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Flights and Larvae

Using optogenetics to teach recombinant DNA and neurobiology

**Josh Titlow,
Heidi Anderson,
and Robin Cooper**

Switching genes between organisms and controlling an animal's brain using lasers may seem like science fiction, but with advancements in a technique called optogenetics, such experiments are now common in neuroscience research. Optogenetics combines recombinant DNA technology with a controlled light source to help researchers address biomedical questions in the life sciences. The technique has gained the most traction in neurobiology—the biology of the nervous system—where specific wavelengths of light are used to control or measure the activity of neurons in transgenic organisms (i.e., those with artificially inserted genes).

These optical recording and stimulation techniques are used in nervous system preparations ranging from individual cells in culture to whole organisms, where the observations and data collected have been used to determine which neurons are involved in specific animal behaviors. In this article, we describe an inexpensive *Drosophila* (fruit fly) optogenetics experiment used to teach principles of the nervous system, genetics, and bioengineering at the high school level. (See sidebar [p. 44] for specific learning objectives and key concepts for the lab.)

Fruit flies receive an algae gene

Algae and other microorganisms have been known to sense and emit light (Foster and Smyth 1980). Advances in molecular biology techniques near the end of the 20th century enabled researchers to determine which proteins were involved in *phototaxis* (movement in response to light), clone the respective genes, and transfer them into new species for research. The proteins themselves are called *channelrhodopsins*, which are transmembrane ion channels that convey a

non-specific ion flux when the channel is activated by a specific wavelength of light.

Channelrhodopsin-2 (ChR2) is sensitive to blue light. By introducing point mutations into the gene, researchers have altered the light sensitivity and optimized ion conduction in these channels, making photo-activation more efficient and easier to use. These advances paved the way for optogenetics to be implemented in teaching laboratories. The basic methods and concepts for using optogenetics in the undergraduate classroom have been developed by Pulver and colleagues (Pulver et al. 2011a; Pulver et al. 2011b; Pulver and Berni 2012). Here we have adapted those exercises to a high school-level module that addresses core disciplinary ideas in the *Next Generation Science Standards* (NGSS Lead States 2013) (Figure 1).

FIGURE 1

Addressing the standards.

This optogenetics activity addresses the following *Next Generation Science Standards* (NGSS Lead States 2013):

HS-LS3: Heredity: Inheritance and Variation of Traits

Scientific and engineering practices:

- Planning and carrying out investigations
- Analyzing and interpreting data
- Using mathematics, information, and computational thinking.
- Obtaining, evaluating, and communicating information.

Disciplinary core ideas:

- LS3.A: Inheritance of Traits
- LS3.B: Variation of Traits

Crosscutting concepts:

- Patterns: Different patterns may be observed at each of the scales at which a system is studied and can provide evidence for causality in explanations of phenomena.
- Cause and Effect: Empirical evidence is required to differentiate between cause and correlation and make claims about specific causes and effects.

Connections to engineering, technology, and applications of science:

- Influence of engineering, technology, and science on society and the natural world: New technologies can have deep impacts on society and the environment, including some that were not anticipated. (HS-ESS3-3)

Expression of ChR2 in *Drosophila melanogaster* is restricted to motor neurons

A huge breakthrough in *Drosophila* transgenics occurred when the yeast GAL4-UAS binary expression system was introduced into the fly genome (Brand and Perrimon 1993; Duffy 2002). Thus the transgenic ChR2 could be expressed in specific subsets of cells using this standard binary expression system. The ChR2 transgene is controlled by the yeast upstream activation sequence (UAS) for a galactose-induced transcription factor (GAL4), meaning that the transgene is carried in every cell but is only expressed where GAL4 is expressed. Expression of the GAL4 gene is controlled by a promoter sequence either from a nearby promoter in the fly's genome or a specific promoter that is added with the GAL4 transgene sequence. By adding a promoter to the GAL4 sequence that is expressed specifically in the nervous system (e.g., a gene that codes for an enzyme involved in synthesis of a neurotransmitter), the GAL4 gene only gets expressed in the nervous system, thus activating expression of the ChR2 transgene specifically in the nervous system.

