Bi 1x Spring
C. elegans optogenetics

1 Overview

In this experiment, you will learn about invertebrate behavior and a new technique called optogenetics. We will work in the common model organism *C. elegans*. Optogenetics is a technique that uses light-activated proteins to activate and inhibit neurons. Selectively activating or inhibiting individual neurons or groups of neurons allows neuroscientists to investigate behavior. The combination of neurons that interact to create behavior are called neural circuits, and systematic perturbation of individual neurons within the circuit allows researchers to piece together how complex behaviors emerge.

We will use optogenetics to probe individual neurons in two different worm strains to study how worms respond to toxins they senses in the environment. Each strain has a light-activated protein in a single neuron of the neural network. When activated, they will cause the worm to exhibit an aversion response—it will reverse direction and move backward. In this experiment, you will use blue light to activate the light-sensitive proteins in the worms and observe whether or not the worms reverse.

2 Background

2.1 Why *C. elegans*?

Caenorhabditis elegans is a tiny nematode worm that lives in the soil. Since Sydney Brenner's work in the 60s, *C. elegans* has been used extensively in biological research. There are many benefits to using this worm. It is very cheap to grow: worms grow on simple agar plates with a few drops of *E. coli* bacteria on top. They also have a conveniently fast regeneration time of three to four days. Most *C. elegans* are also hermaphrodites, meaning they possess both eggs and sperm and can self-mate. The rest, typically 1 in 500, are males. Thus, if we put a single worm on a plate, in three days we can get 200+ worms with the same genotype as the single parent.

C. elegans are small, but they are still relatively large compared to the microbial model organisms we study in Bi 1x. They are one millimeter in length, so they are visible with the naked eye. Therefore, they are easily manipulated using a relatively cheap, low-magnification microscope. In addition, since the worms are not parasitic and the *E. coli* they eat, OP50, has been modified to be nonpathogenic, the entire *C. elegans* lab is a safe place to work. There are many other benefits of *C. elegans* that have been developed and exploited in the last couple of decades. Strikingly, each worm has the same number of cells, and the lineages of these cells, from egg to adult, are completely known. The genome is completely sequenced, and it contains many vertebrate homologues. Imaging is easy, since the worms are transparent. They are also easy to keep and may be frozen and thawed. We will explore some of these advantages in our excursion with *C. elegans*.

2.2 Why study neural circuits in *C. elegans?*

The worm nervous system is a more tractable system to study than other model organisms. With only 302 neurons and roughly 7,000 synapses, scientists have been able to track the development and name each and every neuron. Neuroscientists are actively working at understanding how the worms nervous system generates behavior. We are going to work with a subset of neurons that have a well defined function in controlling locomotion.

2.3 Why use optogenetics?

Optogenetics offers exquisite control to perturb neurons and measure the subsequent response. The technique uses genetically-encoded light sensitive probes to control events on the cellular and molecular level. The functional units in optogenetics are opsins, light sensitive proteins found in the eyes of many organisms. The particular opsin that has been employed in research labs was originally isolated from the eyespot of the green algae *Chlamydomonas*.

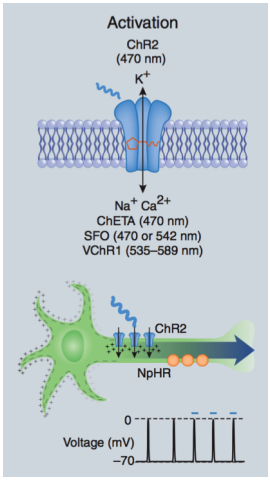

Figure 1: How a channelrhodopsin works. Blue light activates the channel and it opens, allowing positively charged sodium and calcium ions to flood the neuron, simulating an action potential.

Opsins are gated ion channels that open and close when exposed to light signals (see Fig. 1). They allow the passage of charged ions. This is similar to the depolarization or hyperpolarization of a neuron due to non-light stimuli, so we have artificially stimulated the neuron. Because they are activated by a light pulse, researchers can control the exact time that a neuron fires. In some model organisms, the opsin can be expressed in a single cell, allowing very precise probing of neural systems.

