibling and disrupted in mutant (arrowhead). (C, E) Merged *nbt:dsRed*; *sox10:megfp* fluorescence in larvae shown in B and D indicating features scored by students. PLLn position is marked with white arrow, spinal cord is marked with bracket, subset of motor nerves from spinal cord are marked with asterisks. PLLn and motor nerve phenotype are disrupted in *stl159* mutants. Merged image created in Fiji; students scored merged or single channel images depending on research question.

orient the dorsal-ventral and anterior-posterior axis. All images were saved in .czi (Carl Zeiss ZEN software) format.

Zebrafish Image Database for Analysis

Representative images for each larva at each timepoint, illumination (brightfield, dsRed, mEGFP), and magnification were selected for the image collection. Images were renamed consistently to indicate sample number and parameters: The first number in the filename (e.g., 01, 02, etc.) denoted a single larval specimen imaged at multiple timepoints. For example, all images in the database collection labeled “19” represented the same larva; 19-36 for 36 hpf, 19-48 for 48 hpf, and 19-72 for 72 hpf, with magnification and illumination to follow. Magnification is noted second in the filename. At 36 hpf, larvae were imaged at 50x and 80x. At 48 hpf and 72 hpf, larvae were imaged at 32, 50, and 80x. Illumination/transgenes was noted last; mEGFP = *sox10:megfp* (glia) and dsRed = *nbt:dsRed* (neurons). White light, or brightfield, was marked BF.

Google Drive was used to store and distribute images for remote student use, with the entire dataset >10GB. Students were directed to download a subset based on their research question if they lacked hard drive space or fast internet while working remotely. The naming format described above allowed students to selectively download

samples of interest without needing to download the entire image dataset. The database may be available to instructors upon request.

INDEPENDENT PROJECT STRUCTURE

Early in the second semester, students identified a project mentor, developed their research question, and wrote an independent project proposal. The subsequent seven-week project period consisted of four general steps: continued research of literature and zebrafish anatomy, work in FIJI to view images and collect data, data analysis using RStudio, and presentation of their work orally and in a written manuscript (Table 1). During the project period, students met with their project mentor, as well as course instructors and classmates, at least weekly for updates and feedback. Although these steps typically occurred in that order, it was not a strictly linear process. Below are details for each step before and during the project period.

Project Preparation and Development (~3-4 Weeks, Concurrent with other Lab Activities)

Background and Literature Review

Students were encouraged to rely upon primary literature discussing zebrafish nerve analysis suggested by the instructor for their initial inquiry into lateral line

Nervous system features	
Spinal cord	The spinal cord includes cell bodies as well as axons emanating from these neurons and from the brain. Axons are grouped into tracts which run anterior to posterior in the dorsal and ventral parts of the spinal cord. Spinal cord tracts are often more visible with glial markers that enwrap axons than with neuronal markers.
Lateral line nerves (LLn)	LLn are superficial sensory nerves that are relatively easy to assay in larvae, though they are less prominent than the spinal cord. LLn are paired, with one anterior (ALLn) and one posterior (PLLn) on each side (left/right). The ALLn encircle the eye, while the PLLn extend posteriorly from approximately the ear to the tail. In a lateral view, typically only one PLLn is in focus, though the other may be visible through the transparent body. The lateral line ganglia (LLg) are posterior to the ear. The ALLn are near the brain, and the bright fluorescence there obscures the small ALLn. The PLLn are more obvious in these lateral views. The lateral line nerves innervate sensory organs called neuromasts, which are not visible with the <i>nbt:dsRed</i> transgenic marker. Normally, the PLLn develops between the skin and at the meeting point of dorsal and ventral muscle, called the horizontal myoseptum. Any deviations of the main nerve fascicle from the horizontal myoseptum, including axons or glia, are abnormal at these timepoints.
Motor nerves	Motor nerves are comprised of axons with cell bodies in the ventral spinal cord. In early stages of development, there is typically one fasciculated motor nerve emanating from the spinal cord and extending ventrally along each muscle segment. Motor nerves are not as medial as the spinal cord but not as lateral as PLLn; still, it is sometimes possible to see motor nerves on both sides of the transparent animal.
Other major landmarks	
Muscle	~30 segments of body wall muscle appear as chevron shapes pointing posteriorly. The “point” of the chevron is the horizontal myoseptum, a boundary between dorsal and ventral muscle blocks. Body wall muscle serves as the substrate for developing peripheral nerves. Motor nerves extend ventrally from the spinal cord centered on each muscle segment, while PLLn migrate along the horizontal myoseptum, crossing each segment while extending posteriorly.
Yolk	The yolk and yolk extension are prominent and useful landmarks in early zebrafish development. At 36-72 hpf, the yolk is a large ball ventral to the first ~8 muscle segments, with a thinner stretch of yolk extension ventral to the next ~7-8 more posterior segments. These are always ventral to the body wall and thus provide dorsal-ventral orientation. Additionally, the position along the anterior-posterior axis can be determined by checking whether nerves are dorsal to the yolk, yolk extension, or even more posterior. This is critical because zebrafish development proceeds anterior to posterior; therefore, the same anterior-posterior position should be analyzed across age-matched larvae.
Melanocytes	Melanocytes are dark pigment cells migrating throughout the skin of the fish. They are neural crest-derived, as are Schwann cells; migrating crest cells also use muscle as a substrate. While melanocytes are not part of the nervous system, they come from a similar lineage. Melanocytes are dynamic during development but are typically located along the horizontal myoseptum, in patches on the dorsal and ventral aspects, and clustered directly above the yolk.
Notochord	The notochord is the fluid-filled primitive backbone of the fish prior to the development of the skeleton. It provides flexible support to the larva and will eventually be replaced by vertebrae. In brightfield images, the notochord is visible as a thick, depressed “stripe” along the anterior-posterior axis, ventral and parallel to the spinal cord.

