

Bridging optogenetics, metabolism, and animal behavior for student-driven inquiry

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Biotechnology is an ever-evolving field of science critical to improving the quality of human life, particularly in medicine. Optogenetics, an area of biotechnology, involves genetic modification of cells to express light-sensitive ion channels, which allows for the use of light to manipulate behavior. This module utilizes an approach to bridge optogenetics, cellular metabolism, and animal behavior for student-driven inquiry in college courses. *Drosophila melanogaster* larvae modified to express Channelrhodopsin-2 (ChR2) in motor neurons serve as model organisms in this module. Students can connect temperature, metabolic rate, and gene expression through data collection of behavioral responses to light stimuli exhibited by larvae raised at various temperatures. Students can observe the role of cofactors in metabolic processes via larvae that have been fed all-trans retinal, a cofactor to ChR2. Students can analyze and interpret data in order to make a claim about how the two variables investigated (temperature and cofactor) impact the behavior of the target organism. The final activity allows students to form connections to cutting-edge research related to optogenetics, such as work with the GAL4/UAS system. This would provide a foundation for further exploration of such research. The module can be used in in-person, hybrid, or remote settings.

Keywords problem-based learning, behavior, gene expression, optogenetics, physiology, asynchronous learning, hybrid

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Introduction

The ability to express genes in a controlled and specific manner is indispensable for biological research. One of the most powerful and flexible systems for manipulating gene expression is the

GAL4/UAS system adapted from yeast (Kakidani & Ptashne, 1988). This bipartite module uses one transgene with the GAL4 transcription factor coding sequence expressed under the control of a desired promoter paired with a second transgene in which the gene to be expressed is under the control of the

Upstream Activating Sequence (UAS), a DNA enhancer sequence to which the GAL4 transcription factor will bind. Because any UAS responder transgene can be paired with any GAL4 driver transgene, the researcher has tremendous flexibility in expressing the gene of interest in different tissues and under different developmental or environmental conditions. Systems based on this strategy have been developed for use in diverse research animals, including fruit flies, zebrafish, and mice (Deisseroth, 2015; Han and Boyden, 2007; Fenno et al., 2011; Camporeze et al., 2018; Boyden et al., 2005).

The GAL4/UAS system has been used to investigate an incredible diversity of biological phenomena, including to map neural circuits to understand behaviors. One way this has been accomplished is to use GAL4 driver lines that express in various subsets of neurons or muscles combined with UAS-channelrhodopsin. Channelrhodopsin is a light-activated channel that permits influx of cations. When ions enter the neurons, an action potential is stimulated and the neurons fire. This allows researchers to observe the resultant behavior and deduce the function of the neurons in the circuit. The module described here aims to illustrate both the molecular genetic manipulation of gene expression and the physiological and behavioral consequences of optogenetic manipulation of ion flux, tools that are implemented at the forefront of research in biology. In addition to gaining an understanding of the basic principles behind these tools, students can engage in an authentic scientific investigation. This module integrates ideas related to genetics and physiology with practices of science (Bybee, 2011) to give students a true taste of the power of biotechnology. Further, the culminating activity focuses on connecting knowledge gained from the experimental phase to practical applications in neuroscience.

Direct involvement with the practices of science is crucial for students to gain an understanding of both the phenomenon being taught as well as how scientific knowledge is generated (National Research Council, 2012). The module provides opportunities for students to engage in the scientific practices through an experiment involving

optogenetics and the testing of the behavioral responses of *D. melanogaster* larvae stored under various conditions to light stimuli. Students examine how two variables (temperature and cofactor) influence the larvae's responses, organize this information into a table, and then create a graph to visualize and interpret what the data shows. During this part of the module, students engage in the practices of planning and carrying out an investigation, analyzing and interpreting data, and making an argument based on experimental evidence.

The initial activities within this module support students in improving their data and graph literacy, areas for which deficits in students' understanding are well established (Carlson et al., 2010). Embedding the learning of such skills within real-world applications connected to cutting-edge science increases engagement (Premadasa & Bathia, 2013). The second portion of the module that addresses the aforementioned concepts involves review of scientific literature related to the topic of optogenetics. Students look for trends that exist in both numbers of publications per year as well as topics of publications and create graphs based on their findings. Students are able to gain knowledge regarding practical and interesting applications of optogenetics while strengthening literacy skills.

This guide (Appendix A) has been created for teachers and students and includes detailed steps for completion of the module. In addition, the guide provides alternative, cost-effective options for laboratory equipment that may not be available in all classrooms. The guide promotes additional scientific inquiry through suggestions of supplementary variables students can test if time and resources allow. In addition, due to the current situation of COVID-19 worldwide, many instructors are teaching activities which can be conducted remotely. We have provided a means by which this module can be viewed and have some student activity with downloaded content for instructors to use as they see fit.

Student Outline

Objectives

- Students will be able to explain what a transgenic organism is.
- Students will be able to formulate hypotheses on how optogenetics can be used to manipulate locomotion in *Drosophila melanogaster* larvae.
- Students will be able to design an experiment in the use of optogenetics and relate it to associated larval behaviors.
- Students will be able interpret data to explain how temperature and cofactors play roles in metabolic processes.
- Students will be able to argue from the evidence an explanation for the observations.
- Students will be able to describe practical applications of biotechnology, particularly optogenetics.

