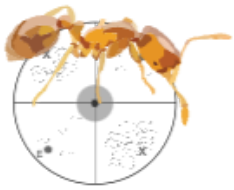
Apr 12, 2024

Chemical ecology in the classroom: chemotaxis assay using C. elegans



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We use this protocol and it's working

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Abstract

There are many natural products in the environment that influence other organisms. How nervous systems sense these compounds to initiate a behavioral response remains an active area of chemical ecology research. We developed a Course Undergraduate Research Experience (CURE) utilizing *Caenorhabditis elegans* chemotaxis assays to test how ant compounds are detected by heterospecific nervous systems. These experiments can be conducted in an undergraduate laboratory course, where new insights into interspecies interactions can be generated through genuine research experiences in a classroom setting.

Attachments

35mmplates_chemotaxi... 199KB

Worms_1080.mp4 496.2MB

exampledata.csv 2KB

Image Attribution

Logo by Lauren O'Connell

Guidelines

This experiment can be designed to test multiple worm strains with the same compound, one worm strain with multiple compounds, or multiple strains and compounds. The Caenorhabditis Genetics Center (CGC) and the Caenorhabditis Natural Diversity Resource (CeNDR) are good sources for wild-type and mutant worm strains.

If using this protocol for teaching, we have found the following works well:
- A max of 20-24 students per class
- One round of training with known attractive and repulsive compounds is beneficial prior to exploring unknown compounds or extracts.
- Students typically start out performing 3 assays each during training, but with experience can reliably run up to 9 assays at once.
- During training and early experiments, increased sample size per group is needed to account for data variation and statistics. For example, all students should perform the training assays to maximize the sample size (20 students = 20 samples per compound tested).
- Worm numbers should be scaled with the experiment by calculating the number of worms needed per assay (300) and accounting for some loss (500 worms per plate needed). For example worms can be grown on an extra large (150 mm) plate and used for steps 2 and 3 by washing with 10 mL of M9 buffer and bleaching in 10mL of bleach solution in a 15 mL falcon.
- Code the compounds or worm strains such that students are unaware of treatment group until they submit their data to the instructor.
- For ant collections, students construct ant aspirators, collect ants outside, and then sort ants in the laboratory. Instructors perform the extraction steps outside of class time.

Materials

Solutions:

M9 buffer: 3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H_2O to 1 liter. Sterilize by autoclaving.

Bleach solution: 9.8mL MilliQ water, 600 µL 5N KOH, 1.2mL 12% sodium hypochlorite.

Chemotaxis plates: 5mM KPO4 (pH 6), 1mM CaCl2, 1mM MgSO4, 2% agar

Chemotaxis buffer: 5mM KPO4 (pH 6), 1mM CaCl2, 1mM MgSO4

LB Broth (Miller, pH 7.0, 5L): 50g NaCl, 50g Tryptone, 25g yeast extract, H_20 to 1 liter. Sterilize by autoclaving.

OP50 E.Coli bacteria grown in LB Media broth: Inoculate 5mL of LB broth with 1 colony of OP50 *E.Coli* and grow overnight at 37°C.

1M *Potassium Phosphate Buffer* (pH 6.0): 108.3g Potassium Phosphate Monobasic (KH_2PO_4, Anhydrous), 35.6g Potassium Phosphate Dibasic (K_2HPO_4, Anhydrous), H_20 to 1 liter. Sterilize by autoclaving. Cool and check pH.

Field work and extraction materials (ant extracts):

Plastic condiment cups and lids
Plastic straws
Mesh filters
Fine tweezers
Whirl-paks (3 per student)
Scissors
Lab tape
Dissection needles
Clay
Methanol
12 mL and 1 mL glass vials w/ PTFE caps
Sharpies for labeling

Laboratory materials (chemotaxis experiment):

Wild type worms (PD1074; Caenorhabditis Genetics Center (CGC) at the University of Minnesota)
Worm picks
micropipettes and tips
microcentrifuge tubes
dimethyl sulfoxide (DMSO)
sodium azide
pasteur pipettes and bulbs
clicker counter
PFTE coated 10 well slide (Polysciences, Inc. 183571)
Chemotaxis templates printed on transparencies (see attached document)

Equipment:

autoclave
incubator

ethanol burner or bunsen burner
nitrogen evaporator
dissection scope

Troubleshooting

Safety warnings

Sodium azide is a health hazard. Steps involving sodium azide should be performed in a ventilation hood. All waste should be discarded as biohazard.

The protocol necessitates the use of an open flame. Care should be taken to avoid burns or fires.

Ethics statement

Check local regulations for collecting samples in nature, as scientific collection permits may be required.