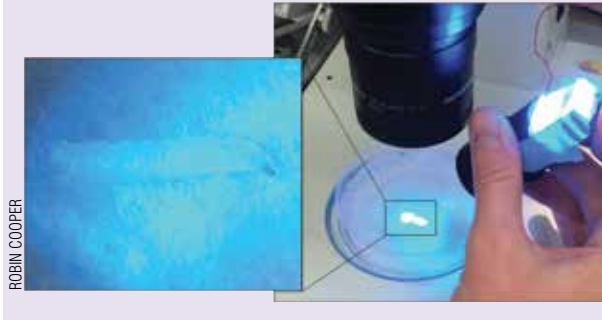
The promoter used to drive expression of GAL4 in the flies used for this activity is called OK371-GAL4. The promoter element is from the *Drosophila* vesicular glutamate transporter gene (DVGLUT) that is expressed almost exclusively in neurons that release glutamate (Mahr and Aberle 2006). Glutamate is the neurotransmitter that stimulates skeletal muscles in insects, as opposed to acetylcholine in vertebrate nervous systems. Therefore in the OK371 > ChR2 line, shining a blue light on the whole animal will activate all of its motor neurons at once, making a visual and observable behavior for students to measure response to stimuli.

Building an LED controller and behavior arena

Drosophila larvae have a thin and translucent cuticle that allows light to penetrate relatively well. To increase penetrance, light from an LED is focused through a 10× ocular objective lens (Figure 2). This is a technique introduced by Pulver et al. (2011a). In our activity, we reduce their light apparatus to

FIGURE 2

Optogenetic activation of fruit fly behavior. A blue LED is focused through an optical objective to activate neurons in third instar *Drosophila* larva (inset). Students observe the behavioral responses to this stimulus through a dissecting microscope.



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the bare minimum components (Figure 3) and provide a set of behavioral experiments that are appropriate for the high-school classroom.

The following materials are needed to perform this optogenetics experiment:

Materials needed for LED setup:

- ◆ Side-emitting LED (488 nm emission [blue])
- ◆ Small heat sink (to disperse heat from the LED)
- ◆ 10× ocular objective lens (taped to the heat sink holding the LED; cover back part of the light with tape)
- ◆ Lab adhesive tape
- ◆ 9V battery
- ◆ 9V battery snap connector
- ◆ Solder (or adhesive to connect the 9V battery wire to the LED)

Materials for each lab station:

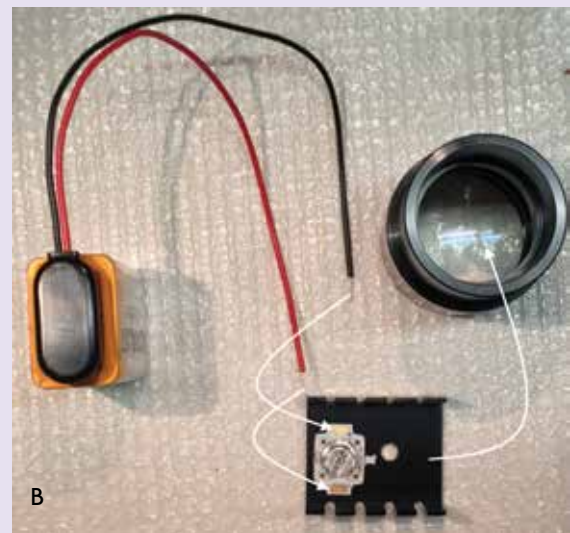
- ◆ Petri dish with lid (8.5 cm dia.)
- ◆ Paper filter (8.5 cm dia.)
- ◆ Flat-tipped toothpick (or small paint brush) for maneuvering the larva
- ◆ Disposable plastic dropper (3 ml)
- ◆ Stopwatch
- ◆ Data sheet
- ◆ Stereomicroscope (10× ocular/4× objective)
- ◆ 3rd instar larvae (OK371>ChR2 fed retinal; the control is the same flies not fed retinal, see below)

FIGURE 3

Building the LED apparatus. An LED is glued to a heat sink and powered by a 9V battery through a snap connector (A). This assembly is then attached to a microscope eyepiece (LED has been left uncovered for display but would normally be covered with tape) (B).



