SUPPLEMENTARY MATERIAL

C. elegans

Caenorhabditis elegans are soil dwelling roundworms that feed on bacteria and grow up to about 1 mm long. In the lab, they can be easily and inexpensively grown in Petri dishes with *E. coli* as a food source. They have become a popular model organism for a number of reasons. They have a short generational time that makes it easy to do aging studies, to do multigenerational studies, and to create large colonies of worms quickly. Although generational time is short, it can be manipulated somewhat by controlling the incubation temperature of the worms. Worms are usually kept at temperatures between 16°C and 25°C. Generally, worms kept at 20°C have a lifespan of about 2–3 weeks and a generation time of about 4 days. Additionally, worms can be frozen and kept alive for long periods of time. This makes research much easier on a scientist's schedule.

C. elegans come in 2 sexes: hermaphrodites and males. Adult hermaphrodites contain 959 cells, and males contain 1031 cells. Of those cells, 302 are neurons! Because the worms have a clear cuticle, their cells can be easily visualized under a light microscope. The nervous system in *C. elegans* consists of a nerve ring (which is analogous to the worm's brain), and 2 nerve cords: a larger ventral nerve cord, and a smaller dorsal nerve cord. The *C. elegans* genome has been completely sequenced and the developmental fate of each of the cells has been mapped. Many mutant strains of worms can be inexpensively purchased from the Caenorhabditis Genetics Center (CGC). Genetic manipulation can also be achieved by feeding worms *E. coli* that contains RNAi, or by injecting DNA into the gametes.

Despite being a relatively simple invertebrate model organism, there is still quite a bit of homology between *C. elegans* and humans in terms of neurotransmission. Worms use glutamate, GABA, acetylcholine, dopamine, tyramine, octopamine, and serotonin as neurotransmitters. Acetylcholine is believed to be the primary excitatory neurotransmitter responsible for controlling motor function in *C. elegans*, however glutamate signaling is still critical for a number of functions. *C. elegans* have glutamate receptors (GLRs) that share a lot of homology with mammalian glutamate receptors. GLRs are homologous to AMPA receptor subunits. NMR receptors are homologous to NMDA receptor subunits. EATs are homologous to vesicular glutamate transporters (VGluTs), and GLTs are homologous to excitatory amino acid transporters (EAATs).

In this series of laboratories, you will learn basic worm maintenance and handling skills, perform fluorescence microscopy on green fluorescent protein (GFP) expressing worms, perform Western blots on worm homogenate to detect glutamate receptors, test receptor mutant worms for behavioral deficits, and design a series of experiments to test

questions of your choice. This work will culminate in a group research project of your choice for which you will report your results in the form of a scientific research article.

Core resources

- Caenorhabditis Genetics Center (CGC) is an NIH supported program housed at the University of Minnesota with the overall goal to promote research on *C. elegans* by acquiring, maintaining, and distributing genetically characterized worm stocks.
    - https://cbs.umn.edu/cgc/home

- The Journal of Visualized Experiments (JOVE) has some really great videos showing techniques and experiments involving *C. elegans*:
    - https://www.jove.com/v/5103/an-introduction-to-caenorhabditis-elegans
    - https://www.jove.com/v/5104/c-elegans-maintenance
    - https://www.jove.com/video/2490/c-elegans-positive-butanone-learning-short-term-long-term-associative
    - https://www.jove.com/video/50069/c-elegans-chemotaxis-assay
    - https://www.jove.com/v/5113/c-elegans-chemotaxis-assay
    - https://www.jove.com/v/5105/rnai-in-c-elegans
    - https://www.jove.com/video/4094/c-elegans-tracking-and-behavioral-measurement

- Wormbook is a valuable online textbook all about C. *elegans:*
    - http://www.wormbook.org/

- C. *elegans* II is another textbook published in an electronic version:
    - http://www.ncbi.nlm.nih.gov/books/NBK19997/

- Other relevant readings:
    - Kowalski, Dahlberg, Juo (2011) The Deubiquitinating Enzyme USP-46 Negatively Regulates the Degradation of Glutamate Receptors to Control Their Abundance in the Ventral Nerve Cord of *Caenorhabditis elegans.* J. Neurosci 31(4):1341–1354.