Expand *C. elegans* stock

1w

1 **Prepare nematode growth medium (NGM) plates to grow worms.** 1h

1.1 Combine the following materials and stir for 00:10:00 1h

- 3 g NaCl
- 20 g agar
- 2.5 g peptone
- 975 mL water

Autoclave and allow to cool to 50 °C

1.2 Add additional ingredients to the cooled media and mix:

- 1 mL 5 mg/ml cholesterol in 95% EtOH
- 1 mL 1M CaCl2
- 1 mL 1M MgSO4
- 25 mL 1M potassium phosphate buffer, pH 6

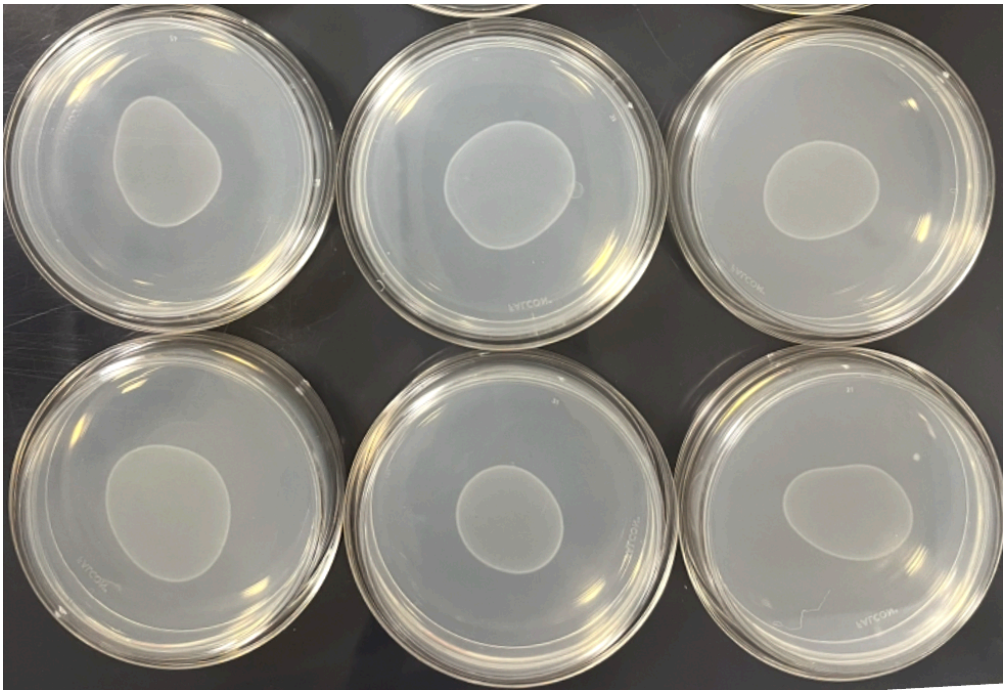
1.3 Plate nematode growth medium (NGM) using aseptic technique (under a flame) by adding 12.5 mL to each 60 mm petri dish.

1.4 Allow the petri dishes to dry with the lid on and face up at room temperature for 48:00:00. After the petri dishes are dry, they may be inverted and stored at 4 °C or seeded with bacteria. 2d

Note

Plates can be stored at 4 °C for several months.

1.5 Add 500 µL of OP50 *E. coli* to the center of the NGM plate and gently swirl the plate to allow the bacteria to form a circle. Allow the bacteria to grow on the NGM plate (right side up) for 24-48 hours at room temperature. The *E. coli* provides nourishment for the worms. Plates may be stored at 4 °C upside down. 2d



Nematode growth medium (NGM) plates seeded with *E. coli*

1.6 Transfer adult *C. elegans* to NGM plates seeded with OP50 *E. coli*. Allow the adult worms to reproduce for 2-4 days at Room temperature . 1h

Note

Strains can differ in their timing of growth. The goals should be to get lots of young adults without letting the plate starve (no bacteria left).

The development of the worms can be controlled by incubation temperature. Worms will mature faster at 25 °C and slower at 16 °C . Plates of different strains were incubated accordingly to have the plates at the correct stage for bleaching.

If you wish to avoid picking individual worms, you can instead use a sterile scapula or spatula to transfer a small (~1 cm^2) chunk of worms to a plate.

Synchronize *C.elegans* by bleaching

45m

2 **Synchronize worms by bleaching 3 to 4 days before the chemotaxis assay.**

Note

Take care to avoid contamination of worms and bleach strains if you notice bacteria, yeast, or mold growing on plates.

2.1 Prepare a fresh bleach solution: 9.8 mL MilliQ water, 600 µL 5N KOH, 1.2 mL 12% sodium hypochlorite. 5m

2.2 Place ~ 60 mL M9 buffer, bleach solution and a new 15 mL conical tube on ice. 2m

2.3 Use a glass Pasteur pipet, transfer the worms from one petri dish to a 15mL conical by washing the adult worms containing many embryos (gravid hermaphrodites) off the NGM plates with approximately 2 mL of M9 buffer. 2m

Note

Glass pipets are preferred, as worms tend to stick to plastic.
Worms pellet better in 15mL conicals more so than 50 mL conicals.