Table 2. Major features and landmarks of zebrafish larvae for neurodevelopment analysis.

(Gilmour et al., 2002; Schröter et al., 2008; Pujol-Martí and López-Schier, 2013; Thomas et al., 2015; D'Rozario et al., 2017) and motor nerve patterning (Westerfield et al., 1986; Birely et al., 2005; Palaisa and Granato, 2007; Kucenas et al., 2008; Banerjee et al., 2015). Additionally, students were given an instructor-prepared guide that described general larval zebrafish anatomy, the background of *stl159*, the image database, working in Fiji, and suggestions for statistical analysis. The relevant parts of this guide are summarized in this paper, and the complete guide is available upon request.

Orientation to Zebrafish Anatomy

To correctly identify siblings and mutants and analyze salient phenotypes, students became familiar with the zebrafish larval features at 36-72 hpf. In particular, they learned to identify relevant aspects of the nervous system

(spinal cord, lateral line nerves, motor nerves) and major landmarks and features of the larvae (muscle segments, yolk and yolk extension, melanocytes, and notochord). Several of these features are shown in Figure 1, with descriptions provided in Table 2. Additional reviews of peripheral nerve and muscle development, cited above, can provide more detail.

Independent Research Proposal

After students finalized a mentoring relationship with their faculty mentor, they reviewed relevant literature in an annotated bibliography. Students were encouraged to study figures that had distinct differences in neural and glial architecture in larval zebrafish. At the initial step of project development, students were not given access to the image database, so they could broadly develop interesting biological questions and methodology and understand the

state of the field. Once students identified common themes in development (mispatterned or absent axons, glia, and melanocytes due to loss of genetic factors), they were given access to the image database and asked how they might apply that theme to this resource and biological question. Their questions are summarized below in “Student Outcomes and Discussion.”

Each student then individually wrote a proposal, generally ~2-3 pages, with an introduction, methods, materials, and literature cited. The introduction provided essential background information along with the central research question and served as a draft of the introduction for the final paper. The methods and materials sections were written in future tense for this assignment, though it also served as a draft for the final paper. This section was reviewed by the instructor and mentor prior to starting full-time research to ensure that students had developed an appropriate research question and defined a plan to answer a novel biological question using the image database.

Working in Fiji (~3-4 weeks)

After students were familiar with the anatomy and biology of their research question, they downloaded and installed Fiji on their personal computers and used the online ImageJ manual for guidance. For getting started and troubleshooting, these sections were particularly helpful:

Basics: <https://imagej.nih.gov/ij/docs/concepts.html>.

Tools: <https://imagej.nih.gov/ij/docs/guide/146-19.html>.

Measure: <https://imagej.nih.gov/ij/docs/guide/146-30.html>.