A



B

Student objectives

- Students apply optogenetics to the study of locomotion and feeding behavior in *Drosophila* larvae.
- Students observe firsthand how optogenetics can be used to activate motor units in a live, genetically modified organism.
- Students practice observational skills and work as a team to obtain measurements of behavior.
- Students input data into spreadsheets and use software to analyze and graph the data.
- Students collaborate within a group to explain important aspects of the experiments to their peers.

Student guidance questions and key concepts

- How does light affect crawling behavior and feeding behavior in the ChR2 larva?
- Were there any long-term effects of optogenetic stimulation on the larva?
- What causes the larva to respond to light?
- What is a channelrhodopsin? In what organism did channelrhodopsin originate?
- Which cells in the fly express channelrhodopsin?
- If you wanted to see what happens when sensory neurons are photoactivated in the fly, how could you generate a fly that expresses ChR2 in sensory neurons?
- What would you expect to happen at the neuromuscular junction if the ChR2 larva was stimulated with blue light for several minutes?
- How could this optogenetics system be used in biomedical research to address a clinically relevant problem?
- In people with severe spinal cord injuries, neural stimulation often helps the spinal cord recover. How could you use optogenetics to photo-activate neurons in a human spinal cord?

Materials for instructors:

- ◆ Apple juice (50 ml for 20 groups)
- ◆ Yeast paste (1 ml yeast/10 ml water)
- ◆ Scoopula (to transfer larvae)
- ◆ Water bottle (to transfer larvae)
- ◆ *Drosophila melanogaster* larvae: OK371 > ChR (expresses channelrhodopsin in motor neurons). The OK371-GAL4 and UAS-ChR2 lines can be obtained separately from the Bloomington Drosophila Stock Center (OK371-GAL4: <http://flybase.org/reports/FBst0026160.html> and UAS-H134R-ChR2-mcherry: <http://flybase.org/reports/FBst0028995.html>). Follow the links to obtain stock numbers for these lines, then on the Bloomington Drosophila Stock Center homepage, navigate to the page for purchasing and enter the stock numbers to make a one-time purchase. To generate larvae with both transgenes, collect three or four virgin adult female flies from one line and place them into a vial of food with two or three males from the other genotype. The progeny will express ChR2 in all motor neurons. Alternatively, contact coauthor Robin Cooper (rlcoop1@uky.edu) to request recombined stocks (i.e., no crossing is needed because the line is homozygous for both transgenes). For more information about obtaining these supplies, see “On the web.”
- ◆ All-trans retinal (cofactor that is fed to the flies >24 hr. before the experiment)

Larval optogenetics experimental procedure

Before class begins, the teacher constructs light apparatuses and cultures the larvae in fly food with retinal (see “On the web” for links to online materials that have detailed instructions for building the LED and preparing the culture medium). Larvae are obtained by allowing the adults to lay eggs directly into the retinal food source one week before experimentation. It will take 5–7 days for the eggs to reach the third instar stage in retinal medium (see online materials for details). We usually spend 90 minutes running this lab: 60 minutes to complete the experiment and answer questions, plus 15 minutes before and after for discussion. An additional 60-minute session is needed to complete data analysis.

Effects of motor unit photoactivation on crawling behavior

Safety note: Remind students not to shine the LED light at other students and to unplug the light when not in use. Instruct students on how to find the focal plane of the light and optimize stimulation (an image of the LED can be seen when the light is in focus). They should be careful not to crush the larvae during handling, a common mistake. We use tooth-

picks with flat tips to transfer larvae. The activity can be completed in one 90-minute laboratory class session or across two 45-minute class sessions.