2.4 Repeat with another 2 mL of M9 buffer. 2m

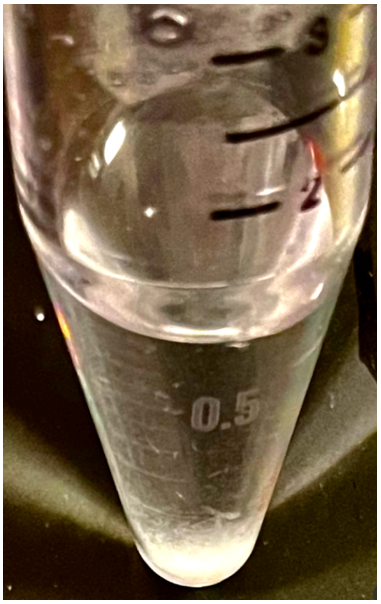
2.5 Centrifuge 800 rcf, 4°C, 00:01:00 to pellet worms. 1m

2.6 Remove the supernatant with a Pasteur pipet (collect as chemical waste). 30s

2.7 Add 4 mL M9 buffer to the 15 mL conical tube and resuspend the worms. 1m

2.8 Centrifuge 800 rcf, 4°C, 00:01:00 to pellet worms. 1m

2.9 Remove the supernatant with a Pasteur pipet, leaving behind 1mL of M9 buffer and the worm pellet. 30s



Worm pellet after transfer from petri plate.

2.10 Add 5 mL of the bleach solution to the 1 mL worm pellet. 30s

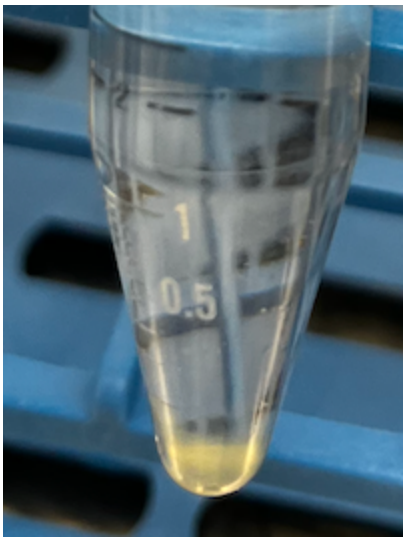
2.11 Vortex for 00:05:00 . 5m

Note

Time can vary depending on strength of the bleach solultion and users should modify as necessary. Once adults dissolve the solution will turn slightly yellow and the tube should be placed immediately on ice or spun in the centrifuge (next step) to avoid over-bleaching.

2.12 Centrifuge 800 rcf, 4°C, 00:01:00 to pellet embryos. 1m

2.13 Remove the supernatant leaving 0.5 mL of worm embryo/ bleach solution. The worm pellet should be slightly yellow. 30s



Yellow colored worm pellet after bleaching.

2.14 Add 12 mL M9 buffer to the 15mL conical tube and resuspend the embryos by shaking. 1m

2.15 Centrifuge 8000 rcf, 4°C, 00:01:00 to pellet embryos. 1m

2.16 Remove the supernatant leaving 0.5 mL of embryo mixture. The embryos will be white or clear in color. 30s

2.17 Repeat 3 more times for a total of 4 washes. 12m

Counting and plating *C. elegans* embryos 1w

3 **Plate the embryos**

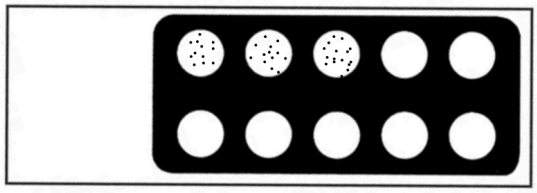
3.1 Vortex the 0.5 mL embryo mixture. 15s

3.2 Add 2 µL of embryo mixture to 18 µL of M9 buffer in a microcentrifuge tube. 30s

3.3 Pipette 1 µL of this dilution into 3 wells of a microscope PFTE coated slide. 15s

Note

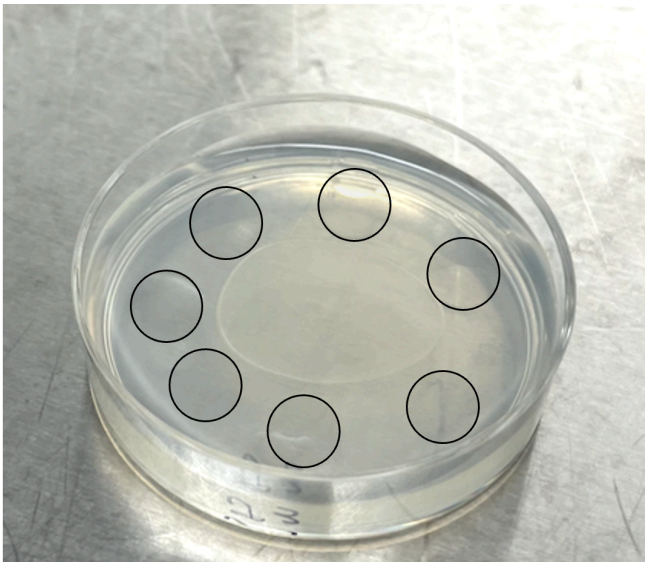
As a cost-saving alternative, the experimenter can use a grease-pen to draw circles on a standard glass slide.