Protocols for Working in Fiji:

Converting .czi Files for Image Analysis

Fiji can open .czi files, which includes image information and critical metadata. Images can be readily opened in Fiji using the Bio-Formats Import Options using default settings. However, because .czi is a proprietary file format with large size, it can be challenging for novices relative to the .tif format. Once students identify the necessary images for their research question, they should save files as .tif by converting to RGB color via the Image menu > Type > RGB Color, then saving as .tif using File > Save As. Students should retain the same general filename with “(RGB)” appended to match the converted files to the originals.

Adjusting Intensity for Image Analysis

Because many features are labeled with *nbt:dsRed* and *sox10:megfp*, and because the image collection served a variety of research questions, a particular feature of interest sometimes needed enhancement with Image > Adjust > Brightness/Contrast. This is due to the relatively short exposure time of the image to avoid capturing the vibration of the living fish in the water (Figure 2A). However, these features can be revealed using the Brightness and Contrast (B&C) feature in Fiji. The B&C pop-up box has a histogram showing the range of pixel intensities. To show fine features, like small processes on motor nerves, the maximum and minimum intensities can be scaled using the sliders in the B&C box (Figure 2B). Though this may saturate the brighter spinal cord, it permits visualization and analyses of dim features like motor nerves and PLLn. While

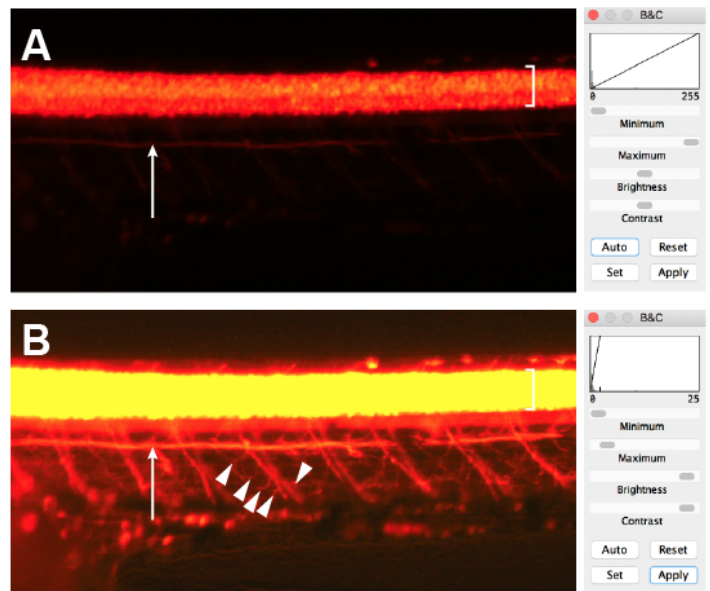


Figure 2. *dsRed* expression in a 72 hpf zebrafish taken at 80x magnification. (A) Raw low-exposure .czi image as it appears in Fiji. The spinal cord (bracket) is prominent, and PLLn (arrow) is visible. Motor nerves are too dim to observe. Histogram (right) is scaled 0-255, with most pixel brightness values to the left. (B) Scaled image shows PLLn (arrow) and motor nerves, including fine branched processes (arrowheads). Spinal cord (bracket) is saturated and should not be analyzed using this image. In the B&C box, the maximum is scaled so the display values correspond to the peaks in the histogram.

gamma adjustment may be used to avoid saturation, non-linear adjustments should not be used to quantitatively measure pixel intensity. This enhancement works best in RGB color mode (not 16-bit or RGB Stack). The scaled image for analysis should be saved as a copy with a new filename.

For intensity measurements, scaled images must be adjusted to the same degree; because images were collected with the same exposure and same conditions, the intensity can be directly compared if all images receive the same linear scaling. For counting, measuring length, etc., it is less critical to adjust maximum intensity to the same identical degree for all images, but they should be at least similar so as not to introduce artefacts. For instance, adjusting brightness in fluorescent images altered melanocyte appearance; thus, melanocytes should be analyzed only using brightfield images.

Counting Features with Fiji

Counting in Fiji is best accomplished with the point tool. Options for the point tool can be accessed by double-clicking in the toolbar. Counting and measuring points is easiest with “Auto-measure” and “Add to Overlay” turned on. Auto-measure keeps a tally of clicked points in the Results window, which opens automatically. Add to overlay essentially converts the point into a multi-point tool while also visually saving the dot position if saved as .tif. Point labels can be turned on and off in with Image > Overlay > Hide Overlay to view both a marked-up and clear image in a single file (Figure 3A). Results can be saved as a .csv for a

record of all measurements, e.g., those for a single specimen (Figure 3B). These values, in addition to serving as a running tally of the counted features, can also be used to mark distance between features, measure position, etc.