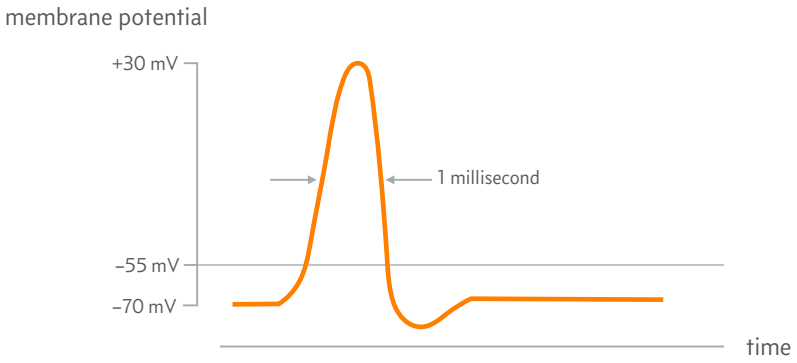

Figure 2: Schematic of an action potential. Membrane proteins open allowing positive ions (Na^+) into the neuron, increasing the membrane potential. This is the depolarization phase. The flux of positive ions causes another class of membrane proteins to open—these proteins allow positive ions (K^+) out of the neurons, thereby restoring the membrane potential to the resting state. This is the repolarization phase.

The specific opsin we are using is channelrhodopsin 2 (ChR2), which is activated by blue light (470 nm). It allows cations to flow into the cell, thereby depolarizing the neuron and causing an action potential. An action potential is a rapid increase in positive ions flooding into the cell (see Fig. 2). This creates a transient increase in the voltage across the cell membrane, called depolarization. This propagates down the axon, and releases a signal to the next neuron. When the next neuron receives the signal, it will also experience an action potential.

2.4 Identifying features in worms

The basic anatomy of a hermaphroditic worm is shown in Fig. 3. We will describe in more detail the important structures of this experiment in what follows.

2.4.1 Head and tail differentiation

In the experiment, it will be important to distinguish the orientation of the *C. elegans* body. This may seem trivial at first but it will be important to get right as we conduct experiments for worm behavior. Worms will generally move forward and will scan their path using their nose. In addition, under the microscope their heads may generally be of a lighter color. The pharynx of the worm may be visible at higher magnification on the head side. Finally, the head is more rounded while the tail of the hermaphrodite usually tapers off to a point. While doing the experiment, keep in mind that worms have no eyes. They have no way of sensing light in their environment.

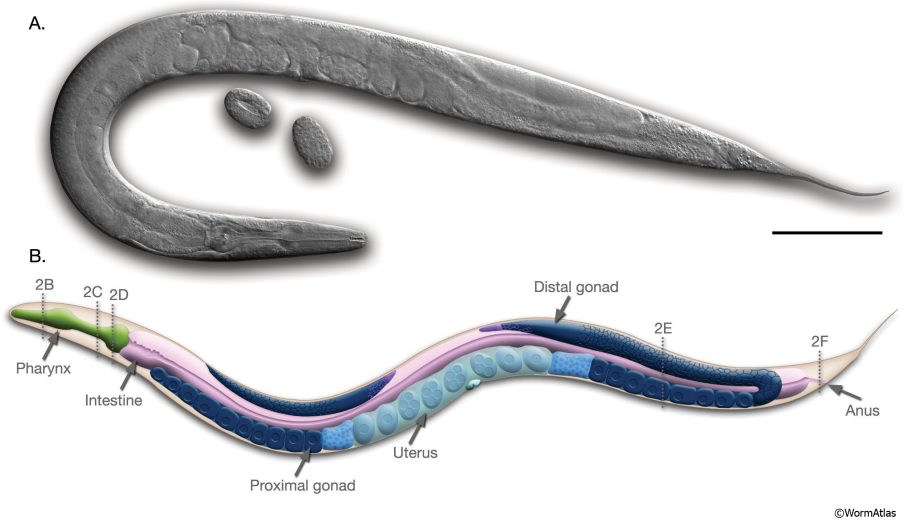

Figure 3: A) Differential interference contrast (DIC) image of an adult hermaphrodite. Scale bar 100 µm. Schematic drawing with anatomical structures. You can ignore the labels with dotted lines, as they refer to another figure in WormAtlas, the source of this image.