3.4 Using a dissecting scope, focus on the slide such that the embryos are visible within a single well.

3.5 Using a clicker, quantify the number of embryos per well and note if there are any worms present. All of the worms should have dissolved in the bleaching step. Add the total number of embryos in the 3 wells and take the average. Multiple the average number of embryos per well by the dilution factor of 10 to get the number of embryos per 1 µL . Divide 500 by the number of embryos per microliter to determine the volume of the embryo solution needed for the next step.

3.6 Pipet approximately 500 embryos around the outer edge of a new NGM 60 mm plate seeded with OP50 *E. coli*. Prepare 1 plate for every 2 chemotaxis assays. 5m

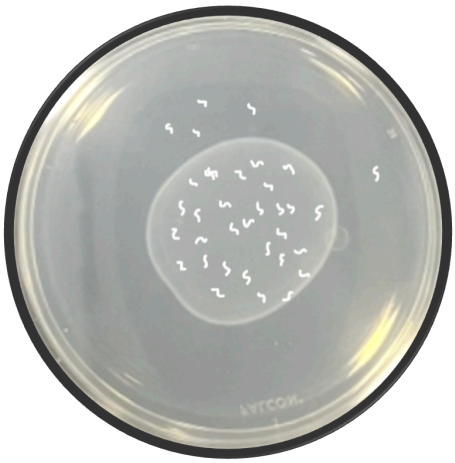


3.7 Young adult worms will be present after 48:00:00 of incubation at 20 °C . 2d

Note

Staging worms is important for the assay. Young adults are ideal. If one strain is maturing faster than another strain, place the faster growing plate at 18 °C .

Have plates come up to room temperature a few hours before the assay.



Building insect aspirators using household objects 11m

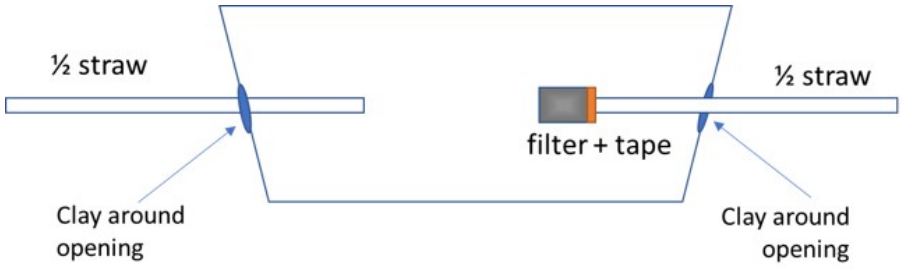
4 Cut a plastic straw in half and tape a small piece of filter around one end of one half of the straw. The filter should cover the open end. Keep the other half of the straw for later. 1m

5 Prepare the plastic cup. 5m

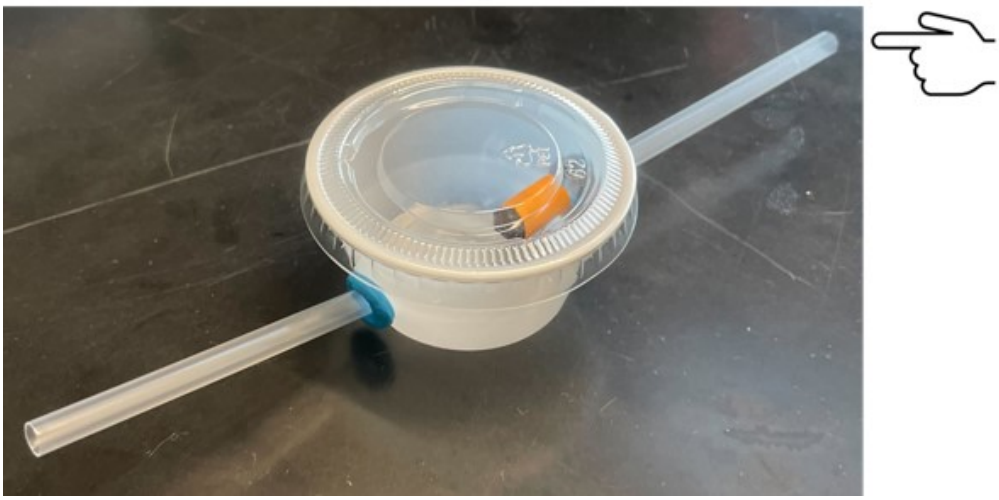
5.1 Make two holes with the dissection needle on either side of the deli cup. The holes should line up across from one another. Make the holes wide enough for a straw using a pen. 2m

5.2 Insert the two halves of the straw into each hole. The filter side of one of the straw halves should be inside of the cup with the open side outside of the cup. 1m

5.3 Line the holes on the outside of the cup with clay by rolling a small log of clay and shaping it into a circle around the hole. 2m



6 Place the lid on top and test the suction by placing your mouth on the open straw end with the filter and sucking in. 1m



Obtaining chemical extracts for use as chemotaxis stimuli

2d

7 This protocol is written for extracting compounds from ants, but other arthropods, plants, microbes, or other biological materials can be used.