Introduction

The behavior of animals is controlled by complex physiological processes involving all the bodily systems. The nervous system is generally considered the master of coordination and initiation of behaviors; however, if a tissue is not responsive to a neural command, then the initiated behavior will not be elicited. Such conditions in the dissociation of the nervous system with targeted tissues occur with various disease states and injuries in animals (i.e., autism, Parkinsonism, muscle dystrophy, and neural injuries). There are various medical procedures utilized to alleviate or dampen these disease states. New approaches are continuing to be investigated such as gene therapy and pharmacological interventions. In addition, additional experimentation in future potential treatments is being addressed. One such novel approach is in the expression of light activated proteins in defined tissue or cells to excite or depress the excitability of defined cells to control a tissue such as a heart or skeletal muscle and even defined neural pathways with the central and peripheral nervous systems (Gunaydin et al., 2010; Deisseroth, 2015).

The introduction of light activated ion channels and ion pumps which can be selectively targeted to tissues and specific neuron types is now a reality in therapy for humans as this approach was used to treat a retinal dysfunction and is being examined for wider uses (Scholl et al., 2016; Sengupta et al., 2016; Towne and Thompson, 2016). Other treatments in regulating neural activity with light sensitive channels will likely be forthcoming due to the ability to target defined neural circuits (Banghart et al., 2004; Fiala, 2013; Klapoetke et al., 2014; Towne and Thompson, 2016). Experimentation for understanding the limitations and control of manipulating cellular activity with this modern technique requires studies in various animal models and cell cultures. Experiments with light sensitive channels have been occurring in worms, insects, and rodents (Nagel et al., 2005; Hornstein et al., 2009; Titlow et al., 2015; Riemensperger et al., 2016; Giachello and Baines, 2017; Quinn et al., 2016; Zhu et al., 2016; Malloy et al., 2017). In order to understand control in expression of introduced genes and how modulating the activation of these light sensitive proteins further experimentation in understanding cellular metabolism and function of the proteins with modifiers of the protein still need to be studied (Higgins et al., 2017; Bender et al., 2016; Blumberg et al., 2016; Dawydow et al., 2014; Deisseroth, 2014, 2015; Gradinaru et al., 2007; Grosenicket al., 2015; Lee et al., 2014). An approach to examine functional activation of light sensitive channels and environmental conditions of the proteins is to observe the extent of behavioral changes in intact animals.

The fruit fly, *Drosophila melanogaster*, is especially attractive for such studies because of the easy manipulation of cell specific gene expression and the reproducible stereotyped behaviors exhibited. Both larval and adult form of *Drosophila* have been utilized as a proof of concept in activation of light sensitive proteins. Since genetic crosses are used for expression and light is used to activate the light sensitive proteins, the term optogenetics is used to describe this technique (Deisseroth, 2015; Han and Boyden, 2007; Fenno et al., 2011; Camporeze et al., 2018; Boyden et al., 2005). The crawling of a larvae or reduced climbing on a tube for adults can be readily quantified for alterations in response to altered neural control of muscle or altered activity of the body muscles themselves. The duration and intensity of these behavioral alterations can also be readily quantified by direct visual observation.

A compound which enhances the channel conductance of light sensitive channels is all trans retinal (ATR). It is a cofactor for the channelrhodopsin (Dawydow et al., 2014). The ATR also helps in preventing the degradation of channelrhodopsin. ATR needs to be introduced to animal models, such as *Drosophila*, as they do not make

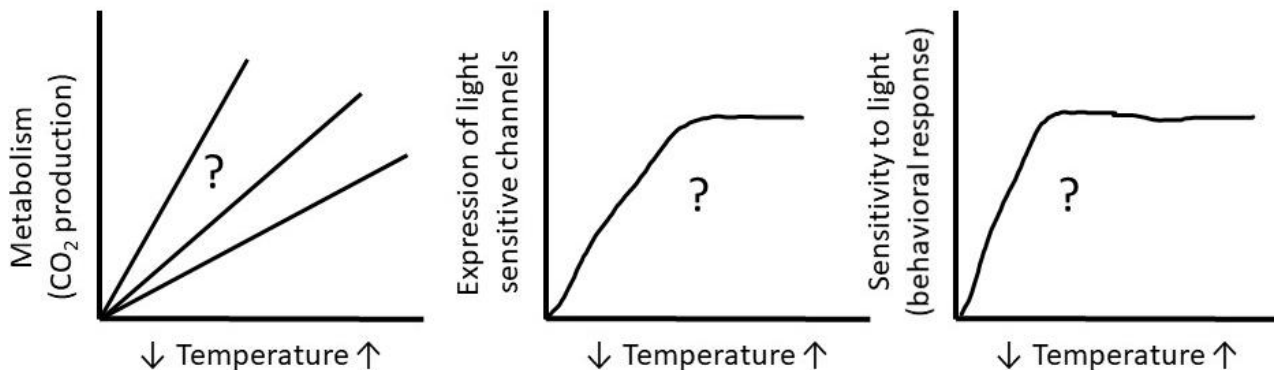
retinal while other animals, such as humans, can synthesize it. Larval and adult *Drosophila* can be fed a food supplement containing ATR (1mM). Some light sensitive channels such as the highly light sensitive channel, ChR2-XXL, can be activated without ATR in the diet (Dawydow et al., 2014). In this protocol we use the light-sensitive channelrhodopsin ChR2.XXL, a hypersensitive variant of ChR2 as well as CHR2 (Zhu et al., 2016; Oswald et al., 2015) with larval and adult *Drosophila* without feeding ATR as well as fed ATR.

