ARTICLE
Using *Drosophila* Two-Choice Assay to Study Optogenetics in Hands-On Neurobiology Laboratory Activities

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Optogenetics has made a significant impact on neuroscience, allowing activation and inhibition of neural activity with exquisite spatiotemporal precision in response to light. In this lab session, we use fruit flies to help students understand the fundamentals of optogenetics through hands-on activities. The CsChrimson channelrhodopsin, a light-activated cation channel, is expressed in sweet and bitter sensory neurons. Sweet sensory neurons guide animals to identify nutrient-rich food and drive appetitive behaviors, while bitter sensory neurons direct animals to avoid potentially toxic substances and guide aversive behavior. Students use two-choice assays to explore the causality between the stimulation activation of these neurons and the appetitive and avoidance behaviors of the fruit flies. To quantify their observations, students calculate preference indices and use the Student's t-test to analyze their data. After this lab session, students are expected to have a basic understanding of optogenetics, fly genetics, sensory perception, and how these relate to sensory-guided behaviors. They will also learn to conduct, quantify, and analyze two-choice behavioral assays.

Key words: optogenetics; fruit flies; two-choice assay; 3D printing; sweet neurons; bitter neurons

Channelrhodopsins are a family of single-component light-sensitive proteins that function as ion channels transporting specific ions across cell membranes in response to light. During this photosensitive process, the all-*trans* retinal chromophore absorbs photons and undergoes an isomerization reaction (Yizhar et al., 2011). Channelrhodopsin-2 (ChR2) is naturally found in the green alga *C. reinhardtii*, where it generates low-intensity photocurrents (Sineshchekov et al., 2002; Nagel et al., 2003). In 2005, ChR2 was successfully expressed in hippocampal neurons and used to control neural activity (Boyden et al., 2005). Since then, multiple channelrhodopsins have been added to the optogenetic toolkit, including anion channels such as halorhodopsins for inhibiting neuronal activities (Yizhar et al., 2011). Optogenetics is a specialized approach that combines optics and genetics to enable precise activation and inhibition of specific cell types genetically. Furthermore, the use of light permits the remote control of neuronal activity in a fashion of high temporal and spatial precision. This capability enables researchers to establish causal relationships between activity patterns and brain function and/or behavior in both healthy and diseased conditions (Boyden et al., 2005; Yizhar et al., 2011; Adamantidis et al., 2015).

Fruit flies are a helpful model organism for investigating neuroscience questions as they are inexpensive and easy to rear, exhibit robust appetitive and avoidance behaviors in response to environmental stimuli, and are highly genetically tractable (Bellen et al., 2010; Venken et al., 2011). The small size and semi-transparent external cuticle of fruit flies facilitate the penetration of light (especially the long-wavelength red light) to reach internal cells, making them well-suited for optogenetic manipulation (Venken et al., 2011; Ash et al., 2017). In contrast to mammalian cells, fly cells lack the ability to produce a sufficient amount of retinal, a cofactor that is necessary to support the functionality of channelrhodopsins; it can, however, be added to their food (Schroll et al., 2006; Yizhar et al., 2011).

Fruit flies have taste receptors binding to many of the same molecules as mammals can detect, making them an excellent model for studying taste detection and sensory-guided behavior. The gustatory receptor neurons in fruit flies are distributed on the labellum, pharynx, legs, and wing margins. Four distinct families of gustatory receptor genes have been identified: the gustatory receptor family, the ionotropic receptor family, the epithelial sodium channel/degenerin family, and the transient receptor potential family. GR5a and GR66a are two members of the gustatory receptor family. Activation of GR5a by sweet substances drives appetitive behaviors (movement towards) to nutrient-rich food, while activation of GR66a by bitter-tasting chemicals guides animals to avoid or move away from potentially toxic food. Since detection and responses to nutrients and toxins are crucial for survival, insights from fruit flies may uncover fundamental strategies used throughout evolution to translate chemical recognition into feeding decisions (Amrein and Thorne, 2005; Scott, 2018).

The fundamental concepts of optogenetics and sensory-guided behaviors can seem abstract when only discussed through lectures. Students may not have opportunities to apply optogenetics to real-world problems, such as distinguishing sensory-guided appetitive or avoidance behaviors. Hands-on learning helps students develop a deeper understanding of the subject matter and allows students to apply their theoretical knowledge in practical scenarios. Several hands-on modules using fruit flies to teach undergraduate students optogenetic fundamentals

have been developed, in which students observe the behavioral responses to light and use the electrophysiology to measure the light-induced changes in membrane potential (Pulver et al., 2011; Titlow et al., 2015). Unlike the published lab sessions that use electrophysiological recordings to examine the action potentials, this module focuses on quantitatively analyzing sensory-guided behavior in the context of optogenetic fundamental principles.