2.4.2 Sex determination

The *C. elegans* population is mostly hermaphroditic meaning these worms possess both male and female reproductive organs. This is useful for experimentation as researchers can isolate one hermaphrodite worm and obtain identical copies of it by self-mating. However, the worms do need a method for genetic diversity and males represent a small portion of the population. At the chromosome level, the hermaphrodites have two X sex chromosomes while the males only on have one X (missing the other one).

How do we distinguish between males and hermaphrodites? Males are smaller than hermaphrodites and do not possess the ovaries that are very prominent in hermaphrodites at higher magnifications. Male *C. elegans* have a fan shaped hook on their tail. This is their sexual organ for delivering sperm. Fig. 4 shows a schematic drawing of male and hermaphroditic worms.

2.4.3 Age determination

In addition to studying worms of the same sex, it is important that we study worms of the same age when measuring phenotypes of mutants. There are methods to synchronize the worms but it is possible to determine the age of the worm by inspection.

Before they reach adulthood, hermaphrodites exhibit four larval stages after hatch-

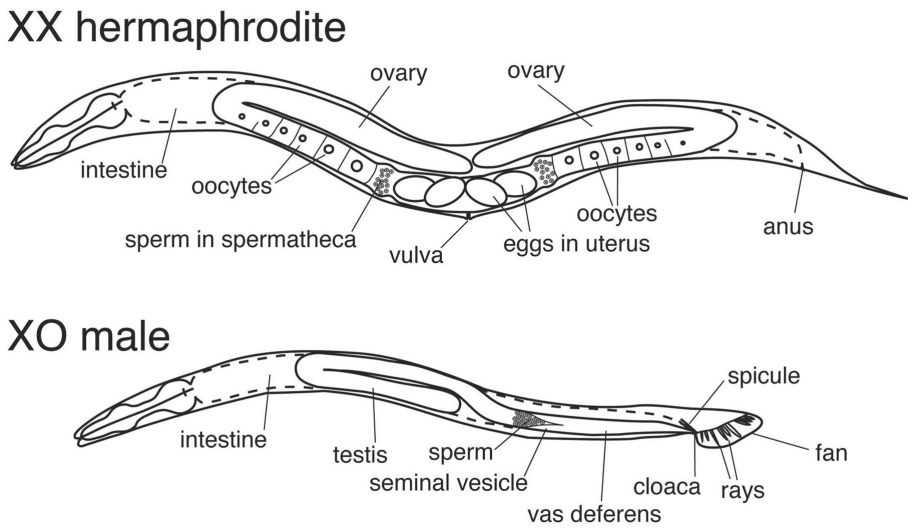

Figure 4: Schematic drawing of the two sexes of *C. elegans* showing important morphological features. Image taken from WormBook.

ing (L1, L2, L3 and L4; see Fig. 5 for the *C. elegans* life cycle). Many experiments are performed on young adults because their age can be known with more certainty than a full-grown adult; older adults looks similar to younger ones. We are therefore interested identifying worms in the last larval stage (L4). If we isolate a group of L4s, we can ensure a sample of young adults for the experiments on the next day. There are a couple of makes for an L4 worm.

> **Size.** Worms get progressively larger until adulthood. The L4s are the largest worms without the visible egg lines in the adults. If there is a diverse population of worms on a plate, the L4s are the ones that are visibility smaller than adults but much larger than the smaller larval stages.
>
> **Vulva marking.** L4 represents the last stage before maturity. This means a lot of action is happening at the sexual organs. The vulva is approximately at the halfway point the body on one side. In L4 worms, there is usually a whitening/darkening spot at the vulva. Conversely, adult vulva are white in color. When picking L4s vulva markings give the best certainty that a worm is an L4.