    - Maricq, Peckol, Driscoll, Bargmann (1995) Mechanosensory signaling in C. elegans mediated by the GLR-1 glutamate receptor. Nature 378(6552)78-81.

Lab 1: Worm Handling

Safety and general practices

Worms feast on *E. coli*! Be sure to wash your hands before leaving lab.

Instructions for using a bottom lit dissecting microscope

- Place worm plate on microscope (keep covered whenever possible)
- Make sure everything is plugged in
- Turn on the power on the external power box
- Adjust light level (9 is probably best)
- Rotate mirror if necessary, to reflect light evenly through the plate
- Adjust intraocular distance for your eyes
- Adjust the zoom using the small upper knob
- Adjust the focus using the large lower knob

Part I: Observations

What general patterns of behavior do you notice? How do the worms move? Do they cluster in any particular areas? How do they overlap with the bacterial lawn? Can you identify worms for each of the 4 larval stages and adults? Additionally, can you discriminate between a male and a hermaphrodite? This is sometimes difficult as there are often many more hermaphrodites than males. Indeed, it is estimated the ratio of hermaphrodites to males is ~500 to 1!

Part II: The Worm Pick and Micro-Sterilizer

Worms can be moved from one plate to another through the use of a "worm pick" tool. A worm pick is a thin platinum wire that has been flattened and bent to make a very small, thin spatula. You will be provided with a commercially available worm pick. These unique picks have a pre-flattened platinum wire securely fastened to a handle. It's important to be as sterile as possible to avoid contaminating the worm plates. The wire part of the worm pick should be sterilized before and between each use using the infrared micro-sterilizer. Proper use and handling of the pick and the micro-sterilizer will be demonstrated for you.

Part III: Tutorial on worm transportation

Your instructor will show you a brief tutorial video (https://www.jove.com/v/5104/c-elegans-maintenance beginning at 3:45, Handling *C. elegans*) on how to transport worms from one dish to another. Using the worm pick, practice moving one worm from the seeded plate (the plate with *E. coli*) to another clean (unseeded) plate. You might find it helpful to dip your pick in the sticky *E. coli* before retrieving a worm. Observe your worm to make sure it is alive. Remember to sterilize your pick after transferring each worm. This skill will take practice! Expect to be uncoordinated at first. All scientists that work on small organisms such as fruit flies and worms spend time developing their ability to manipulate small things under a microscope. Spend some time moving single worms around the plate and from plate to plate until you get the hang of it. **Do not move on to the next part before you are completely comfortable with this exercise.**

Part IV: Worm Poke Practice

Make an eyelash tool by attaching an eyelash or eyebrow hair to a wooden toothpick using Super Glue or tape. Hold it in front of a moving worm until it bumps into the tool. Observe/record the worm's behavior.

Part V: Slide Mounting Practice

Use the microscope to locate a larval stage 4 (L4) worm on the plate. L4 hermaphrodites can be distinguished by the presence of a small white half-circle patch in the worm midsection. Apply a tiny drop of mounting medium to the slide. Transfer a single worm using the worm pick to the dot of medium on the slide. Apply coverslip, carefully avoiding air bubbles. Locate your mounted worm under the microscope and mark its location with a marker.

Part VI: Chunking technique

Worm maintenance is important when working with *C. elegans*. One way to maintain a worm stock is 'chunking'. Generally, you will not be responsible for maintaining the stocks for this lab, but we want you to be exposed to this technique. After you have completed all of the other tasks, practice the chunking technique. First, sterilize a scalpel and wait a few moments for it to cool. Cut a ~5mm x ~5mm square of agar from a section of the plate that contains worms, and place it face down on another plate with a bacterial lawn. Observe the chunked plate under the microscope to watch the worms migrate away from the chunk. Store plate in the incubator and check next week to see if you were able to avoid contamination.