For readily observed behavioral changes we express CHR2-XXL in motor neurons as well as body wall muscles. Activation of these tissues will cause the muscles to contract and would result in slight changes in locomotion to extreme activation causing a paralysis of any movement. Thus, the behavioral change is used as an index to quantify.

In these experiments, the expression of channelrhodopsin is controlled by use of the Gal4/UAS binary expression system that consists of two elements, yeast Gal4 transactivator and the upstream activating sequence (UAS), a DNA enhancer to which GAL4 protein binds. Combining the two drives the expression of a UAS-reporter or effector gene wherever there is GAL4 protein (Brand and Perrimon, 1993; Duffy 2002). GAL4 drivers are constructed by placing the transcriptional regulatory sequences (enhancers) from a given gene next to the GAL4 protein coding sequence. Those regulatory sequences then confer upon GAL4 the expression pattern of that gene. The use of different GAL4 driver transgenes can therefore permit the expression of UAS-channelrhodopsin in different tissues of the animal. The pattern of gene expression that is instructed by a particular GAL4 driver transgene can be observed using a separate UAS-reporter gene. In this experiment, we will use a UAS-GFP (green fluorescent protein) transgene to detect the locations of expression for each GAL4 driver. GFP is a protein derived from jellyfish that gives off green fluorescence when illuminated with strong blue light.

Gene expression in *Drosophila* is temperature dependent as it is for many organisms including humans (Fast and Rosenkranz, 2018; Haltenhof et al., 2020). The expression and processing of genes and the translation of associated proteins can account for a significant amount of the cellular metabolism. In addition, cellular metabolism in producing ATP and other forms of energy is temperature dependent which then relates to cellular activity such as maintaining homeostatic balance in ionic composition (Clarke 2004; Gillooly et al., 2001; Suarez 2012). Thus, an added activity to this exercise is to address how temperature may affect the expression and sensitivity of the *Drosophila* to light with and without being fed ATR but also at varied temperatures.

An illustration in the theme of this exercise is shown in the following figure.



Methods and Data Collection

Part A: Safety and Initial Set-Up for Experimental Phase

The LEDs are very bright light and can damage your eyes. **DO NOT** look directly into the LED or shine onto other people.

The ATR (all-trans retinal) is a compound that can absorb through skin. **DO NOT** get on your skin. If you do come into contact, wash with soap and water as soon as possible. It will not burn your skin but absorption into the body should be avoided.

Part B: Procedure for Experimental Phase 1

1. Obtain vials with larvae that are the progeny of crosses between a UAS-GFP (Green Fluorescent Protein) reporter and each of the GAL4 driver lines that will be used for optogenetic studies in Experimental Phase 2. As positive and negative controls respectively, also use mhc-GFP transgenic flies and the UAS-GFP line without GAL4. Gently rinse food and larvae from the vial into a petri dish using a water bottle and brush. Transfer larvae to an apple juice plate and do not leave immersed in water, as they will drown.
2. Use a fluorescence stereomicroscope to observe the location of green fluorescence in the larvae from each cross. GFP will be visible under blue light illumination (~488nm) when observed through a green filter (~515nm). If available, use a camera to capture images of representative larvae from each cross. As an alternative, a cell phone camera pointed over an ocular lens of the microscope may be used. To slow larval movements for photography, the petri dish may be chilled in advance and placed in a shallow dish of ice to remain cold.
3. Describe and compare the pattern of fluorescence observed for each of the GAL4/UAS-GFP crosses and for the mhc-GFP and the UAS-GFP strain without a GAL4 driver.

Part C: Procedure for Experimental Phase 2

1. Before beginning, dim the lights in the room as much as possible while still being able to see the larvae. Locate the bottle containing larvae that have been fed food without ATR and have been stored at room temperature. Using the scoopula, remove one scoop of the food and place it into a medium-size Petri dish.
2. Starting slowly and adding more as needed, use your water bottle to dilute the food so that you can locate the larvae. You can use your paintbrush to mix the water into the food so that it looks like the dishes in the figure.
3. Locate the medium-size Petri dish labeled **Room Temp/No ATR**. Use the plastic dropper to place 1-2 drops of apple juice on the Petri dish. Swirl the dish so that the apple juice spreads throughout the dish.
4. Use the small paintbrush to transfer 3 larvae to the dish containing the apple juice. Use paper filter to wick away excess water from the larvae so that movement is not inhibited. Place this dish into the dark area within the cardboard box.
5. Use the LED light to shine the light directly on one larva for 10 seconds. One person in your group should shine the light while the other operates the timer. After 10 seconds, turn the light off, but continue to time how long it takes for the larvae to resume moving.
6. Record your observations on the data table located on Page 6.
7. Repeat steps 1-6, but using the bottle of larvae and Petri dish that are both labeled **Room Temp/ATR**.
8. Repeat steps 1-6, but using the bottle of larvae and Petri dish that are both labeled **Incubator/No ATR**.
9. Repeat steps 1-6, but using the bottle of larvae and Petri dish that are both labeled **Incubator/ATR**.