In this lab, we used the Gal4/UAS system to express the CsChrimson channelrhodopsin, a red-shifted cation channel, by *Gr5a-Gal4* and *Gr66a-Gal4* in sweet and bitter neurons, respectively (Brand and Perrimon, 1993; Dunipace et al., 2001; Chyb et al., 2003; Klapoetke et al., 2014). This red-shifted channelrhodopsin was used for two reasons, 1) flies cannot detect red light, thus avoiding potential confounds from innate sensory processes, and 2) due to the long wavelength of red light, it penetrates deeper into tissue with less scattering compared to a shorter wavelength light (de Salomon and Spatz, 1983; Venken et al., 2011; Ash et al., 2017). Utilizing 3D printing, we created a choice-chamber apparatus known as the "fly maze" that enables students to conduct two-choice assays and quantitatively evaluate fly behavior (Dudai et al., 1976). In the hands-on part of the lab, students perform the two-choice assay and observe flies' responses to red light (i.e., activation of relevant neurons). This behavioral analysis allows students to draw causal links between the light-induced activation of sweet and bitter neurons and how they regulate appetitive and avoidance behaviors. To quantify the results, students calculate preference indices and use the statistical Student's t-test in Microsoft Excel to analyze their data.

The pedagogical objectives of this lab module include understanding the concept of optogenetics, employing the GAL4/UAS system to express optogenetic tools in specific cells, using a fly maze to conduct two-choice assays, calculating preference indices to determine appetitive or avoidance behavior, and performing the statistical Student's t-test. In addition, this lab module helps students understand animals' sensory perception and their sensory-guided behaviors in response to environmental stimuli when different sensory neurons are activated.

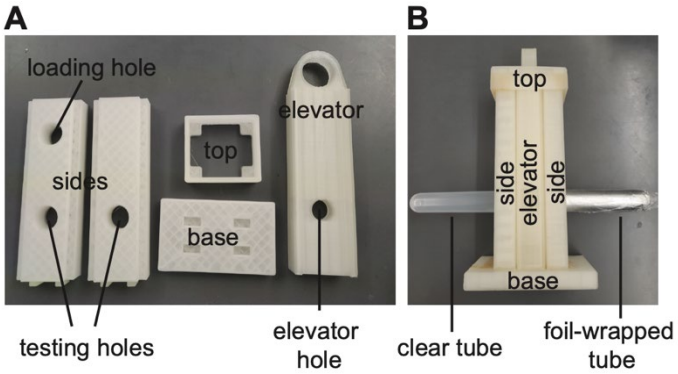


Figure 1. Fly maze. (*A*) A fly maze consists of five parts: two sides, one top, one base, and one elevator. One side has a loading hole and a testing hole, and the other one only has a testing hole. There is an elevator hole in the elevator. The distance between the testing holes and the sides' bottom is equal to the distance between the elevator hole and the elevator's bottom. (*B*) The assembly of the fly maze. Two testing tubes, one clear and one foil-wrapped, are inserted into the testing holes.

MATERIALS AND METHODS

Context for the Module

This exercise serves as the concluding module for Virginia Tech's *Neuroscience Laboratory 1*, the inaugural experimental course designed for neuroscience students. The *Introduction to Biology* is a prerequisite to this course, and the lab must be taken in parallel with the *Introduction to Neuroscience* lecture series. Before this optogenetic exercise, students have completed the following six hands-on modules over eleven weeks: (1) Macroscopic gross anatomy using sheep brains, (2) Immunohistochemistry and microscopic imaging using mouse brains, (3) Neuron computer simulation, (4) Neuromodulation and neuropharmacology using crickets, (5) Neural coding and microscopic surgery using cockroaches, and (6) Conduction velocity using earthworms and human subjects. These modules prepare students for the upcoming optogenetics module by covering topics such as the electrical properties of neurons, chemical-electrical gradients, ion channels, ion flow, circuit activation, and sensory-triggered behavioral responses.