Note: *Many of these skills will be used throughout Unit I. Please feel free to use any extra time at the end of lab to hone your worm handling skills.*

Lab 2: Fluorescence Imaging in *C. elegans*

In this laboratory, we will take fluorescent images of 2 worms: one wild type, and one worm with GFP tagged GLR-1 (GLR1::GFP). Next week, each student will turn in a figure with legend illustrating your results. Use the microscope to locate a larval stage 4 (L4) hermaphrodite worm on the seeded plate to image. L4 hermaphrodites are smaller than adult hermaphrodites (the largest worms on the plate), with a very tapered tail and a distinguishing white oval with a small black dot in the middle of the animal that will develop into the vulva (structure used to lay eggs). Carefully label your slide to indicate which side will contain which strain of worm. Apply a tiny drop of mounting medium to one side of the slide. Using the worm pick, transfer the correct worm strain to the mounting solution. Apply coverslip, carefully avoiding air bubbles. Using a black marker, place a tiny dot on your coverslip near your worm. This will to help you quickly locate your worm when you transition to the fluorescence microscope. Repeat with the other worm strain. Let stand at room temperature for ~5 min. Keep your slides covered with aluminum foil to prevent exposure to light.

Lab 3: *C. elegans* Behavior

C. elegans responds to a gentle touch to the nose by initiating backward locomotion. (Kaplan and Horvitz, 1993). In Journal Club you learned about the contribution *glr-1* has on normal neuronal signaling in *C. elegans* (Maricq et al., 1995). When *glr-1* is deleted, it's found that mechanically stimulated mutant worms lose their ability to withdraw backwards. Yet these same worms maintain their ability to withdraw normally when exposed to chemical repellents. Working in small groups, this week you will have the opportunity to work with these genetically mutated worms and investigate through behavioral testing the implications of this deletion. You should come to lab prepared to perform two tests for behavioral differences between wild types and *glr-1* deletion mutants. Each student will conduct the nose touch reversals test, and each group will set up and run 4 chemotaxis behavioral tests using a chemo-attractant (NaCl in this case) and a chemo-repellant (octanol). Below are the 2 protocols. Note that these experiments may require advance preparation and a waiting time, so please plan accordingly. In two weeks, each student will turn in a figure with legend illustrating the cumulative class results for the following two assays.

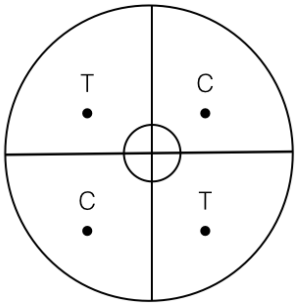
Chemosensory Stimulation:

Chemotaxis Protocol

Materials per group:

- 5 cm unseeded plates (4 chemotaxis plates/team)
- Plates with synchronized worms (you will be blind to the genotype)
- M9 buffer
- Chemotaxis assay buffer
- 2 & 1000 ⌈L pipette
- Pasteur pipettes
- Microcentrifuge tubes
- 2% (~0.56M) sodium azide solution in ddH_2O
- 0.2% (34.2mM) NaCl solution in ddH_2O
- 30% octanol in ethanol
- 70% ethanol and ddH_2O

Procedure:



1. Prepare 5 cm unseeded plates using the template provided. Divide the back of the plate into four equal quadrants. At the intersection point draw a 0.5cm radius circle. In each of the quadrants draw a dot that is equidistant from the center and from the other points. Label the dots alternately T (for test) and C (for control).

2. *Harvest worms:* Transfer 1 ml of M9 buffer onto surface of worm plate. Swirl and tilt to ensure all of the worms have become unstuck from the plate, and pipette the worm and M9 solution into a 1.5 mL micro centrifuge tube. Repeat as necessary.

3. *Wash worms:* Leave the tubes undisturbed for two minutes on the bench to let the worms settle at the bottom of each tube. You should see a "worm pellet" as a white clump settled at the bottom of the tube. Carefully remove most of the buffer, trying not to disturb the worm pellet. Add 1 mL M9 Buffer and *gently* flick the bottom of the tubes to resuspend the worms. Being careful not to disturb the worm pellet, pipette off most of the supernatant and discard.

4. *Wash worms (2nd time):* Add 1 mL M9 Buffer to each tube and let the worms settle at the bottom of the tubes for one minute. Being careful not to disturb the worm pellet, pipette off most of the supernatant. The washes enrich for larger animals (adults), which is what you want.

5. *Wash worms (3rd time):* For the final wash, add 1 mL of Chemotaxis Assay Buffer to each tube and allow the worms to settle to the bottom of the tubes for about one minute. Pipette off most of the supernatant.