2.5 Worm bioinformatics

The *C. elegans* was the first multicellular organism sequenced, so every gene is documented. This makes for easier experimentation in that there is a lot of information about various strains and mutations. This information is centralized on one database: `http://www.wormbase.org`. We can search for genes of interest as

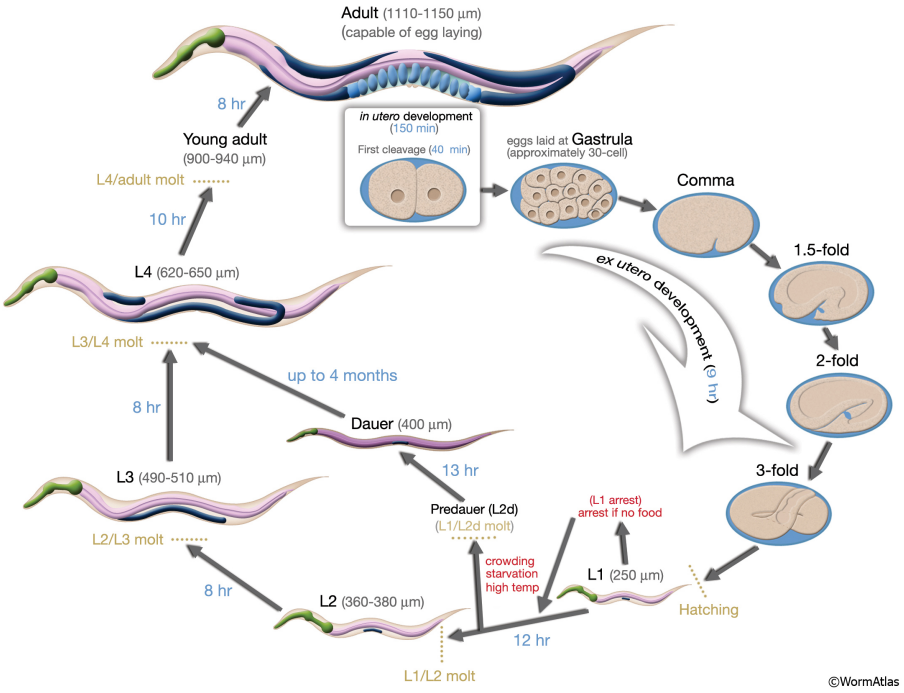

Figure 5: The life cycle of *C. elegans*. Image taken from WormAtlas.

well as the existing strains of *C. elegans* and have a comprehensive understanding of the genetics in play. We will be using some of these powerful techniques to understand some of our strains.

2.6 The Reversal Neural Circuit

A neural circuit is a series of interconnected neurons that create a pathway to transmit a signal from where it is received, to where it causes a behavioral response in an animal. An example is the neural circuit involved in reversals in *C. elegans*. This circuit consists of three types of neurons: sensory neurons receive stimuli from the environment, command interneurons integrate information from many sensory neurons and pass a signal to the motor neurons, and motor neurons control worm behavior, such as reversals.

There are six neurons acting in a circuit that responds to environmental cues and triggers a reversal (Fig.6). These include four sensory neurons (ALM, AVM, ASH, and PLM). Each sensory neuron is sensitive to a different type of stimuli. For example, the sensory neuron we are studying (ASH) is sensitive to chemosensory stimuli such as toxins, while another neuron (PLM) is sensitive to mechanical stimuli (touch) in the posterior part of the worm's body. The sensory neurons send signals that are integrated by two command interneurons (AVA and AVD). Each sensory neuron can provide an impulse to the command interneurons at any time. In order for

the command interneuron to fire and activate motor neurons, the sum of the stimuli at any point in time must exceed a certain threshold. Once the stimuli from one or more sensory neurons has induced an action potential in a command interneuron, that signal is passed to motor neurons which will modulate worm behavior.

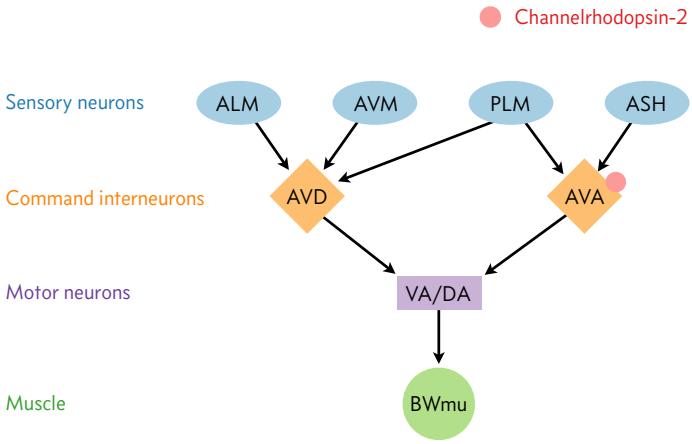

Figure 6: Neural network that controls reversals in *C. elegans*. All neurons are named with their 3-letter abbreviation. A red dot indicates that we put a channelrhodopsin in the ASH neuron.