Fly Maze and Light Source

The fly maze consists of five parts that were created by 3D printing using Clear Resin (RS-F2-GPCL-04, Formlabs) or other hard plastics (Figure 1A). If there are no local 3D printers, online services, such as SHAPEWAY, are available to print the apparatus. The computer model files for these parts are provided in the supplementary materials (3D_models.blend) and on GitHub (https://github.com/zhuoaprilfu/3D-Model-for-Fruit-Fly-Maze?search=1). After assembly (Supplement Movie 1), two testing tubes (60818-664, VWR), one clear and one foil-wrapped, were inserted into testing holes on the sides (Figure 1B).

The optical stimulation light source (Vilinsky et al., 2018) (Figure 2) for the optogenetic assays is comprised of a triple red (627 nm) LED starboard (07007-PD000-F, LEDSupply) mounted on a star-shaped heat sink (882-100AB, Wakefield-Vette) with thermal adhesive tape (LXT-T-12, Luxeon Star). A triple secondary LED optic (10507, LEDSupply) was mounted on the LED starboard with liquid adhesive (46040, Loctite). Power was supplied with a 1000 mA LED driver (3021-D-E-1000, Luxeon Star). The part list for the optical stimulation light source is provided in Supplement Table 1.

Drosophila Strains and Maintenance

The strains used in the experiments were *Gr5a-Gal4* (Chyb et al., 2003) (BDSC: 57592), *Gr66a-Gal4* (Dunipace et al., 2001) (BDSC: 57670 should work), and *UAS-CsChrimson* (Klapoetke et al., 2014) (BDSC: 55136) as previously described.

Using the following protocol, *Gr5a-Gal4* and *UAS-CsChrimson* were crossed to create a stable line of *Gr5a-Gal4;UAS-CsChrimson* (referred to as *Gr5a>CsChrimson*),

and *Gr66a-Gal4* and *UAS-CsChrimson* were crossed to create a stable line of *Gr66a-Gal4;UAS-CsChrimson* (referred to as *Gr66a>CsChrimson*). Flies were raised in a cornmeal medium and habituated to a 12:12 hour dark:light cycle at 22°C. 1 L cornmeal medium contained 1 L dH_2O, 79 g dextrose, 7.5 g agar, 24 g flaked yeast, 57 g cornmeal, 2.1 g methal-4-hydroxybenzoate (dissolved in 11.1 mL ethanol), 6 g sodium potassium tartrate tetrahydrate, and 0.9 g calcium chloride. Each vial (32-113RL, Genesee) was filled with about 10 mL medium and capped with a plug (49-102, Genesee). To achieve convincing results, we recommend placing 15-30 flies in each vial. In our experiments, ten males and ten females were placed in each vial (parents were removed after one week). Two weeks later, new flies from the progeny (ten males and ten females for each vial) were sorted into new vials to expand the stock. Each vial could create two to four new vials. Two days before the behavioral assay, flies were transferred to all-*trans* retinal (ATR) food. Each vial contained ten males and ten females. 1 L ATR food contained 1 L dH_2O, 50 g D-sucrose, 7.5 g agar, and 40 μmol all-*trans* retinal (R2500, Sigmal-Aldrich). One day later, *Gr5a>CsChrimson* flies were starved for 20-24 hours by flipping them into sucrose-free ATR food. Flies were kept in the dark in ATR food until the behavioral assay.

Two-Choice Assay

The distance between a red light and the clear tube was 11 cm (Figure 2). The light intensity at the clear tube surface was about 40 klux. A timer was set for one minute. The elevator transported flies from the loading hole to testing tubes and was lifted to align the elevator and loading holes. Using a funnel, flies from one vial were flipped into a clear tube and loaded into the loading hole. The elevator was pushed down to position the elevator hole in the middle of the loading and testing holes. Alternatively, the elevator was positioned to allow the elevator hole in the middle of the loading and testing holes, and the flies were loaded from a testing hole.

Next, the red light was turned on and the elevator was pushed to the bottom position while the timer started. When one minute had elapsed, the elevator was lifted to move the elevator hole to the middle of the loading and testing holes so that the openings of clear and foil-wrapped tubes were blocked.