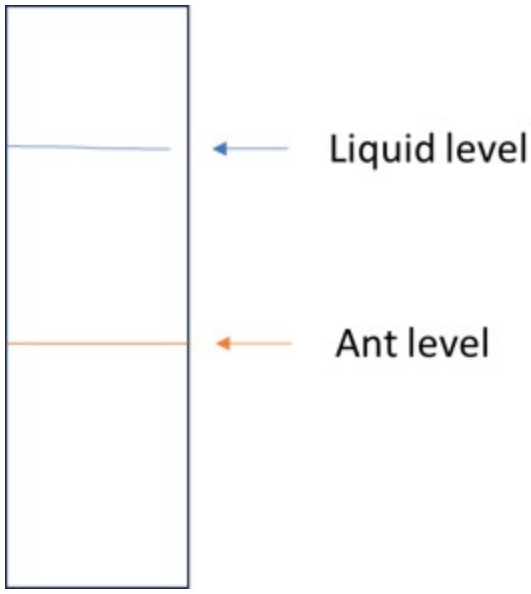
8 Place the collected ants in a freezer for 00:15:00 until immobile. 15m

9 Wearing gloves, sort ants by species into medium weighing boats. 15m

10 Prepare chemical extract. 1d

10.1 Label a 12 mL glass vial with ant species name and fill with 8 mL of methanol (>95%). 2m

10.2 Add ant species to their corresponding glass vial until the volume of ants is halfway up the liquid line within the glass vial. 10m



10.3 Once ants are added, place glass vials in the -80 °C freezer for 24:00:00 . 1d

10.4 Pipette the methanol to a clean glass vial, leaving the ants behind. 20m

Methanol was moved from the vial containing ants (left) to a new vial (right).

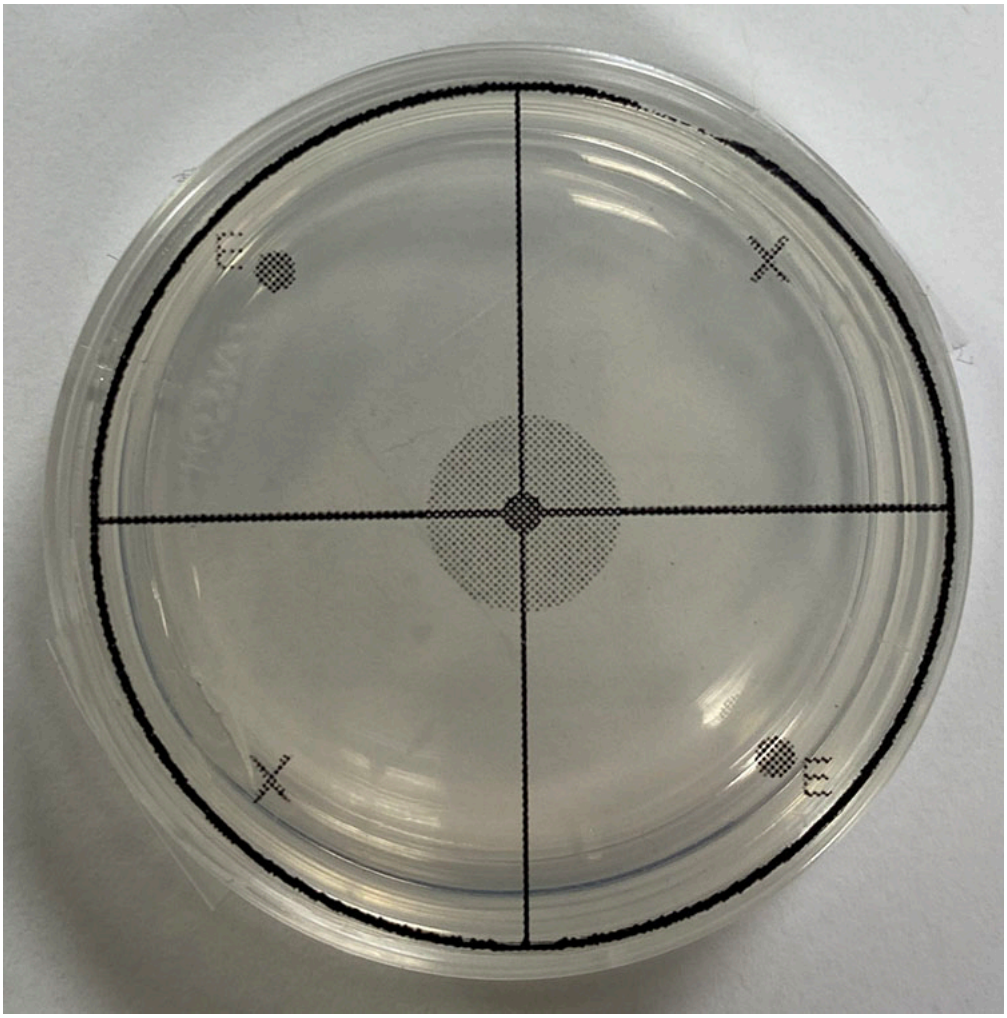
10.5 Evaporate the methanol-only samples under a constant flow of nitrogen gas using a nitrogen evaporator. 5h