6. *Treat chemotaxis plates with sodium azide:* Sodium azide is an anesthetic that causes paralysis in *C. elegans.* Place 2 µL of 2% sodium azide solution to all four spots (T and C). Leave the lids off of the plates until all of the sodium azide has soaked into the agar. *Do not add the sodium azide too far in advance as it may diffuse through the agar, changing the localized concentration at each spot.*

7. *Place worms:* Resuspend the worms by flicking the bottom of each tube. Using a Pasteur pipette place one drop (~2 µl) of the worms in the center of the plate. Check each plate under the microscope to confirm that you transferred ~75-100 worms (if necessary, you can add more worm suspension to the plate).

8. *Remove excess liquid:* Working under a microscope *CAREFULLY* use a Kimwipe to touch the edge of the worm drop. The goal is to wick away the excess liquid from the drop so that the worms will be able to crawl away from the center of the agar. Do not dab at the agar as you may kill the worms.

9. *Add chemicals*: Place 2 µl of the test substance (either NaCl or octanol**) on the T dots. If working with NaCl, place 2 µl of water to control spots. For the octanol plates, place 2 µl of 70% ethanol to control spots. **Immediately cover plates to avoid rapid evaporation of ethanol.** Begin timing a 1-hour incubation.

10. *Incubate plates:* After ~10 minutes make sure the worms are not all clumped at the origin. If they are, disperse them with a pick. Allow the plates to incubate undisturbed at room temperature for the remainder of the hour.

11. *Chill plates:* If after 1-hour incubation you are not ready to begin counting your chemotaxis plates, place them in the refrigerator to chill the worms to stop them from moving.

12. *Count worms:* Count the number of worms in each quadrant that completely crossed the inner circle. Record your counts and add them to the cumulative data file on the Instructor's computer.

13. *Data analysis:* Calculate the chemotaxis index for each of the four test groups using the following equation:

Chemotaxis Index = (# Worms in Both Test Quadrants - # Worms in Both Control Quadrants) / (Total # of Scored Worms)

This will yield a chemotactic index between -1.0 and +1.0. A +1.0 score indicates maximal attraction towards the target and represents 100% of the worms arriving in the quadrants containing the chemical target. An index of -1.0 is evidence of maximal repulsion.

***** *Wear gloves and safety glasses when working with sodium azide*

****** *Wear gloves when working with octanol.*

Mechanical Stimulation:

Nose Poke Protocol

Materials:

- Eyelash tool
- Plates with well-fed worms, 1 plate WT & 1 plate *glr-1* [*kp4*] mutants (you will be blind to the genotype)
- Worm pick
- Infrared Micro-sterilizer

Procedure:

1. Select a worm to test.

2. Place the eyelash tool in front of the worm so that it will bump into it.

3. Record whether the worm responded or not (yes or no). A trial is scored as a success (yes) when the animal either halts forward locomotion **or** initiates backward movement following the stimulus.

4. Repeat for a total of 10 trials per worm, waiting 5-10 seconds between each trial.

5. Incinerate each worm after the 10 trials are complete.

6. Repeat for a total of 5 worms per genotype.

7. Calculate the percent positive score and add your results to the cumulative data file on the Instructor's computer.

Lab 4: Western Sample Preparation from *C. elegans*

Rationale:

Western blotting is a technique that is used to detect specific proteins in a sample. In our case, the sample will be prepared from whole worms. Samples are made by combining large quantities of worms with a gel sample buffer containing beta-mercaptoethanol (®-met) and sodium dodecyl sulfate (SDS), and then heating to 90°C. Native proteins in an organism have a 3-D quaternary structure based on the sequence of amino acids and how they interact with each other. ®-met breaks up any disulfide bonds that can exist between cysteine residues. SDS is a detergent that linearizes the protein and coats it evenly with negative charge. Heat also helps to denature proteins.

In a linear and uniformly charged form, proteins can be run through a polyacrylamide gel matrix and separated by size. This step is called electrophoresis. Electrical current is run through the gel, and negative charged proteins will run from the negative side to the positive side (in this case, down) of the gel. Smaller proteins will be able to move more quickly, and larger proteins will move more slowly. Therefore, smaller proteins will end up further down the gel than larger proteins.