Data Analysis

As part of the graphical and data literacy portion of this module, we recommend having students create a graph of the data that they have collected that can be shared during the discussion portion. We suggest giving them the freedom to be as creative as they would like with this process. Any errors within their graph can be addressed during discussion.

This is also a great opportunity to incorporate technology for students to utilize to create their graphs. Some suggestions include Graphical, Infogram, and Plotly.

Discussion

Discussion of Experimental Phase

Suggestions for discussion questions or considerations as this investigation is being completed include:

1. How did the fluorescence differ in the larvae from the various UAS-GFP crossed in the first experiment?
2. Why would you expect no fluorescence in flies carrying only the UAS-GFP transgene?
3. How did light affect crawling behavior in the larvae?
4. What causes the larvae to respond to light?
5. What is channelrhodopsin? In what organism did channelrhodopsin originate?
6. What evolutionary advantage might channelrhodopsin have provided to organisms that developed it ?
7. Did a diet containing ATR change larvae behavior? What about temperature? Why would temperature affect metabolism in larvae? How did the students interpret that data to reach the conclusions?
8. Do you think the response would be the same or different if we did the same experiment on adult fruit flies?
9. Which cells in the larvae express channelrhodopsin?
10. What would you expect to happen at the neuromuscular junction if larvae were stimulated with blue light for several minutes?
11. How does temperature relate to enzyme function at very high and low levels of temperature? How does temperature alter movement of molecules in a solution?

Keywords for Literature Gathering Phase

For the literature gathering portion of this module, we have created a list of twelve suggested keywords to use based on a classroom of 24 students.

Optogenetics	Channelrhodopsin	Drosophila melanogaster
Transgenic organism	Biotechnology	Neurobiology
Recombinant DNA	Gene therapy	Neuroscience
All-trans retinal	Genetically-modified organisms	Blue light

Directions for Literature Gathering Phase

1. Go to <http://www.pubmed.gov>.
2. Search for your assigned keyword.
3. On the right side of the page, click “Download CSV” to obtain the number of publications produced per year.

4. Use the data you collected to document your findings by creating a graph of your choosing.

Guiding Questions for Literature Gathering Phase

1. Did the number of publications based on your topic per year consistently increase or decrease? Are there any trends you can find?
2. Are there any explanations for the trends you found?
3. Sort your search results on PubMed by “Best Match” and review the titles of publications from the first three pages of your search results. Are there any trends within the topics related to your search term?

Discussion for Literature Gathering Phase

Discussion questions or considerations for the literature gathering phase of this module include:

1. What are some of the trends you noticed in numbers of publications per year? Are there explanations for the trends?
2. What are some of the trends you noticed within the titles of the publications?
3. After reviewing scientific publications about optogenetics, what are some ways that you think it could be used in biomedical research to address a clinically relevant problem?
4. 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?
5. After seeing how the optogenetics was able to be used to manipulate larvae behavior, what are some of the limitations that make it more complex to utilize optogenetics on humans?

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Materials

- One bottle of larvae that has been stored at room temperature without ATR
- One bottle of larvae that has been stored at room temperature with ATR
- One bottle of larvae that has been stored in incubator without ATR
- One bottle of larvae that has been stored in incubator with ATR
- Assembled LED light
- Medium-size cardboard box
- 2 medium-size Petri dishes (one should be scored on the bottom)
- Small paint brush
- Apple juice
- Water bottle
- Plastic dropper
- Paper filter
- Scoopula
- Instructional packet

Notes for the Instructor

This report is primarily a teaching tool for advanced undergraduate students in physiology and genetic courses that participate in experimentation. The significance of the exercise is that it can lead to deeper levels of discussion in the mechanism behind how channelrhodopsin functions as an ion channels (i.e. properties of ion channels) as well as how temperature influences metabolism and gene regulation which can all be very complex and difficult to address without some reading of primary literature. One may wish to leave it in terms of generalities depending on the level of the course the participants are enrolled in.

In addition, learning how the GAL4-UAS tool is used in *Drosophila* to express particular genes in defined tissues. There is a free lab manual for *Drosophila* online (*Drosophila Workers Unite* by

Michele Markstein <http://marksteinlab.org/dwu/>). This web site includes basic information on *Drosophila* care and crossing lines, as well as a description of the GAL4/UAS system. In addition to science-based approach, this module lends itself to incorporation of engineering practices by potentially having students construct an apparatus for providing LED lights. By utilizing the assembly instructions provided they can learn to design and solder electronic parts. The students also learn how the all-trans-retinal (ATR) alters the sensitivity of the channel rhodopsin to light. The ATR is a cofactor for the which increases the sensitivity to light and increases single channel conductance (Dawydow et al., 2014).