10.6 Once dry, add 8 mL of dimethyl sulfoxide (DMSO) to the glass vials to resuspend your chemical extracts. 15m

Prepare chemotaxis plates 32m

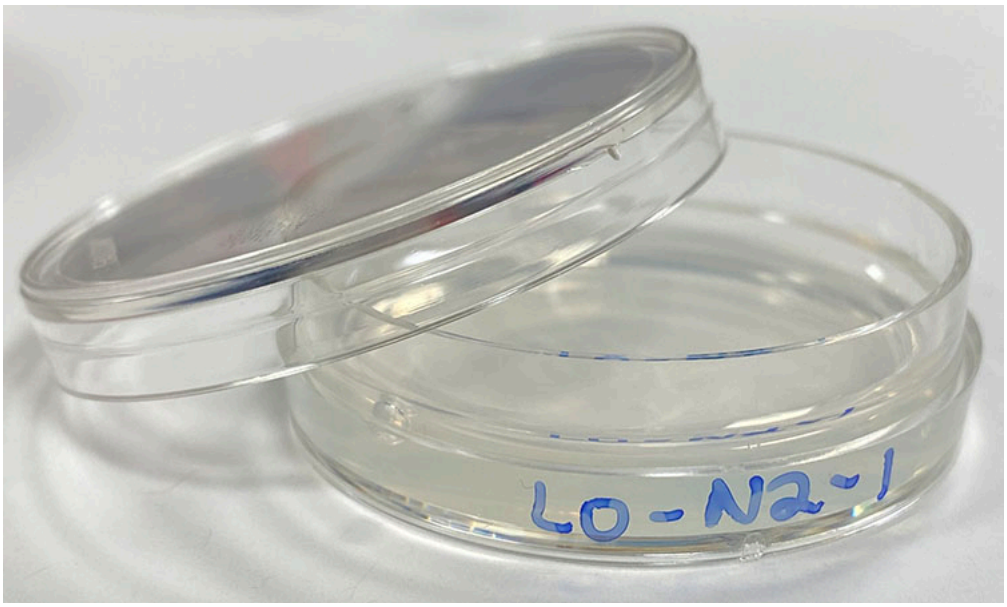
11 Label chemotaxis plates. 2m

11.1 Tape a transparent assay label to the bottom plate (the side with the agar). 1m



Chemotaxis assay template taped to the bottom of a 35 mm chemotaxis plate.

11.2 With a fine marker, label the side of the bottom plate with the compounds to be tested and the experimenter's initials. If possible, code the compounds such that the experimenter is unaware of treatment. 1m



Label the plate with experimenter initials, worm strain, and plate replicate number.

12 Take the lid off a chemotaxis plate and apply compounds by adding 5 µL of experimental compounds to each of the dots marked "E" and 5 µL of solvent to each of the "X" marks. Place the lid back on the plate. Repeat for other chemotaxis plates. 2m

13 Incubate chemotaxis plates for 00:30:00 to establish a chemical gradient. Chemotaxis plates should remain flat, upright, and with lids on. 30m

Prepare worms for chemotaxis 20m

14 Take a moment to look at the worms under a dissection scope. If applicable, note any phenotypic differences (behavior, movement, body shape, etc) between wild type and other strains such mutant lines or wild isolates. 5m

15 Label a 1.5 mL tube with the worm strain and experimenter's initials. Find the 0.1 mL marking on the tube and use a marker to make the mark more visible. This will let you know how much liquid to take out at the end. 1m

16 Harvest young adult worms from the NGM worm embryo plate prepared in Step 3.7. 5m

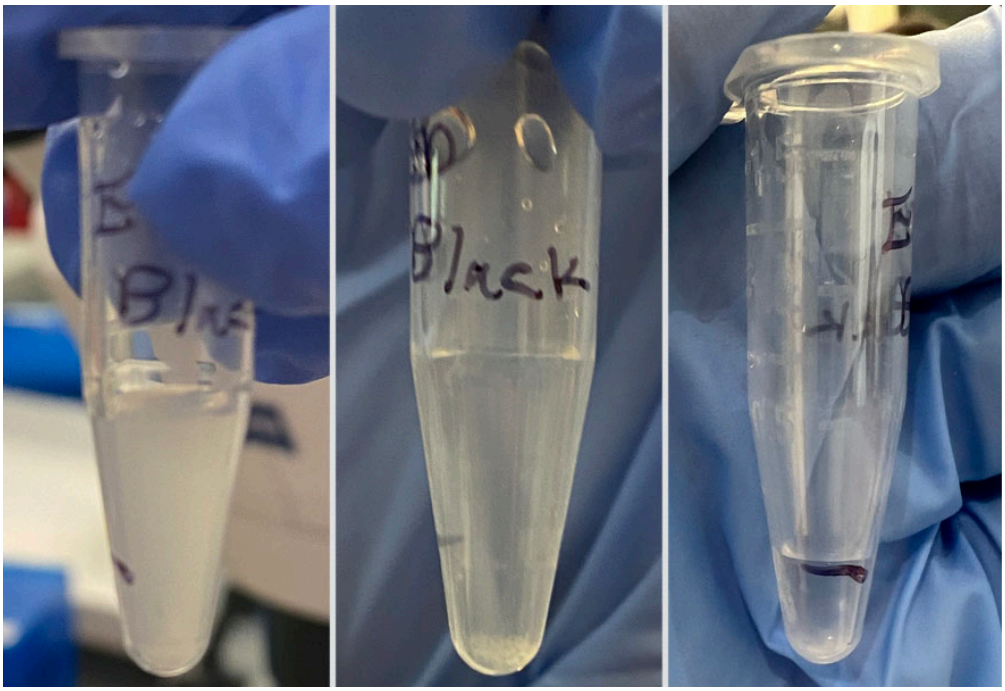
16.1 With a P1000 micropipette, add 1 mL of chemotaxis buffer to the worm plate. Gently swirl the plate. 1m