In this experiment, we will use optogenetics to dissect the function of individual neurons in this circuit. We will work with two optogenetic worm strains. The AVA strain has channelrhodopsin (ChR2) expressed only in the AVA sensory neuron (indicated in Fig. 6 with a red dot). When we shine blue light on this strain, we will activate the ChR2, which will allow sodium and calcium cations to flow into the neuron, simulating an action potential. We will ask if this cause the worm to exhibit aversion behavior and reverse? If not, what does it say about the influence of sensory neurons on the circuit? We will also work with an ASH strain that has channelrhodopsin expressed only in the ASH command interneuron (not shown in the example in Fig. 6). When we activate the ASH neuron with blue light, does it reverse? We are interested in the difference in the reversal responses between activation of the AVA vs. ASH neurons.

3 Protocol

The protocol consists of two parts. First, you will get accustomed to handling *C. elegans*. This is typically called "worm picking." You will perform an ontogenetic assay in which we will score various mutants in terms of their reversal response.

While practicing handling worms, make sure you can identify the head and tail of a worm and tell whether it is wriggling forwards or backwards. This will be key when

you perform the optogenetic assay. You should also be able to identify L4s, young adults, and adults.

3.1 General worm picking procedure

Worm picking can be fun but also frustrating. The only way to get better is by practicing. Here is the general guideline for worm picking. Please read the entire procedure before beginning. This method should be done right even if we are not conducting an experiment because come experiment time, it will be important that you make no mistakes in terms of mislabeling, contamination, or mass worm murder. This procedure may be confusing as you read it, but we will go through it step by step in lab to ensure everyone is comfortable with picking.

We will pick wild type worms (called N2) and worms exhibiting the dumpy phenotype. The differences will be obvious in lab!

1. Obtain the plate of worms and a new transfer plate. Make sure the transfer plate is clean, has food, and has no worms on it. Label the new plate with the correct strain. Be sure to label the bottom of the plate, **not the lid**!
2. Look at the plate of worms under the stereomicroscope. Get the scope in focus and at a comfortable magnification.
3. Light your alcohol candle.
4. Flame the metal section of the worm pick on the oil candle to sterilize it. **This must be done every time to ensure no cross contamination of plates.**
5. The bacterial lawn is sticky, so it can be used as glue for picking. Move the plate such that an edge of the bacterial lawn is in your field of view.
6. Find your worm pick under the scope. You can do this by waving the pick under the scope an inch above the plate and then moving it down.
7. Gently move your pick through the edge of the bacterial lawn to get some sticky "glue" on your pick. Be sure not to push your pick down into the agar. **You should never puncture the agar**, as worms will burrow into the holes and be lost.
8. Identify the worm you want to pick and align in the center of the scope.
9. Under the scope, the pick will appear to have relatively flat end. To pick the worm from the plate, use a circular brushing motion, so that the worm sticks to the "glue."
10. Now that the worm is on the pick, put the transfer plate under the scope, use the same type of technique to place the worm onto the new plate. As a hint, it easier to plate the worm on a region without the food. Thus, try to place it just outside the middle circle of food. Touch your pick "glue" onto the plate and wait for the worms to squirm out onto the agar of the plate.

11. After worm has been transferred. Flame the pick!
12. Ask lots of questions, get lots of help—picking worms is not easy at first.
13. If you hear a popping sound when you flame the pick, it is possible you just burned a worm.