Measuring Features with Fiji

Measuring is a two-step process; first, by carefully drawing a line along the feature (Figure 3A,C), then selecting Analyze > Measure (Figure 3B, D). The Results window will indicate the length of the specified line. New lines will also be recorded in the same Results window, as well as re-measurements of the same line. Fiji will use file metadata to provide values in microns. The Pixel width value can be validated or adjusted in the Image menu > Properties. To test that the pixel-to-micron conversion is correct, measure an image of a micrometer at the same magnification; if incorrect, calculate the correct ratio to set as Pixel width.

Drawing a new line will replace any previous line selections. However, lines can be added to an overlay with Image > Overlay > Add Selection. This allows a record of the lines drawn, and as with the point overlay, it can be toggled off with Image > Overlay > Hide Overlay.

Creating Image Figures with Fiji

Polished figures of representative images of the observed phenotypes should be alongside the quantification in presentations and papers. Zebrafish micrographs are typically represented with anterior to the left and dorsal toward the top. Images that are representative of the wild-type and mutant phenotype for any parameter can be corrected to the same orientation using Image > Transform > Flip Horizontally / Flip Vertically / Rotate options. To represent both dsRed and mEGFP fluorescence in a single image (e.g., Figure 1C, 1E), use the Image > Merge Channels function in Fiji.

Data analysis (~2-3 weeks)

Chi Square to Test Mendelian Ratio

Most student projects predicted phenotypic differences between mutant larvae and wild-type siblings. Therefore, most students began analysis by determining if they had identified the expected proportion of mutants. Students independently binned larvae as “wild-type” or “mutant” based on differences in melanocyte or nerve patterning and manually calculated χ^2 Goodness-of-Fit tests to determine whether they had the expected ratio of siblings to mutants. For a recessive mutation inherited in Mendelian fashion, students predicted 25% would be mutant, or 7-8 out of 30.

Analysis with RStudio

Students in BIOL 109-110Y use R programming for data analysis beginning in the first weeks of the course. By the independent project period, they are familiar with datasheet setup using Google Sheets, uploading and importing data as .csv files into their projects in rstudio.cloud, employing descriptive and inferential statistics, and creating data visualizations via *ggplot2*. Students analyzed their data based on their questions of interest, typically using Student's t-test to determine differences between wild-type and

mutant groups at a single timepoint, or 1-way or 2-way ANOVA to compare across timepoints.

Scientific Communication (1-2 weeks)

Oral Presentation

In the final class, students gave 15-minute presentations with their partner, with time to field questions from the audience. The presentation included background material with research question, a brief explanation of methods, results including images and quantification with emphasis on biological findings, and a discussion to connect their results with the field. To prepare their presentations, students were given explicit instruction on how to focus their work and present information concisely. Students were graded using a rubric that was appropriate for both remote and in-person projects.

Final Paper

For the final assignment, students turned in a complete manuscript of their work in the style of a journal article submission. Manuscripts included introduction, methods, results, captioned figures, discussion, acknowledgements, and references. Additionally, students wrote a brief response to the feedback they received on their project proposal, which was generally rewritten to serve as the introduction and methods sections of their final papers. This served as an exposure to writing a “Response to Reviewers” as part of the article publication process.

Grading of the Independent Project

As noted in Course Structure, the independent project was incorporated into a year-long course. Within the project, students were assessed on their annotated bibliography (10 points), project proposal (100 points), weekly progress reports (10 points each), oral presentation (60 points) and final paper (100 points). Additionally, students received scores for participation and upkeep of the lab notebook throughout the semester, including the project period.

STUDENT OUTCOMES AND DISCUSSION

Development of Independent Questions

The pedagogical goal was to develop inquiry-based remote research projects that were driven by students' interests and agency. Students were encouraged to choose biologically sound question(s) based on the literature and predict what nerve patterning phenotypes could result with different types of genetic disruptions. For some questions, students predicted a difference across wild-type siblings and mutants, while for others, they would stay the same. For instance, students predicted that central nervous system patterning might stay the same, while sensory or motor nerves would be mispatterned, if a gene specifically disrupts peripheral nervous system patterning. This encompasses multiple testable hypotheses. Highest emphasis was placed on ensuring that the data they were choosing to collect would tell a biological story.