16.2 Tilt the plate to allow the buffer and the suspended worms to collect at the lower edge of the plate. 30s

16.3 Pipette the solution up and over the agar surface and allow the worms and liquid to collect in the bottom. Repeat to get as many worms off the plate as possible (recommended 3 times). 3m

16.4 Pipette all the buffer and worms off the plate and transfer to the 1.5 mL tube. 30s

17 Wash the worms with chemotaxis buffer. 8m



The liquid will be cloudy at first, due to bacteria from the plate, but will become clearer with successive washes.

17.1 Place the tube containing the worms suspended in buffer in a tube rack and incubate 00:02:00, allowing the worms to fall to the bottom of the tube. 2m

17.2 Carefully remove consecutive batches of 200 µL of the buffer from the tube. This is recommended so as to not to disturb the worm pellet. Discard the liquid in a liquid waster 30s

container.

17.3 Gently add 900 µL of chemotaxis buffer to the tube. *Pro tip:* do not resuspend worms with a pipette between washes, as worms stick to the plastic tip. 30s

17.4 Repeat this wash process twice for a total of three washes. 5m

18 After the final wash, leave roughly 100 µL of buffer on top of the worms by pipetting out the buffer.

Set up the chemotaxis assay 42m

19 Add 2 µL of 0.5 M sodium azide to the four compound (E & X) spots on each chemotaxis plate. Take care to not put sodium azide in the center dot. Change pipette tips each time and being careful not to poke the agar. 2m

Note

The purpose of adding sodium azide is to paralyze the worms after they have made a chemotaxis choice, so they can be easily counted later.

Safety information

Sodium azide is a hazardous compound. Perform this step in a ventilation hood and dispose of used pipette tips and tubes as hazardous waste.

20 Agitate the worms by flicking the tube. Use a micropipette to transfer worms to the center spot of each plate. The goal is to add roughly 100 worms to each plate. *Pro tip:* use the same pipette tip for adding the worms to each plate.
If four chemotaxis assays are being run, then transfer ~25-30 µl per plate. Scale up or down depending on the number of plates. 5m

20.1 *Optional:* If after 5 m, the worms are still swimming in a puddle in the middle, you may need to remove excess liquid. To do this, touch the edge of a filter paper or kimwipe to the worm drop center. This is best done looking through a dissecting scope. The goal is to remove excess liquid so that the worms can crawl away from the center. This must be 5m

done carefully because worms can stick to the Kimwipe. The goal is to have roughly 100 worms per plate.

21 Incubate chemotaxis plates face up and lid on for 00:30:00 at room temperature. 30m

Worm quantification and data visualization 10m

22 Using a dissecting scope, focus on a chemotaxis plate such that the worms are visible within a single quadrant. 30s

23 Using a clicker, quantify the number of worms on the E or X quadrants on each plate. It is recommend that both X or both E quadrants are counted first and then the other two can be counted. 2m

23.1 *Counting tips:* if the worm is on a line it is counted in the total number of worms, but not assigned to the S or E quadrants. Worms in the center circle or on the plastic rim of the plates are not counted at all. 30s

24 Calculate the Chemotaxis Index (CI) for each plate, where positive values suggest attraction and negative values suggest repulsion: CI = (Number of worms in the two experimental quadrants – Number of worms in the two solvent quadrants) / Total number of worms on the plate. 2m

25 Plot the chemotaxis index values for each plate, with the chemotaxis index on the y-axis and compound on the x-axis. 5m