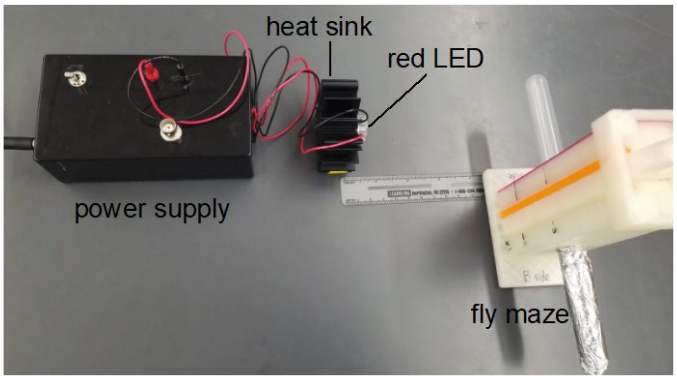


Figure 2. Red light source. Red LED is mounted on a heat sink and powered by a 1000 mA LED driver. During the experimental procedure, the red LED is placed 11 cm away from the clear testing tube.

To count the number of flies in the clear and foil-wrapped tubes, the fly maze was first tilted to the side of the clear tube. Flies inside were tapped to its bottom. The clear tube was removed from the testing hole, and flies inside were flipped into another tube using a funnel. This tube was then capped and placed in ice. After about one minute, the flies stopped moving, and the number of flies was counted. Next, the fly maze was tilted in the opposite direction, and the flies in that chamber were tapped to the bottom of the foil-wrapped tube. The foil-wrapped tube was removed from the testing hole, and flies inside were counted as flies in the clear tube. Finally, a clear tube was inserted into the loading hole. The elevator was lifted to align the elevator and loading holes, and the flies that stayed in the elevator hole were tapped into the clear tube and counted. This process was recorded and provided on GitHub (https://github.com/zhuoaprilfu/3D-Model-for-Fruit-Fly-Maze?search=1).

The preference index was calculated using the following formula: preference index = (fly number on the “red” side (in the clear tube) - fly number on the dark side (in the foil-wrapped tube and the elevator hole)) / (the total number of flies). This process was repeated for three vials of *Gr5a>CsChrimson* flies and three of *Gr66a>CsChrimson* flies. Student’s t-test was performed using the Excel formula: *p* = T.TEST(array1,array2,tails,type). Array1 was the data set of preference indices from *Gr5a>CsChrimson* flies. Array2 was the data set of preference indices from *Gr66a>CsChrimson* flies. Two-tailed distribution was used, and the tails was set to 2. The type was set to 3 for the unequal variance t-test.

RESULTS

In each semester, we offered three to four *Fly Optogenetics* sessions, including morning (10:00 am) and afternoon (2:00 pm) sessions. Since the morning sessions were four hours earlier than the afternoon classes, and *Gr5a>CsChrimson* flies were starved the evening before the experiments, we separated data from the morning and afternoon sessions to detect any potential effects of the different starvation periods on the behavior. Here, we report data from the most recent semesters: spring 2022, fall 2022, and spring 2023.

Fly vials were labeled with red (*Gr5a>CsChrimson*) and black (*Gr66a>CsChrimson*) lines. Students were not told the genotypes of flies and were tasked with identifying their genotypes based on their collected behavioral data. After collecting the data, students calculated preference indices to quantify whether flies preferred the red light or the dark side. The preference index ranged from -1 to 1, where the negative value indicated a preference for the dark side, a positive value indicated a preference for the red-light side, and a value close to 0 indicated no preference between the dark and red-light sides. Subsequently, the students were instructed to use the Student’s t-test to determine whether there were any significant differences between the preference indices of *Gr5a>CsChrimson* and *Gr66a>CsChrimson* flies.

CsChrimson is a red light-activated channelrhodopsin. *Gr5a>CsChrimson* flies expressed CsChrimson in sweet