At the beginning of the project, some students felt simultaneously overwhelmed by possibilities and boxed-in by the image collection. Students developed very creative ideas for testing causation of patterning defects that would

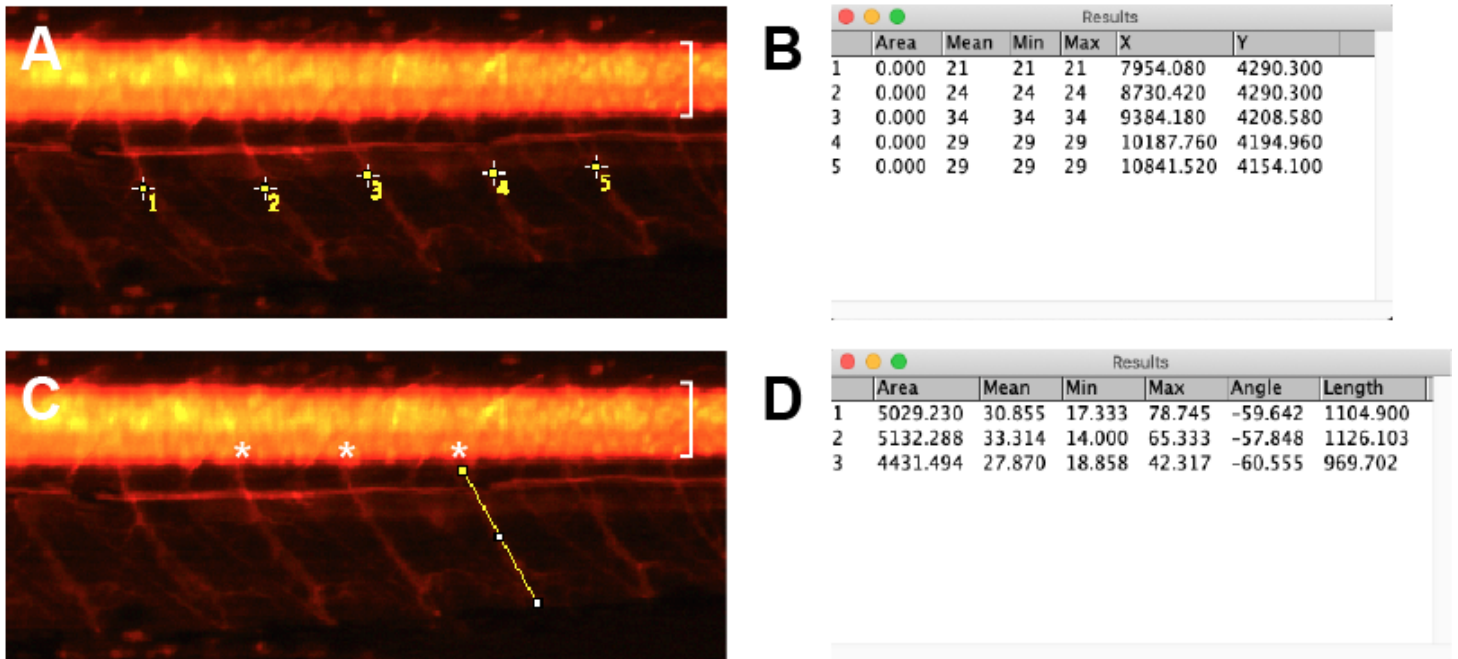


Figure 3. Counting and measuring features in Fiji. (A) *nbt:dsRed* expression in 72 hpf larvae. Spinal cord is indicated with bracket. Five motor nerves are counted with the multi-point tool as indicated. (B) Results window shown for five points on motor nerves. Mean, Min, and Max report fluorescence brightness; because this is a point (with area 0), they are all the same, as there can be only one fluorescence value for a single point. X and Y are the coordinate position for the point in the image. Clicking additional points in the image would number those points and add values to the same Results window. (C) The image shown in A is marked to measure motor nerve length. Three motor nerves (exit points marked with asterisks) were measured, with the third marked with a yellow line. (D) Results window for three motor nerve measurements. Mean, Min, and Max report fluorescence intensity. Length and angle are line-specific features that represent useful data for analysis. The first two results are for lines not represented in the image, as they disappear when new lines are drawn.

have required bench work, which was not possible in the remote setting. To mitigate disappointment and promote feasibility and success, students worked with the instructor to tailor similar but testable questions.