3.2 Optogenetics assay

3.2.1 Setting up the experiment

1. Once a ChR2 is activated, it can take up to 10 minutes until it can be reactivated. Therefore, **it is important to keep the worms in the foil boxes at all times. It is also important to make sure that white or blue light is NOT shining on the worms while you are working with them**, other than the brief pulse of blue light to activate the channelrhodopsin. This means using filters on the stereoscopes that only allow red light through. The plates that the worms are on are also light-sensitive. They contain a co-factor that isomerizes to an inactive form when exposed to light. Be sure to keep the plates in the dark.
2. Make sure the filters are properly installed on the stereomicroscopes.
3. Obtain nine blank food plates from the TAs. You will use three for each strain of worm.
4. Get a plate of each of the worm strains. There are three strains: WT (wild type, the control), AVA and ASH. Label new plates clearly, on the bottom, as the lids are interchangeable. Pick one adult worm of each strain onto each of the three plates. All worms on the plate should be adults, but keep an eye out in case there are undifferentiated L4's.
5. **Place the worms in their foil box and keep them here until it is time to run the experiment.**

3.2.2 Scoring reversals

When stimulating the opsins, it is important that the worms are moving forward, and have been moving forward for a couple seconds. Worms reverse naturally a couple of times per minute, and if you try to activate the ChR2 when the worm is reversing, you will get false positives.

For the experiment, start with your wild type control strain.

1. Take the first worm and place it under the microscope. You can zoom in to whatever magnification you are comfortable with. However, keep in mind that if you zoom in too much, it will be harder to watch the worm reverse as it will quickly leave the field of view.

2. Once you have the worm in your sight, briefly close the LED circuit so that the blue light flashes. The light should be left on for no more than 1 sec.
3. Record whether or not the worm reverses.
4. Repeat for the last two replicates.

Repeat the procedure for the two experimental strains.

This concludes the portion of the experiment for which you will take data. However, once you are done, it would behoove you to continue some experimentation. Try leaving the light on for different lengths of time. Does this affect the response you get from either strain? While doing this, keep in mind that each opsin needs some "rest" time before it can be re-activated. Mark in your notebook whether you observe a difference for each strain.

4 Assignment

Problem 0 (Summary)**.**
Write a summary of this experiment, its objectives, and conclusions between one paragraph and one page in length.

Problem 1 (Contamination (**Prelab**))**.**
Contamination of plates happens easily and is frustrating to worm biologists. What are the possible sources of contamination? What are some of the ways in which we can prevent these contamination sources? Think about the food (the worms eat a very weak strain of *E. coli* OP50), the method of worm transfer, and the way they are stored in 60 mm petri dishes.

Problem 2 (Basic Neuroscience (**Prelab**))**.**
Define an action potential. Explain how activation of opsins influences neuronal activity.

Problem 3 (Reproduction rates)**.**
One of the advantages of using *C. elegans* as a model organism is that worm stocks are cheap and easy to maintain. Given one hermaphrodite, it can conservatively give 200 progeny over its lifetime. It is often enough to start with a couple of worms and end up with more than enough in a week. Given no constraints, **estimate** the number of worms we can obtain in one week if we start with five hermaphrodites. What the are experimental constraints that limit this number?

Problem 4 (Accessing organized biological information)**.**

With the explosion of information about genetic sequences in the past couple of decades, biologists have created searchable databases to organize and share this information. For *C. elegans*, the bioinformatics goldmine is http://www.wormbase.org/. In this problem, you will familiarize yourself with this tool, which is an integral part of working with this model organism.

a) Look up the gene *him-5* on WormBase. What phenotype would you see in a *him-5* knockout worm? Please explore the various tabs available on WormBase and explain the mechanism stated in the overview and beyond.

b) Although *C. elegans* is an invertebrate, it has a lot of gene homology with humans and other organisms. Look up the gene *sod-1*. Read the description and look for the homology section (there is a navigational bar on the left). What human gene shares homology with *sod-1*? How can this gene be used for medical research based on this homology?

Problem 5 (The Experiment: The neural circuit)**.**

a) Which worm strain gave the most pronounced behavioral response when the ChR2's were activated? Support your answer with a statistical analysis.

b) Based on the diagram of the neural circuit and your knowledge of neurons and networks, provide a hypothesis to explain the behavior.

Problem 6 (Another type of opsin)**.**
In addition to the channelrhodopsin ChR2 that we used in this experiment, there is another commonly used opsin called halorhodopsin. Halorhodopsins are essentially the opposite of channelrhodopsins in that they let negatively charged ions such as chloride into the cell, thus suppressing action potentials.

a) How could halorodopsins be used to probe the neural circuit we studied in class?

b) Propose ways in which both types of opsin could be used in biomedical engineering to address diseases.

Problem 7 (Organizing your results)**.**
Include any data or observations not specifically asked for in the other problems.