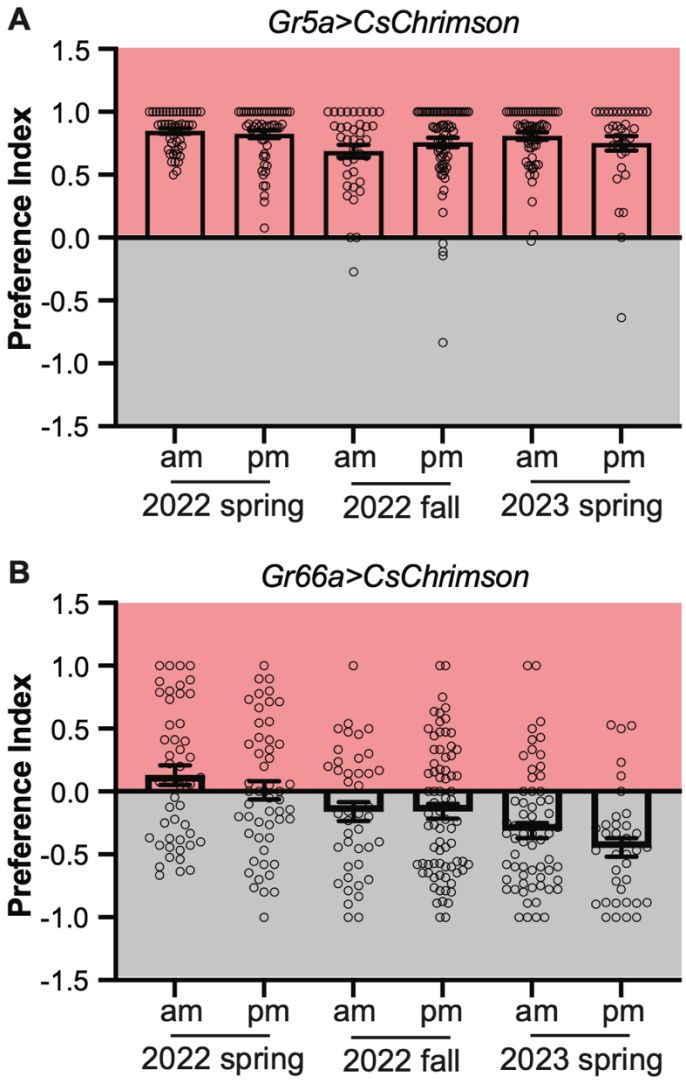


Figure 3. The preferences of *Gr5a>CsChrimson* (*A*) and *Gr66a>CsChrimson* (*B*) to the red light in the two-choice assay. *Gr5a>CsChrimson* and *Gr66a>CsChrimson* express CsChrimson in sweet and bitter neurons, respectively. *n* = 36-75; data represent means ± SEM.

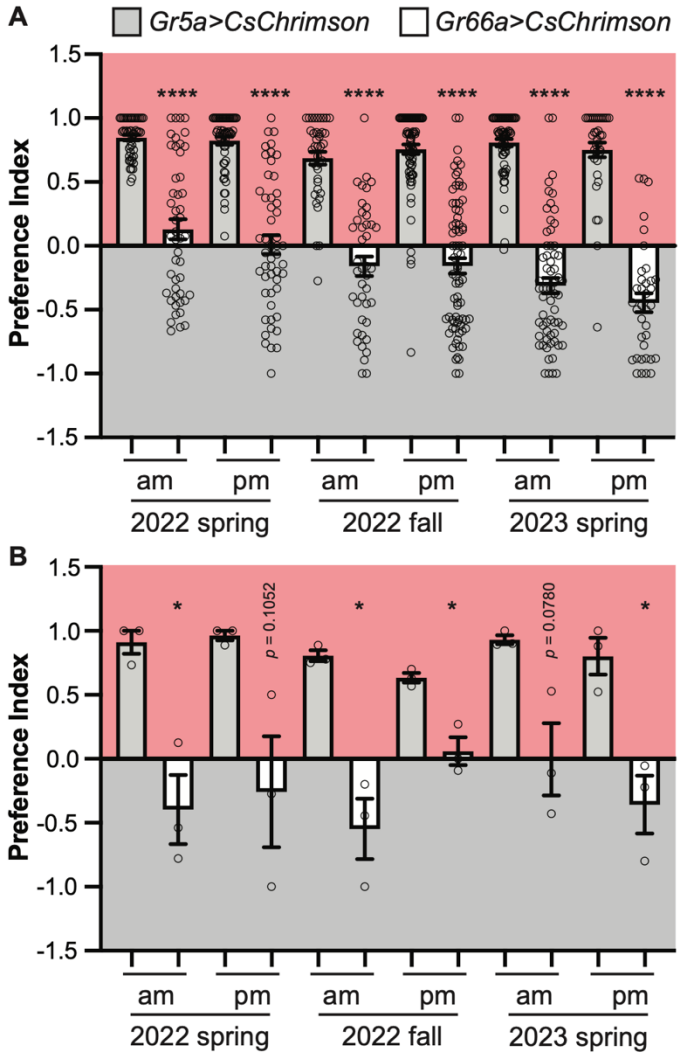
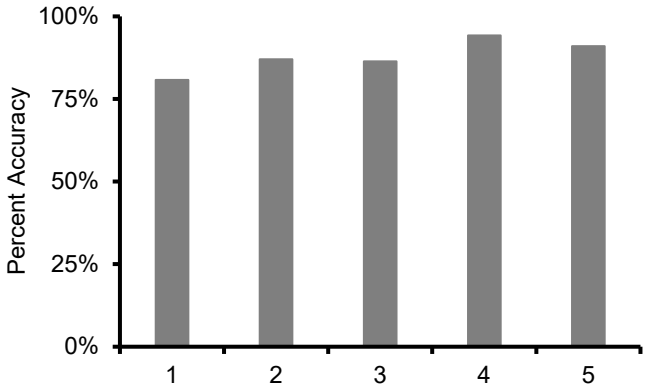