After electrophoresis, the next step is transfer. The proteins are transferred out of the gel, and onto a piece of specialized paper membrane called PVDF. This time, the movement is out of the plane of the gel, rather than down through the gel. PVDF binds proteins with high affinity, so they remain stuck there.

Now, the membrane can be probed with antibodies for our proteins of interest. Immunoglobulin G (IgG) antibodies are produced by the immune system to bind to foreign proteins and mark them for degradation. If you inject a foreign protein into an animal, it will begin production of antibodies directed against this antigen, which can later be collected and purified for laboratory use. However, antibodies are not always completely specific and can stick to other proteins. Therefore, a blocking step is used to help reduce nonspecific binding of antibody to the membrane. One popular blocking solution is milk, because it has lots of protein that can bind up things on the membrane that are just generally "sticky." Once the membrane has been incubated with milk, antibodies (in a milk solution) can be added to probe the membrane for proteins of interest. In this case, we will be using 2 primary antibodies: one directed against GFP, and one directed against actin. Detecting GFP will allow us to detect GLR-1::GFP worm strain, as well as a purified GFP peptide we will use as a positive control. Probing for actin, a major component of the eukaryotic cytoskeleton, will serve as a proxy measure for how much tissue has been loaded into each well: more worms = more actin. Using 2 different antibodies can also help us troubleshoot issues if there is a problem detecting GFP.

After the primary incubation, membranes are thoroughly washed of any unbound primary antibody, and then probed with secondary antibodies. The secondary antibody step is important for 2 reasons. First, multiple secondary antibodies can bind each primary antibody, and thereby allowing for signal amplification. Second, the secondary antibodies we have purchased come conjugated to fluorophores, so that a fluorescence scanner can detect them. This allows us to visualize where our proteins of interest appear on the membrane. Secondary antibodies are raised in a different animal than the primary antibodies are raised in. For example, if you use a primary antibody raised in rabbit, you might use a secondary antibody raised in goat. This secondary would be goat-anti-rabbit, and should detect all protein that comes from rabbit. Since we will have 2 different primary antibodies, each raised in a different species, we will have 2 different secondary antibodies, each directed against a different species, and each with a different fluorophore.

For this experiment, each student will turn in a figure with legend illustrating the results of your Western blot experiment.

Protocol[1]:

Each group of students will make 2 samples: one GLR1::GFP worm sample and one wild type (N2) worm sample. (You will be provided with *A. victoria* GFP protein diluted at 1:100 in sample buffer to serve as a positive control.)

Worm Collection:

1. Pick up 3 large plates/genotype of nearly starved worms. This should give you 50-100 µL of packed worms after the washes described below.

2. Wash the worms off the first plate by pipetting 1mL M9 medium on the plate, swirl gently and suck the liquid off using a Pasteur pipette. Transfer M9/worm solution to a clearly labeled Eppendorf tube.

3. Gently centrifuge your samples for about 10-15 secs to pellet the worms making sure to balance the centrifuge with another Eppendorf tube. Carefully draw up the supernatant using a Pasteur pipette and discard. Be very careful not to disturb the worm pellet. If the pellet becomes disturbed, spin again.

4. Repeat once or twice more to ensure you have retrieved nearly all the worms from the plate.

[1] Protocol adapted from *Making Protein Gel Samples from Worms* developed by Michael Koelle, Yale University.

5. Repeat with the remaining 2 plates of worms. Continue to add the worms (of the same genotype) to the same Eppendorf tube, spinning and discarding supernatant.

6. Wash the worms 3x more with M9, spin at 3,000 rpm, and remove as much liquid as possible and discard the supernatant. Use a P200 pipette to remove the last little bit of supernatant.

7. Estimate the volume of packed worms you have collected and add an equal volume of gel sample buffer (1:1) and place on ice.

Heating and Storage:

8. Heat to 90°C for 10 min on a heating block or water bath to dissolve worms.

9. Spin the tube 10 min at 10,000 rpm to pellet debris. (**This time you will save the supernatant and discard the debris!)** Carefully draw supernatant and transfer to a new, clearly labeled tube.