Below are some of the questions that students asked:

In what ways is the PLLn mispatterned in mutants?
What fraction of mutants exhibit stalled PLLn vs. incorrect extension?
How far does the PLLn deviate from its expected position in mutants?
Where do melanocytes aberrantly migrate in mutants?
How does motor nerve extension differ in mutants?
To what extent are motor nerves defasciculated in mutants?
Do the above phenotypes improve, or worsen, across developmental timepoints?

Additionally, the dataset permitted additional analysis the students did not pursue (in some cases, they developed the question but ran out of time to test it):

Do glial cells migrate differently from axons in mutants?
Are muscle segments abnormally patterned in mutants?
Is length of animal and/or head size normal in mutants?
Are there fewer axons or glial cells in mutants?

Student Observations and Outcomes

Because students did not know *a priori* which samples were siblings or mutants, they developed skills in observation that resulted in the discovery and characterization of novel phenotypes. They created detailed quantification phenotypes (e.g. numbers of mispatterned melanocytes, distance of PLLn from horizontal myoseptum) and developed measurement and analysis techniques to accurately convey the phenotypes they observed. In one case, a student tried three different techniques to quantify motor nerve defasciculation (counting branches, measuring area, and analyzing plot intensity profiles) to determine which approach best reflected the observed defects.

Though the learning curve to understand zebrafish anatomy and genetics was steep, students responded positively to the opportunity to make independent discoveries, especially when they validated their lecture course experiences. As an example, several students remarked that they were delighted to realize they had identified mutants in a proportion predicted by Mendelian inheritance, reflective of the genetics lessons in their introductory biology courses. They were excited to see that basic biological principles were reflected in their own research and could serve as the foundation for more complex hypotheses.

All students who participated in this project indicated an interest in potentially majoring in neuroscience when they

selected a project mentor and research area. However, they were all novices to studying neuroscience at the collegiate level, and the introductory biology courses generally do not teach neuroscience principles. Therefore, this research experience allowed these novice neuroscientists to explore research while learning the details “on the fly” – reminiscent of how graduate students often tackle a new area of research in laboratory rotations. Because the students had varying backgrounds of neuroscience education and exposure, they were encouraged to exercise metacognitive skills to identify their gaps in knowledge and independently find answers to their questions using the literature as well as through discussions with each other and their instructor. Additionally, because this study focused on nervous system architecture rather than function, basic comparisons of patterning between wild-type and mutants were relatively straightforward for novice students.

The ability to assay a central image database while working remotely provided a reasonable balance of independence and shared experience. Several students independently chose to pursue similar research questions; however, they answered their questions in different ways, based on how they saw the phenotype in the image. They were also able to compare notes on identification of mutants; one partner pair chose to separately assign samples as wild-type or mutant, then bring their notes together to determine a “final” assignment. Though students generally had consistent findings (a testament to their observation abilities and knowledge), it was useful for them to note that the same phenotypes could be validly scored in different ways.

To promote collegiality and collaboration among remote students, the instructor facilitated a group meeting with all remote students to discuss their research questions and strategies. This meeting revealed common issues across groups, such as software difficulties and concerns with mutant identification. In addition to being an efficient use of time, as issues were resolved together rather than individually, the students were able to develop a sense of camaraderie and agency in troubleshooting each other's questions. More meetings would likely have been beneficial, though scheduling was too difficult.

Some challenges for remote students were insurmountable. Students sometimes felt isolated without easy or quick solutions to their issues. Troubleshooting with Fiji and R programming was particularly difficult, and students lacked access to other computers (e.g. their research partner's, a computer lab) that they would have had on campus. Most technical problems, however, were overcome with additional time and effort.

When developing their projects, several students noted that they wished they had the opportunity to test some of their predictions in person, like testing a drug treatment of a candidate pathway or testing expression of candidate genes. This is not strictly a limitation of the remote aspect of the research; vertebrate animal approval and training would likely take up a large part of the project period and yield less useful data.

Opportunities for Modification or Extension

Because image analysis can be done entirely on a

computer, this lab module was well-suited for remote projects. On-campus students could, however, complete similar projects by first learning how to use the microscope to image samples themselves. While creation of a full image dataset would likely not be possible with novice microscopists, students would learn the importance of imaging parameters, including specimen orientation, magnification, and camera exposure. Students would also appreciate the amount of time that goes into collecting images for a statistically useful sample number. Finally, including students in image collection would allow an additional dimension to experimental design, as students could selectively monitor their feature of interest, rather than using a “one size fits all” image collection.