There are a number of fun *Drosophila* lines which can be ordered to use to examine specific behaviors. These could be quite fun for student engagement in a course. See https://bdsc.indiana.edu/stocks/teach/teach_optogenetics.html

If time and resources allow, there are many other variables that could be tested in addition to those listed in the procedures. Some suggestions include:

- More variations of temperature
- Various wavelengths of light, such as red light, green light, etc.
- Adult fruit flies
- Longer or intermittent periods of light stimulus
- If microscopes are available, body wall movements per x seconds could be observed as part of data collection
- If microscopes are available, mouth hook movements can also be examined with and without light stimuli. See the following link for more information: <http://web.as.uky.edu/Biology/faculty/cooper/Education-optogenetics/Home-optogenetics.htm>
- There are many free resources available online. Students could possibly search for their own variables to test.

In addition to science-based approach, this module lends itself to incorporation of engineering

practices by potentially having students construct an apparatus for providing LED lights. By utilizing the assembly instructions provided (Appendix A), they can learn to design and solder electronic parts.

~~To facilitate discussion among students, the italic font below (see Appendix) represents potential talking points or considerations for both phases of the module.~~

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Appendix A

Data Collection for Experimental Phase 2

CHR2XXL x 24B (body wall muscles)	Response to light stimulus (no Response, head wagging, rolling, stopping, etc...)	Number of seconds to resume the 1st body wall contraction after the light is turned off.
Room temp/No ATR larva 1		
Room temp/No ATR larva 2		
Room temp/No ATR larva 3		
Room temp/ATR larva 1		
Room temp/ATR larva 2		
Room temp/ATR larva 3		
Incubator/No ATR larva 1		
Incubator/No ATR larva 2		
Incubator/No ATR larva 3		
Incubator /ATR larva 1		
Incubator /ATR larva 2		
Incubator /ATR larva 3		
CHR2XXL x D42 (Motor neurons)	Response to light stimulus (no Response, head wagging, rolling, stopping, etc...)	Number of seconds to resume the 1st body wall contraction after the light is turned off.
Room temp/No ATR larva 1		
Room temp/No ATR larva 2		
Room temp/No ATR larva 3		
Room temp/ATR larva 1		
Room temp/ATR larva 2		
Room temp/ATR larva 3		
Incubator/No ATR larva 1		
Incubator/No ATR larva 2		
Incubator/No ATR larva 3		
Incubator /ATR larva 1		
Incubator /ATR larva 2		
Incubator /ATR larva 3		
CHR2XXL- only no genetic crosses. These are serving as controls	Response to light stimulus (no Response, head wagging, rolling, stopping, etc...)	Number of seconds to resume the 1st body wall contraction after the light is turned off.
Room temp/No ATR larva 1		
Room temp/No ATR larva 2		
Room temp/No ATR larva 3		
Room temp/ATR larva 1		
Room temp/ATR larva 2		
Room temp/ATR larva 3		
Incubator/No ATR larva 1		
Incubator/No ATR larva 2		
Incubator/No ATR larva 3		
Incubator /ATR larva 1		
Incubator /ATR larva 2		

Incubator /ATR larva 3		
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Remote learning

If the class is in a remote learning, then the following information can be used of recorded behaviors of larvae.

CHR2XXL x 24B (body wall muscles)	Response to light stimulus (no Response, head wagging, rolling, stopping, etc...)	Number of seconds to resume the 1st body wall contraction after the light is turned off.	YOUTUBE link
Room temp/No ATR larva 1			https://youtu.be/GSS6ZCulKEc
Room temp/No ATR larva 2			https://youtu.be/GSS6ZCulKEc
Room temp/No ATR larva 3			https://youtu.be/BxjBEz6D-nl
Room temp/ATR larva 1			https://youtu.be/s9nBs5B1ajw
Room temp/ATR larva 2			https://youtu.be/s9nBs5B1ajw
Room temp/ATR larva 3			https://youtu.be/s9nBs5B1ajw
Incubator/No ATR larva 1			https://youtu.be/rBL4MuC2lml
Incubator/No ATR larva 2			https://youtu.be/rBL4MuC2lml
Incubator/No ATR larva 3			https://youtu.be/X7yOu7Tlhg4
Incubator /ATR larva 1			https://youtu.be/jQK82q8s1h0 and https://youtu.be/F_Vn2jq1smc
Incubator /ATR larva 2			https://youtu.be/famkMsaRM58
Incubator /ATR larva 3			https://youtu.be/Bq6hd9U-qqs and https://youtu.be/XBAIIJSyqQc
CHR2XXL x D42 (Motor neurons)	Response to light stimulus (no Response, head wagging, rolling, stopping, etc...)	Number of seconds to resume the 1st body wall contraction after the light is turned off.	YOUTUBE link
Room temp/No ATR larva 1			https://youtu.be/Wi820nWwP1g
Room temp/No ATR larva 2			https://youtu.be/pzDHGf_Nz_o
Room temp/No ATR larva 3			https://youtu.be/fWqW2hufMBY
Room temp/ATR larva 1			https://youtu.be/-IV-As_4kRM