10. Immediately store your samples at -20°C until our next lab.

Lab 5A: Western Blotting I

Running Gels:

1. Spin your samples for 10 minutes @ 10,000 RPM, and begin setting up your gel box.

2. Remove white tape from the bottom of the gel.

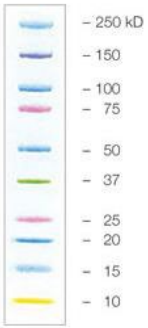
3. Place buffer core in buffer chamber. Insert gel tension wedge behind buffer core in the unlocked position. Insert gel cassette in front of buffer core and a 'dummy' gel on the other side. Pull the tension lever towards the front and lock gel/core/dummy gel into position. Fill inner chamber with **1x Running Buffer** all the way. Wait a moment to make sure the buffer does not leak into the outer chamber. Remove comb from the gel top in one fluid upward motion. Use a transfer pipette to flush out the wells by pipetting buffer in and out of each well while the chamber is full. Fill the outer chamber about 75% of the way up with **1x Running Buffer**.

4. With a P200 pipette, load ladders and samples using long gel loading pipette tips. Be sure to draw from the top of your sample to avoid any pelleted debris in the bottom of the tube. Load ladders and samples in the following volumes and order leaving lane 1 empty:

   Lane 2 > **3uL** rainbow ladder
   Lane 3 > **20uL** GLR1::GFP worm sample
   Lane 4 > **20uL** WT worm sample
   Lane 5 > **4uL** GPF protein
   Lane 6 > **3uL** rainbow ladder

   Note: *Try to pipette slowly so that the samples settle in the wells and don't spill out.*

5. Attach lid making sure gold connectors are aligned properly. Set voltage to 120 V and hit run. You should see tiny bubbles rising up from the wire if current is running properly. Run your gel until dye front is near the bottom of gel and the rungs of the ladder are well separated (~1.5 hrs). The rainbow molecular weight marker should look something like this as it separates:



6. While your gel is running, you can begin to prepare for the transfer.

Transferring Gels:

1. Approximately 20 minutes before the gel is finished running begin to prepare for the transferring. **Gloves should be worn during the transfer process.**

2. Exercise great care when handling the PVDF membrane. Cut a small notch off of the upper left corner of the membrane. Incubate membrane in methanol for 2 minutes. Remove the methanol and incubate in **transfer buffer** for 20 minutes.

3. In the Pyrex dish, soak blotting pads in **transfer buffer**.

4. At the end of the run, press stop, turn off power, and remove gel box cover.

5. Carefully remove gel cassette handling it by the edges. Empty buffer chamber into waste container.

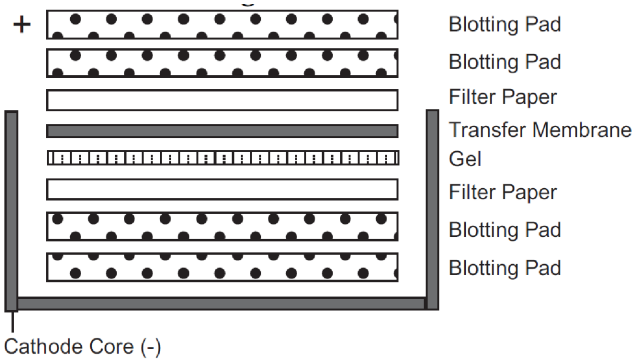
6. Lay the cassette face down on the edge of the bench top. Carefully insert the gel knife between the two plates and push up and down on the knife handle to separate the plates (you will hear a cracking sound – that's okay). Continue to crack open the cassette on all sides until the plates are completely separated. **Very carefully remove the top plate allowing the gel to remain on the bottom (face) plate.**

7. Using the gel knife cut away the 'comb' and make a small notch on the upper right-hand corner of the gel. Place a piece of pre-soaked filter paper on top of gel with bottom edge just above the 'foot' of the gel. Roll out any trapped air bubbles making sure to keep filter paper saturated with transfer buffer.

8. Turn gel plate over onto your gloved hand. Use the knife to push the gel foot out of the slot in the plate, and **carefully** separate the gel from the faceplate. Carefully cut away the gel foot using the gel knife. You can use one of the gel cassette plate as a cutting surface.