Because this was a capstone project for a year-long lab course, students had already developed skills in experimental design, observation, and statistical analysis. If students do not already possess those skills, image analysis offers attractive opportunities to teach all three in an expanded module. Students can learn to identify control and experimental groups and apply the appropriate statistical tests via images alone. Additionally, observational techniques can be superbly taught with image samples. Instructors can develop guided questions and discussion that allow students to identify meaningful landmarks during zebrafish development.

This pilot used a novel mutant in the instructor's lab; in addition to the mutant discussed here, instructors can develop projects related to their own area of expertise. This provides a superb mechanism by which teaching-research faculty can work with students to generate data that leads to publication with student authors. Alternatively, instructors can use existing developmental mutants with different fluorescent transgenic reporters to create a novel exploration for students. For instance, *sox10/colorless* mutants have broad mispatterning of neurons, glia, and pigment cells (Dutton et al., 2001), and there are a wealth of markers for neurons and glia (Förster et al., 2017). This can become an attractive opportunity for an instructor to synergize teaching and research or build a full course-based undergraduate research experience (CURE). Using established mutants, however, might interfere with true inquiry and developing novel questions. It also may necessitate crossing fluorescent transgenes into mutants, which can be time-consuming for an instructor.

Instead of mutants, instructors may consider perturbing nervous system development and structure via varying concentrations of small molecule compounds (Zon and Peterson, 2005). For instance, ethanol and lithium chloride are relatively inexpensive compounds that produce nervous system patterning defects (Ross and Bonner, 2012; Faccioli et al., 2019). Drug treatments are an especially useful alternative for instructors unable to house multiple mutant strains, as only a single strain carrying transgenic reporter genes is needed. Additionally, drugs can be easily applied in combination to test the interplay of multiple factors. The major limitation, therefore, becomes the time to build an image collection.

Given the relative ease of using zebrafish in the classroom relative to other vertebrate models, students may

be incorporated into the steps involving zebrafish breeding and raising embryos to the relevant embryonic/larval stage. This gives students additional control in their experimental design and parameters to pursue. This would be a particularly attractive option for instructors expanding this module into a full course, as well as for courses with upper-level (rather than beginning) students that are studying on campus. Instructors are cautioned to follow institutional animal protocols (IACUC) to ensure that students may handle zebrafish adults and larvae.

Fiji and R/RStudio are freely available software that are often used in laboratories at research institutions, so gaining proficiency at the undergraduate level sets up future researchers for success. Instructors can build image analysis related to their area of research into courses. Zebrafish researchers can use *in situ* hybridization or immunostaining images (Cunningham and Monk, 2018); cell culture and other model systems could be used as well. Instructors who want to teach these skills, but lack animal research or microscopy, can take advantage of freely available repositories (e.g., Gigapan at <https://gigapan.com/>, Allen Brain Atlas at <https://portal.brain-map.org/>) or natural history collections to teach Fiji-based image analysis to undergraduate students. Use of selected images could also allow a focused image analysis module over a shorter period of time, e.g., 2-3 weeks instead of 7-8, particularly if students are not developing independent questions.

SUMMARY

Image analysis is a useful skill for undergraduates to develop in introductory laboratories. Zebrafish larvae are particularly attractive subjects for image analysis, as established transgenic reporters marking neurons and glia can be readily imaged at multiple developmental timepoints in living animals. Instructors can collect images in a single repository that multiple students can use to develop independent questions of interest. The data students collect can be quite robust and amenable to basic statistical tests and data visualizations. Importantly, the entire module can run with students in a remote setting, with free software and remote guidance from the instructor.

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Received July 2, 2021; revised September 7, 2021; accepted September 20, 2021.

This work was supported by National Science Foundation CAREER Award #1941664 to SCP. The author sincerely thanks Dr. Jennifer McMahon, Director of Introductory Biology Labs at Kenyon College, for valuable feedback during the development and execution of the laboratory module and careful reading of this manuscript; the Department of Biology faculty, particularly BIOL 109-110Y instructors, for feedback during the execution of the laboratory module; Rachael Penick, Becky Gallagher, and animal care staff at Kenyon College for zebrafish care and husbandry; and Darcy Blankenhorn for technical assistance.

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