Room temp/ATR larva 2			https://youtu.be/17ziO1RF-Z8
Room temp/ATR larva 3			https://youtu.be/ViHyQ2qH7_s
Incubator/No ATR larva 1			https://youtu.be/uDko1_c8RHY
Incubator/No ATR larva 2			https://youtu.be/TGmRfiKpWhY
Incubator/No ATR larva 3			https://youtu.be/fnAKAynjaWk
Incubator /ATR larva 1			https://youtu.be/m-FOr7he8c
Incubator /ATR larva 2			https://youtu.be/fMxwm3nfq5Q
Incubator /ATR larva 3			https://youtu.be/fMxwm3nfq5Q
CHR2XXL- only no genetic crosses. These are serving as controls	Response to light stimulus (no Response, head wagging, rolling, stopping, etc...)	Number of seconds to resume the 1st body wall contraction after the light is turned off.	
Room temp/No ATR larvae 5			https://youtu.be/GmyVFbbB9i0
Room temp/ATR larvae 6			https://youtu.be/rFa03rk0jCk
Incubator/No ATR larvae 5			https://youtu.be/PzzQZ03BH1g
Incubator /ATR larvae 5			https://youtu.be/EtPU2JILNXQ

Discussion for Experimental Phase

- How did the fluorescence differ in the larvae from the various UAS-GFP crossed in the first experiment?
The specific tissues that are fluorescent should vary with the GAL4 drivers used. Each GAL4 driver includes transcriptional regulatory sequences (enhancers) from a particular gene with specific expression. Specific lines are recommended to give fluorescence in motor neurons and in body wall muscle, but there are many other available GAL4 driver lines that can be used to test other neuron subsets or other tissues.
- Why would you expect no fluorescence in flies carrying only the UAS-GFP transgene?
The UAS-GFP line contains the coding sequence for the fluorescent protein in its genome, but there is no expression of the gene without GAL4 protein present.
- How did light affect crawling behavior in the larvae?
We suggest allowing students to communicate their results at this point.

4. What causes the larvae to respond to light?

The larvae are offspring of fruit flies that have been genetically modified to express channelrhodopsin. The young larvae (instar stages 1, 2 and early 3rd) are negatively phototactic. They tend to go away from light, even the intense blue light. Late 3rd instar are positively phototactic toward moderate white light, but less so for the intense blue light. The natural positive phototactic behavior promotes the larvae to crawl out of the food up the side of containers to then form a pupa out of the food. (An example of this can be seen in nature in a rotting fruit; otherwise, the pupa might drown.) The light is absorbed by the retinal which then promotes opening the ion channel (ChR). This excites the motor neuron and the muscle will be stimulated; the channel lets Na⁺ and Ca²⁺ ions into the cell and it depolarizes the neuron. The neuron fires action potentials due to activating the voltage gated Na⁺ channels from the depolarization. The neuron then relays transmitter on to the muscles. In Drosophila the chemical is glutamate. This excites the muscles cells and the body wall will contract. As long as the light is on, this process will likely keep happening. Once the light is off then the cells will reset the ionic balance by exchanging and pumping ions across the membrane and the original state is regained.

5. What is channelrhodopsin? In what organism did channelrhodopsin originate?

A light-sensitive ion channel. A gene coding for the light sensitive ion channel was taken from algae and then put into other genomes to examine the possibilities of controlling the cells with light. This is now able to be accomplished in isolated cell types, rodent cells in the animal as well as transgenic crosses of Drosophila and other model animals. The "History" section of the following link provides useful information: <https://en.wikipedia.org/wiki/Channelrhodopsin>

6. Did a diet containing ATR change larvae behavior? What about temperature? Why would temperature affect metabolism in larvae?

All-trans retinal (ATR) is a cofactor to channelrhodopsin. In turn, metabolism is necessary for making proteins such as channelrhodopsin. All the enzymes and cellular machinery are controlled by temperature. For example, one reason to store milk or food in a refrigerator or in a freezer is to keep the bacteria from multiplying on your food. With larvae being ectotherms, temperature in the external environment would play a role in metabolism rate.

7. Do you think the response would be the same or different if we did the same experiment on adult fruit flies?

Larval cuticles are transparent and thinner than those of adult fruit flies. We suggest allowing students to form hypotheses to discuss based on this. This could even be tested if time and resources allow. Fruit flies would be placed into test tubes or vials instead of Petri dishes. Some very sensitive channelrhodopsins have been constructed just so they can be used in adult flies. Because of this, very little light through the cuticle will activate the CHRXXL fruit fly.

6. Which cells in the larvae express channelrhodopsin?

The light affected locomotion in the larvae, which is controlled by motor neurons. Motor neurons are where channelrhodopsin is expressed in the larvae. This is because of what genes are expressed for this particular fly line. The channelrhodopsin is co-expressed in all neurons that use glutamate as a neurotransmitter. In this case, this is mostly motor neurons.