Figure 4. Comparison of the preference indices of *Gr5a>CsChrimson* and *Gr66a>CsChrimson* to the red light in the two-choice assay from all student groups (*A*) or from a single group (*B*). Data represent means ± SEM. (*A*) The same data set as Figure 3. *n* = 36-75; Mann-Whitney test (these data don't pass the Shapiro-Wilk normality test); ****, $p < 0.0001$. (*B*) In each semester, we used the data from the student group who first submitted a complete dataset. *n* = 3; unpaired, unequal variance Student's t-test (these data pass the Shapiro-Wilk normality test); *, $p < 0.05$; *p* values were labeled when higher than 0.05.

taste receptor neurons. In the two-choice assay, most *Gr5a>CsChrimson* flies moved to the clear tubes, and the mean preference indices of these flies were positive and close to 1 (Figure 3A). The red light opened the CsChrimson cation channels, which activated sweet taste receptor neurons in *Gr5a>CsChrimson* flies. The activation then guided starved flies to the red light, demonstrating an appetitive behavior driven by sweet neurons. This behavior was robust and consistent across morning and afternoon sessions in all three semesters (Figure 3A).

Gr66a>CsChrimson flies had CsChrimson expressed in bitter taste neurons. Activation of CsChrimson by red light led to depolarization of the bitter neurons, which typically guided avoidance behaviors. Consequently, these flies were expected to exhibit negative preference indices. The data did not, however, always align consistently with the anticipated outcomes. The mean preference indices obtained from the 2022 spring and fall semesters were similar and close to zero (Figure 3B), indicating that these flies have no preference between the red-light and dark sides. These results were unexpected. In both morning and afternoon sessions of the 2023 spring semester, however, negative preference indices were observed, suggesting flies prefer the dark side and bitter neurons guide an avoidance behavior (See the detailed discussion in Discussion). These results were consistent with the expected outcomes. Notably, we did not observe a difference between the morning and afternoon sessions within the same semester.

Although *Gr66a>CsChrimson* flies displayed unexpected and inconsistent preference indices, their preferences were significantly different than *Gr5a>CsChrimson* flies (Figure 4A). These clear differences allowed students to successfully identify the



1. Understanding the principle of optogenetics
2. Using the Gal4/UAS system to express optogenetic tools in specific fly cells
3. Using a fly maze to conduct two-choice assays
4. Using preference indices to determine sensory-guided appetitive and aversive behaviors
5. Using Microsoft Excel to perform an unpaired two-tailed Student's t-test

Figure 5. Pedagogical outcomes. Students took quizzes designed to examine whether the learning goals were achieved and the accurate rate was calculated. Data were collected from 69 students in the 2023 spring semester.

genotypes of flies (Figure 4B). Because of the significant annual student enrollment (approximately 200 students per year), the small number of instructors and teaching assistants, and the constrained hours available for students to complete each lab module, we did not include control experiments in this lab module.

After the two-choice assay, students were allowed to use red laser pointers to observe the appetitive and avoidance behaviors. When the red laser points to *Gr5a>CsChrimson* flies, most flies stay under the laser and extend their proboscises. Conversely, when the laser points to *Gr66a>CsChrimson* flies, they quickly move away.

Although we did not collect formal student feedback for this module, teaching assistants reported that students were excited to have this hands-on opportunity to learn optogenetics and observe fly's immediate response to light. After attending the lecture on the fundamentals of optogenetics and fly genetics, as well as participating in the hands-on activity of performing two-choice assays and analyzing the results, students were required to take quizzes (including true-or-false and multiple-choice questions) to test whether the pedagogical goals were achieved. Quizzes questions were designed to examine students' understanding of (1) the principles of optogenetics, (2) the Gal4/UAS system, (3) the usage of a fly maze to conduct two-choice assays, (4) determining sensory-guided behaviors based on preference indices, and (5) calculating an unpaired two-tailed t-test in Microsoft Excel. For each question, 81% of the students or higher answered the question accurately (Figure 5).