9. Wet the surface of the gel with transfer buffer and position PVDF membrane (notch-matched to the gel) directly on top of the gel. Place another pre-soaked filter paper on top of the membrane and again roll out any air bubbles.

10. Place the bottom piece of your XCell blot module in the Pyrex container of **transfer buffer** with the soaking blotting pads. Build your sandwich (bottom up) as shown below. **Make sure the filter paper/gel/transfer membrane/filter paper assembly is placed in the blot module exactly in the order as shown (<u>gel below transfer membrane</u>)!**



11. Place the cover of the blot module on the cathode core box and place in the same box used for electrophoresis. Place the tension wedge and lock the cathode box in place. Fill the inner chamber with **transfer buffer** and the outer chamber most of the way with DI water.

12. Replace lid and turn on power supply and set to **30 V** and run for **1 hour**. While the transfer is running, you can begin to prepare blocking solution and antibody dilutions.

Primary Antibodies:

Check your dilution calculations with your instructors before proceeding*

1. Blocking and diluent solution:
   - Prepare 20 mL of 3% milk in TBST
     - 10 mL will be used to block your blot
     - 5 mL will used to prepare your primary antibody solution
     - 5 mL will be stored in the refrigerator and for your secondary antibody solution

2. Primary antibody solution:
   - Prepare a total volume of 5 mL:
     - rabbit anti-GFP at 1:2500
       and
     - mouse anti-actin at 1:2500
       in 3% milk TBST

3. Once the transfer is finished, carefully disassemble the sandwich. You should see that the marker has moved out of the gel and onto the membrane.

4. Trim the membrane by using a razor blade to cut off parts past your markers and place in 10 mL 3% milk in TBST blocking solution. Incubate membrane in blocking solution at room temperature for 1 hour on a rocker.

5. Clean up your station. Pour all transfer buffer into the hazardous waste container in the hood.

6. At the end of the blocking step, pour off blocking solution and add primary antibody solution and incubate at 4°C overnight on a rocker.

Lab 5B: Western Blotting II

Secondary Antibodies:

1. The next day, pour off primary antibody solution.

2. Do a quick TBST rinse, and then wash 3x in TBST for 5min each on a rocker.

3. Secondary antibody solutions **(remember to check your dilutions with your instructors)**
   Prepare a total volume of 5 mL of:
   - Alexa fluor 488 goat anti-rabbit at 1:5000
     and
   - Alexa fluor 647 goat anti-mouse at 1:5000
     in 3% milk TBST

4. Incubate in secondary solution for 1 hour at room temperature. During this incubation, your instructors will demonstrate how to use the BioRad Imager (for instructions see Appendix II).

5. Do a quick TBST rinse, and then wash 3x in TBST for 5 min each on a rocker.

6. Rinse 5X quickly in ddH_2O.

7. Make a foil sandwich for storing the membrane: Place 2 Kimwipes on a piece of aluminum foil. Place your membrane on the Kimwipes. Add 2 more Kimwipes. Fold the foil over the top to cover. Allow blot to dry for 10-15 mins.

8. Write your name, date, the proteins being probed, antibodies used, and other necessary information on a piece of tape and tape it to the foil.

9. Your blot is now ready to image*!

***Note**: When imaging your blot, the membrane should be placed in the imager in the face up position. This time the notch will be in the upper right-hand corner of the membrane. This is because during the transfer step, the protein moved out of the gel onto what is now the face of the membrane. Additionally, your sample lanes will appear in the reverse order.*

Relevant Reagents:

NGM
https://www.usbio.net/media/N1005/nematode-growth-medium-lite-ngm-lite-powder

M9 Buffer (1 liter)
3 g KH_2PO_4
6 g Na_2HPO_4
5 g NaCl
1 ml 1 M $MgSO_4$
H_2O to 1 liter
Sterilize by autoclaving.

Chemotaxis Assay Buffer (100 mL)
68.05 mg KPO_4
11.1 $CaCl_2$
12.04 $MgSO_4$
H_2O to 100 mL

TBST (1 liter)
2.423 g Tris
8.766 g NaCl
1 mL Tween 20
H_2O to 1 liter
Adjust pH with HCl to pH 7.4–7.6