Students separate into groups of two to four and prepare their own behavioral arena by (1) placing a paper filter (8.5 cm diameter) into a petri dish (8.5 cm diameter) and (2) dampening it with a few drops of apple juice. This will provide a moist surface for the larvae to crawl on, and the smell of apple juice encourages the larvae to crawl. The teacher distributes two or three control larvae (OK371>Chr2 not fed retinal) to each group (extras are given in case students mishandle the larvae, though they could also record from multiple animals if time allows). Students first observe the larvae under a dissecting microscope, noting how they crawl and what happens when the blue light is shined on them to look for changes in behavior (Figure 4).

Next, one student looks into the microscope and counts the number of crawling strides a larva executes in 15 seconds, while another student keeps track of the time, recording it on the data sheet as “# of crawling strides / 15s” under the column for “Before stimulation.” The same measurement is then repeated, this time with blue light focused on the larva. The number of strides is recorded in the column for “During stimulation.”

Immediately after counting the strides in blue light, the students should take an additional measurement to determine if the light had a lasting effect. This value is recorded as “After stimulation.”

Effects of motor neuron photoactivation on feeding behavior

A second behavioral experiment that can be conducted under the same premise is to investigate feeding behavior by counting the number of mouth hook movements that occur in 15 seconds before, during, and after the light stimulus. Tell students that the mouth hooks are the black cuticle structures at the anterior segment of the animal and that they move much faster than body contractions. They should observe 30–50 movements in 15 seconds.

Students use a dropper to form a small drop of yeast solution on the lid of their petri dish. The solution should be rather dilute, as the students will need to observe the larva while it is submerged in the solution. Students carefully transfer the larva into the yeast solution and observe its feeding behavior under the microscope. As they did with the crawling strides, they will count the number of mouth hook movements that occur in 15 seconds, then again while focusing blue light on the larva, then again immediately after the light is removed. After recording these measurements, the data sheet should be complete, and the students can answer the questions either alone or as a group.

FIGURE 4

Student measuring photoactivated behavioral changes.



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Analyzing data

Students analyze the data in a second session to characterize the effects of the stimulus. If time allows, the groups can test multiple larvae and analyze their own data. Alternatively, the data from each group can be pooled, and then each student can analyze the pooled data set. The crawling data are arranged into six columns in a spreadsheet, “Before,” “During,” and “After” for all of the control values and experimental values. The feeding data are also arranged into the same six columns. Students graph the means and variance for each set of data and then apply statistical tests. One statistical test would be to determine if the light had a significant effect on the behavior. Chi-square analysis is a good option for statistical analysis of these data, as it is part of the AP biology curriculum.

Analysis of data from 19 groups of AP biology students is shown in Figure 5. As expected, the students observed a striking decrease in crawling speed, and there was no lingering effect of photoactivation on crawling (Figure 5A). Feeding behavior was also significantly reduced by photoactivation of glutamatergic neurons (Figure 5B), a result that has not been described in the scientific literature. Within their small groups we ask students to explain how the experiments were controlled and to think about other questions that could be addressed with this methodology.

Assessment

There are several nuances to this data set, enough to allow each group to give an oral presentation or written report on a different aspect of animal behavior and optogenetics. Within the crawling stride data, one group could discuss the “Before stimulation” data set, noting how this measurement serves as an appropriate control, and why the retinal treatment could have caused differences in the animals’ baseline behavior. The second group could discuss how the light stimulus affected the behavior, comparing the experimental “Before stimulation” data set to the “During stimulation” data set. In doing so, students should come to understand how the crawling speed changed, how the light caused this change, and how they were able to tell that effect was due to the transgene and not the blue light in general. The third group could then present the “After stimulation” data set. They would state whether the crawling speed returned to normal, or whether the effect of the light persisted after the light was turned off. Then they should give explanations for why they observed that particular result. The other three groups could then present the analogous data sets from the feeding behavior experiment.

Activity modifications and extensions

This activity can easily be modified to incorporate a student inquiry component by allowing students to experiment with the animals before they are given a protocol. Students are told that this is new technology and they need to determine (1) if it is working and (2) if the stimulations have any lasting effects on the animals. The assignment is to develop hypotheses and design experiments to address these two points. This option hasn’t been explored, so it will be interesting to see how students approach this topic. Other possibilities are increasing the stimulus time and taking “After stimulus” measurements at later time points, e.g., immediately after, five minutes after, one day after, and so on.