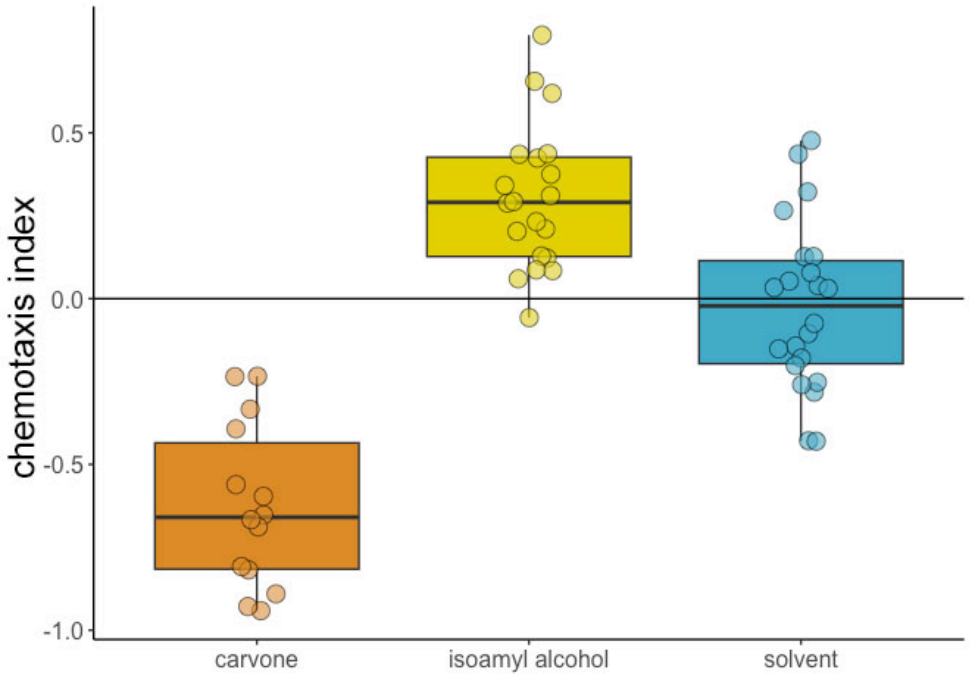
26 Boxplots are useful for highlighting variation between assays without a treatment group. An example dataset (see attached csv file) is shown, where undergraduate students learned how to perform this assay using a known repulsant (carvone), a known attractant (isoamyl alcohol), and solvent (control).

This boxplot was generated in R using the following code:

```
library(ggplot2)
library(wesanderson)

data ← read.csv(file="exampledata.csv", row.names=1)

ggplot(data, aes(y=CI, x=compound, fill=compound)) + geom_boxplot() +
  geom_jitter(position=position_jitterdodge(0.2), shape = 21, size = 4, alpha=0.6) +
  theme_classic() + theme(legend.position="top", axis.text=element_text(size=12)) +
  scale_fill_manual(values = wes_palette("FantasticFox1", n = 3)) +
  geom_hline(yintercept=0)
```



Students learn the chemotaxis assay using a known attractant (isoamyl alcohol) and repulsant (carvone) before running the assay on student-collected stimuli (extracts from ants).

Protocol references

Aspirator instructions were obtained from: **https://www.natgeokids.com/uk/home-is-good/make-a-super-pooter/**

Instructions for cultivating worms are available at Stiernagle T. 2006. **Maintenance of C. elegans.** WormBook: 1-11. PubMed ID: 18050451

This chart can be used to estimate worm growth times:
https://www.wormatlas.org/hermaphrodite/introduction/IMAGES/introtable2leg.htm

The original inspiration for this pipeline came from the NeuroPlant team at Stanford University. A preprint on their project is here: Fryer E, Guha S, Rogel-Hernandez LE, Logan-Garbisch T, Farah H, Rezaei E, Mollhoff IN, Nekimken AL, Xu A, Selin Seyahi L, Fechner S, Druckmann S, Clandinin TR, Rhee SY, Goodman MB. **An efficient behavioral screening platform classifies natural products and other chemical cues according to their chemosensory valence in *C. elegans.*** Preprint DOI: https://doi.org/10.1101/2023.06.02.542933

Publications from undergraduate courses using this protocol:
Lopez JS, Ali S, Asher M, Benjamin CA, Brennan RT, Burke MLT, Civantos JM, DeJesus EA, Geller A, Guo MY, Haase Cox SK, Johannsen JM, Kang JSJ, Konsker HB, Liu BC, Oakes KG, Park HI, Perez DR, Sajjadian AM, Torio Salem M, Sato J, Zeng AI, Juarez BH, Gonzalez M, Morales G, Braden N, Fiocca K, Pamplona Barbosa MM, O'Connell LA. 2024. **Pavement ant extract is a chemotaxis repellent for *C. elegans*.** 2024. micropublicationBiology. 10.17912/micropub.biology.001146

Alfonso SA, Arango Sumano D, Bhatt DA, Cullen AB, Hajian CM, Huang W, Jaeger EL, Li E, Maske AK, Offenberg EG, Ta V, Whiting WW, Adebogun GT, Bachmann AE, Callan AA, Khan U, Lewis AR, Pollock AC, Ramirez D, Bradon N, Fiocca K, Cote LE, Allee MD, McKinney JE, O'Connell LA. 2023. **Argentine ant extract induces an osm-9 dependent chemotaxis response in C. elegans**. micropublication Biology. 10.17912/micropub.biology.000745

Ellington CT, Hayden AJ, LaGrange ZB, Luccioni MD, Osman MAM, Ramlan LIE, Vogt MA, Guha S, Goodman MB, O'Connell LA. 2020. **The plant terpenoid carvone is a chemotaxis repellent for *C. elegans.*** microPublication Biology. doi: 10.17912/micropub.biology.000231