7. What would you expect to happen at the neuromuscular junction if larvae were stimulated with blue light for several minutes?

We suggest allowing students to form some hypotheses to discuss. One possibility is that this could even damage the neurons with too much stimulation. The light-sensitive channels may even quit responding to the light over time as the ATR is degraded. This could be tested if time and resources allow.

8. What would you expect to happen if we completed the same experiment and increased the temperature by 10 degrees? And 10 more degrees after that?

Eventually, the temperature would reach a point where the larvae would die. We suggest allowing students to form some hypotheses to discuss. This could even be tested if time and resources allow. The reverse (decreasing temperature) could also be considered and tested.

Discussion for Literature Gathering Phase

1. What are some of the trends you noticed in numbers of publications per year? Are there explanations for the trends?

We suggest letting students share their findings and graphs at this point. Likely, the majority of topics will exhibit increases in publications per year which signifies the continuing prevalence in the scientific community. One point that would be great to include here is that adult fruit flies and larvae serve as the models for this module and many other scientific experiments mentioned in publications due to being relatively easy to manipulate their genome as well as a quick reproductive rate.

2. What are some of the trends you noticed within the titles of the publications?

A large majority of publications will likely be medically related, which is a great segue into the next question and discussion of how optogenetics is being considered as potential treatment for Parkinson's disease, epilepsy, blindness, and many other medical conditions.

3. After reviewing scientific publications about optogenetics, what are some ways that you think it could be used in biomedical research to address a clinically relevant problem?

See question 2.

4. 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?

One might be able to inject a virus to express light-sensitive ion channels in motor neurons. These neurons might be able to be excited with light to control subsets of skeletal muscles.

5. After seeing how the optogenetics was able to be used to manipulate larvae behavior, what are some of the limitations that make it more complex to utilize optogenetics on humans?

Larvae have thin cuticles which made stimulation relatively easy. Due to our skin being much thicker, using optogenetics to directly stimulate neurons would be much more invasive. Currently, fiber optics are used to stimulate deeper brain regions. Other caveats for expressing light-sensitive proteins in mammalian tissue include alteration of ion levels within cells which affects ability to get results consistently and repeatedly. Changes in ionic balance can have long-term consequences for cellular function.

Preparations Needed by the Instructor

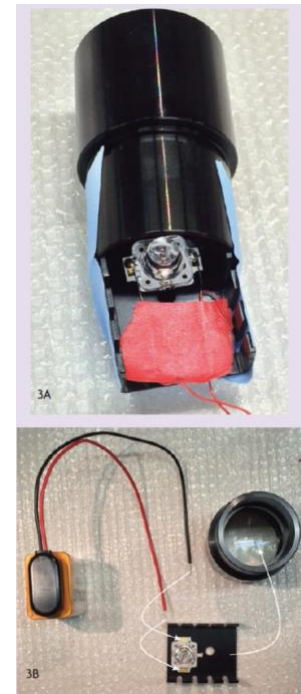
*Materials needed for LED Assembly**

- Side-emitting LED (Blue light - 460nm minimum and 490nm maximum)
- Small heat sink
- 10x ocular objective lens (ensure it is wide enough for heat sink to fit inside)
- Two 6-inch pieces of electrical wire (preferably in different colors)
- Heat sink glue
- Electrical tape
- 9V battery
- 9V battery snap connector
- Solder

**Note: These materials can be found at ledsupply.com or on Amazon.*

Directions for LED Assembly

1. Before beginning the soldering process, determine which lead on LED is positive and which is negative. The LED will have markings (+/-) that specify which is which. Using a soldering iron and solder (found in most physics labs), solder one end of the electrical wires onto the LED. Using different colors will assist with keeping track of which wire is connected to the positive lead and vice versa.
2. Solder the battery snap connector wires to the loose ends of the wires. Ensure the poles on battery connection match with LED +/-.
3. Place electrical tape over the area where the wires have been soldered together to prevent contact with other metal objects.
4. Using heat sink glue, attach the back of the LED to the heat sink.
5. Place the heat sink device into the back a 10x eyepiece objective, which will allow the light beam to be focused.
6. Attach the battery.



Materials Needed for Larvae Preparation

- One bottle of larvae that has been stored at room temperature with or without ATR
- Assembled LED light
- Medium-size cardboard box
- 2 medium-size Petri dishes (one should be scored on the bottom)
- Small paint brush
- Apple juice agar or just apple juice to taint the dish
- Plastic vials with corn meal fly food
- Water bottle
- Plastic dropper
- Paper filter
- Scoopula
- Instructional packet
- Fluorescent microscope. Can use a dissecting microscope modified for GFP visualization of larvae.
- Incubator (water bath can be used if an incubator is not available - see instructions above)

Using ATR

Storage Information: ATR (500mg; available from Sigma-Aldrich, St. Louis, MO, USA) is dissolved in 17.6 ml absolute ethanol to make 100mM stock solutions. Then, 100 μ l of 100mM stock solution is transferred to small tubes, wrapped with aluminum foil and kept in a -20°C freezer. The ATR should be kept away from light, since it is light sensitive; it would be degraded and become ineffective if it is exposed to light for a long time.