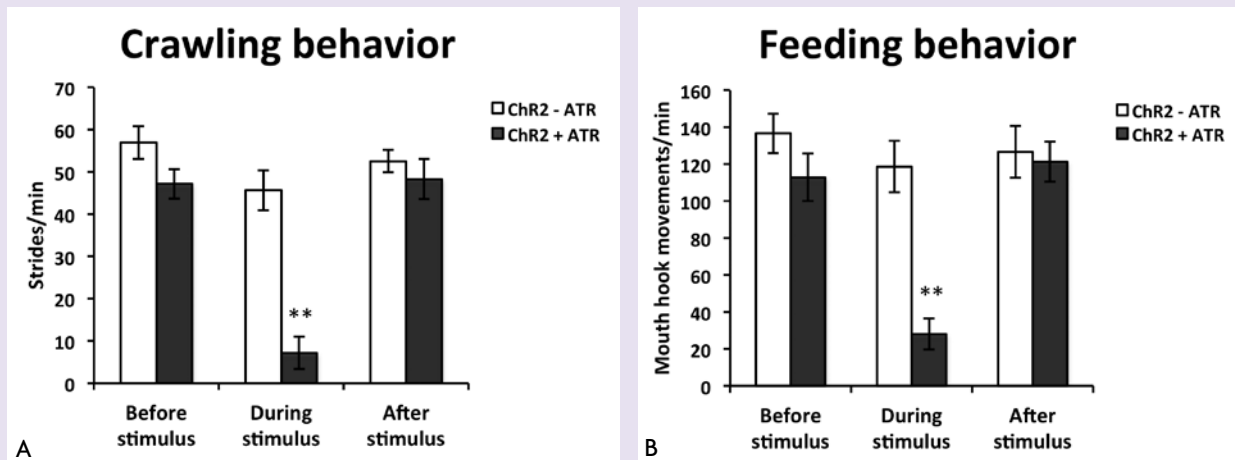
There are many behavioral assays for larvae that could be combined with the stimulus to determine if activating this large group of neurons impairs mechanosensation, phototaxis, chemotaxis, or olfactory learning. This is a typical discussion point we bring up in a group session after the experiments. We ask students to consider how these experiments could be applied to biomedical research questions, involving, for example, epilepsy. During a seizure, large groups of neurons (often glutamatergic neurons) are activated randomly to cause uncontrolled motor activity. By simulating this in fruit fly larvae, researchers could dissect the brains to determine what effects the seizure had on the biology of those neurons.

We have performed this module with two sets of AP biology students, one that was already familiar with genetics and basic neurobiological principles and another that was not. Both groups were able to generate results and comprehend the biological effect of the LED. Therefore, we think that non-AP introductory high school biology students will likely be able to perform the experiments



FIGURE 5

Analysis of optically modified larva behavior. These data were pooled from 19 groups of AP biology students (mean \pm SEM [Standard Error of the Mean]). Crawling behavior (A) and feeding behavior (B) were significantly reduced during light stimulation in animals fed the cofactor (Two-way ANOVA, $**p < 0.0001$). The light did not affect isogenic larvae that were not fed the cofactor, and the 15-second stimulus did not have a persistent effect on either behavior after the stimulus was removed.



but with emphasis placed on data collection methods, the insect's anatomy/physiology, and a more general understanding of genetically modified organisms rather than transgenics and neurophysiology.

Conclusion

Fruit flies are commonly used in high school laboratories to teach principles of genetics. This activity scaffolds on those basic principles by applying fruit fly genetics to concepts in neurobiology and physiology. We find that students have fun controlling fruit fly behavior with LEDs and gaining practical experience with a genetically modified organism. The lab also covers several NGSS standards. ■

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On the web

Datasheet, supplies list with prices and order numbers, links to the *Drosophila* Bloomington Stock Center to order specific fly lines, links to important freely available articles on using optogenetics and *Drosophila* in the classroom, and links to videos to demonstrating optogenetic behavior: <http://joshtitlow.wordpress.com/lights-larvae/>.

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