DISCUSSION

In this lab course, we used the fly gustatory system to help students understand 1) the fundamental principles of optogenetics, 2) an important tool in fly genetics (Gal4/UAS), and 3) how to perform and analyze two-choice behavioral assays. Our post-lab quizzes suggested that over 81% of the learning objectives of this lab module were achieved.

As mammals, the gustatory system in fruit flies drives both appetitive behaviors to detect sweet-tasting nutrient-rich food and aversive behavior to avoid bitter-tasting toxic substances (Amrein and Thorne, 2005; Scott, 2018). The expression of optogenetic channels in GR5a and GR66a neurons triggers opposite responses to light. Similarly, other sensory systems also drive appetitive and aversive behaviors to guide animals toward the environmental stimuli beneficial to their survival and move away from harmful ones. Therefore, the expression of optogenetic tools in specific sensory receptor neurons can test their behavioral consequences. As the ability to detect and respond to various environmental stimuli is essential for survival, this lab module offers undergraduate students an opportunity to enhance their understanding of sensory perception and sensory-guided appetitive and aversive behaviors.

The results obtained from *Gr66a>CsChrimson* flies were unexpected and inconsistent. The initial hypothesis was that activation of CsChrimson by red light would guide avoidance behaviors, resulting in negative preference indices, but the preference indices ranged from about zero to slightly negative (Figure 3B). A possible reason for these unexpected results is related to how flies were loaded into the fly maze. We asked students to load the flies from the testing hole rather than the loading hole because of the difficulty in tapping flies into and closing the loading hole, especially for inexperienced operators, resulting in flies escaping and being smashed. By loading flies from the loading hole, flies are placed at the boundary between the dark and red sides. When the light is on, this placement likely increases their chances of locating the dark and more favorable side.

While the two-choice assay provided robust behavioral results, this module could be expanded in several ways to further students' understanding of optogenetics and its experimental applications, as outlined below.

1. Following the red-light two-choice assay, the same flies can be used to perform a two-choice assay under a different wavelength of light or under ambient light. This control experiment would determine whether the observed appetitive or avoidance behaviors are triggered by the red-light stimulation. Additionally, this experiment would give students an opportunity to use the paired sample t-test or two-way ANOVA for the statistical analysis.
2. If time and resources allow, introducing additional groups of flies can teach students how to set up control experiments. These control flies may include *wild type, Gr5a-Gal4, Gr66a-Gal4*, and *UAS-CsChrimson* that are given food supplemented with ATR prior to the experiments, as well as *Gr5a>CsChrimson* and *Gr66a>CsChrimson* that have not been supplemented with dietary ATR.
3. Another widely used fly optogenetic tool is GtACR (Mauss et al., 2017). Activation of GtACR1 by green or GtACR2 by blue light allows Cl^- to flux into the cell, thereby inhibiting cellular activity. When GtACR is expressed by

Gr66a-Gal4, bitter taste receptor neurons cannot be activated in the presence of green or blue light, and flies don't avoid bitter chemicals. Similarly, when GtACR is expressed in sweet taste receptor neurons by *Gr5a-Gal4*, flies cannot be attracted by sweet chemicals in the presence of green or blue light. Therefore, incorporating GtACR into the module would not only help students better understand optogenetic principles and sensory-guided behaviors but also allow them to explore the concepts of necessity and sufficiency of specific cells for certain behaviors.

4. The 3D-printed fly maze provides a rapid, binary test that allows students to easily and accurately count fly numbers in the light and dark sides, giving them a quantitative behavioral test for responses to sensory stimuli. If 3D printing is not possible, the fly maze can be replaced by a clear tube, half of which is wrapped in foil paper. Students can compare the total number of flies with the number of flies on the clear side to calculate the preference index. This fly maze also enables students to observe flies' responses to red light, but counting the number of flies is challenging.

This lab module provides a hands-on experience to allow students to understand the fundamental principles of optogenetics, animals' sensory perception, and their sensory-guided behaviors. Students also get an opportunity to learn the basic concepts of fly genetics and engage in a quantitative study of appetitive and avoidance behavior.

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