***Purchasing Information: Fly food can be made or can be purchased in packets.

<https://bdsc.indiana.edu/information/recipes/bloomfood.html>

Preparation of fly food supplemented with ATR: In order to prepare fly food supplemented with 1mM ATR, 10ml fly food is dissolved in the microwave. The food is left to cool, then 100 μ l of 100mM ATR is mixed well with the fly food, or 100 μ l of absolute ethanol is mixed with food as a control. The food vial should be wrapped in aluminum foil and the food left until solidified (flies may stick to wet food).

Directions for larvae preparation*

1. Place roughly a tablespoon of fly food that does not contain ATR into a vial or plastic bottle.
2. Gently tap around 8-10 fruit flies into the bottle and close the lid. Place in a dark area at room temperature.
3. Repeat steps 1-2. Instead of storing at room temperature, this bottle should be stored in an incubator at 28 degrees Celsius.
4. Repeat steps 1-2, with fly food containing ATR and storage at room temperature.
5. Repeat steps 1-2, with fly food containing ATR and storage in the incubator.

**Note: This process should be completed 48 hours prior to student experimentation. On the day of the experiment, it is recommended that adult flies are disposed of to prevent them from accidentally being released into the classroom. The larvae will be located in the food. We suggest creating enough vials and bottles of each group to allow students to participate in transferring the larvae to Petri dishes. This will allow them to gain some hands-on experience with processes that mimic what occurs in a real research laboratory. We also recommend labeling the bottles and Petri dishes ahead of time with the conditions the larvae are stored in (room temp/ATR, incubator/no ATR, etc) so that students can easily pair them during the transferring process.*

Additional Preparation Considerations

If unavailable, a water bath could be used in lieu of an incubator.

1. Prepare flies into vials as described in larvae preparation instructions on Page 4.
2. Take a rectangular piece of styrofoam and make a hole in the middle about the same size as the vial containing the larvae. The bottom of the vial containing the food should be submerged in the water after being inserted into the styrofoam.
3. Place rubber bands around the portion of the vial above the styrofoam so that the vial cannot slip through the hole.



If you would like to maintain fly stock for future experiments, place adult flies into new tubes containing fly food. Alternatively, move a chunk of food containing larvae into new tubes. When the food begins to look black with too many dead flies, move adults to a new vial. Ten adults (some male and some female) are sufficient to keep a new

vial going. If tubes are kept in a cool location (18-20 degrees Celsius), development is slowed, and flies will not eat as much food.

For ordering fly lines

There are some procedures where the fly lines obtained can be directly examined without having to make F1 generations with selective crosses.

We used a recently created ChR2 line which is very sensitive to light called $y^1 w^{1118}$; PBac{UAS-ChR2.XXL}VK00018 (BDSC stock # 58374) (Dawydow et al., 2014). Virgin females from w^* ; P{UAS-H134R-ChR2}2 were crossed with males of D42-Gal4 (BDSC stock#8816), TRH-Gal4 (BDSC stock#38389), Gad1-Gal4 (BDSC stock# 51630, or ppk-Gal4(BDSC stock# 32078) line to express ChR2-XXL variant in motor neurons, serotonergic neurons, GABAergic neurons or Type IV sensory neurons, respectively.

One can obtain a similar line from Bloomington Drosophila Stock (P{UAS-H134R-ChR2}2 stock BDSC #28995).

Fly lines for channel rhodopsin expression

$y^1 w^{1118}$; PBac{UAS-ChR2.XXL}VK00018 (BDSC stock # 58374) Virgin Female. Highly sensitive to blue light or [P{UAS-H134R-ChR2}2](#) stock BDSC #28995) Virgin Female. Less sensitive to blue light than ChR2.XXL lines

Line	Sex	Outcome
Cross with below lines		
D42-Gal4	Male	ChR expressed in motor neurons.
TRH-Gal4	Male	ChR expressed in serotonergic neurons
Gad1-Gal4	Male	ChR expressed in GABAergic neurons
ppk-Gal4	Male	ChR expressed in Type IV sensory neurons
24B non stubble	Male	ChR expressed in body wall muscles and heart. 24B-Gal4 (III) (BDSC stock # 1767)

Fly lines for GFP expressing lines

For single transgene controlled expression of GFP in muscles, the mhc-GFP stock #38462 can be obtained from the Bloomington Drosophila Stock Center [$y[1] w^*$; P{w[+mC]=Mhc-GFP.F4-453}2]. As a negative control, a strain carrying a white mutation may be used, such as stock #3605.

For crosses to GAL4 drive lines to illustrate varying tissue expression, use virgin females from a UAS-GFP expressing line (UAS-GFP line $y[1] v[1]$; P{ $y[+t7.7] v[+t1.8]$ =UAS-GFP.VALIUM10}attP2 Bloomington Stock 35786) to the males of the same GAL4 driver lines used in the crosses with the channelrhodopsin transgene.

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