Chapter 21

Acute Behavioral Responses to Pheromones in *C. elegans* (Adult Behaviors: Attraction, Repulsion)

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Abstract

The pheromone drop test is a simple and robust behavioral assay to quantify acute avoidance of pheromones in *C. elegans*, and the suppression of avoidance by attractive pheromones. In the pheromone drop test, water-soluble *C. elegans* pheromones are individually applied to animals that are freely moving on a large plate. Upon encountering a repellent, each *C. elegans* animal may or may not try to escape by making a long reversal. The fraction of animals that make a long reversal response indicates the repulsiveness of a given pheromone to a specific genotype/strain of *C. elegans*. Performing the drop test in the presence of bacterial food enhances the avoidance response to pheromones. Attraction to pheromones can be assayed by the suppression of reversals to repulsive pheromones or by the suppression of the basal reversal rate to buffer.

Key words *C. elegans*, Pheromone, Ascaroside, Long reversal, Repulsion, Suppression of reversal, Attraction

1 Introduction

The pheromone drop test is adapted from a more general drop test for acute avoidance of water-soluble repellents [1, 2]. *C. elegans* moves forward through sinusoidal locomotion and occasionally changes direction by making a transient reversal or by making a sharper acute turn. A long reversal, often followed by a sharp turn of the entire body into an omega shape, is a common escape strategy upon encountering repellents. In the pheromone drop test, a population of animals is transferred to a test plate on which they crawl freely. A volume of a few nanoliters of pheromone dissolved in buffer is delivered to the side of each animal while it moves forward. As the drop touches the animal, it surrounds the entire animal by capillary action and is sensed by the amphids, the *C. elegans* sensory organs in the anterior. If the worm senses a repulsive stimulus, it makes a long reversal. The presence or the absence of the long reversal response in each animal is recorded, and the fraction

Kazushige Touhara (ed.), *Pheromone Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1068,
DOI 10.1007/978-1-62703-619-1_21,

of animals with a long reversal response among the total number of animals tested is calculated.

The known *C. elegans* pheromones belong to a family of small molecules with an ascarylose sugar backbone, and are called ascarosides. Some of the identified ascarosides including ascr #3 (C9) are repulsive to wild-type hermaphrodites in the drop test [3]. For a negative control and basal level correction, buffer alone is also tested. For positive controls, a high-osmolarity glycerol solution can be used as a repellent, and isoamyl alcohol, an attractive odor, can be used as an attractant.

2 Materials

Prepare all solutions using ultrapure water that is distilled, filter purified with 0.2 μm membrane, and autoclaved.

1. M13 buffer: 30 mM Tris–HCl, pH 7.0, 100 mM NaCl, 10 mM KCl. To make a 10× M13 stock, weigh 3.634 g Tris–HCl, 5.844 g NaCl, and 745.5 mg KCl, and dissolve in water. Adjust pH to 7.0, and make up to 100 mL with water. Filter through a 0.2 μm membrane. Store at room temperature. Dilute 1:10 in water with pheromone immediately before use.
2. Pheromone ascaroside stock: *C. elegans* pheromones are not commercially available as of this writing, but methods for purification and synthesis of individual ascarosides have been developed (*see* chapter by Rebecca Butcher). Dissolve each synthesized ascaroside in ethanol to the final concentration of 10 mM. Tightly seal with Parafilm in small aliquots in glass vials and store at 4 °C.
3. Glycerol solution: Prepare 1 M glycerol. Weigh 9.209 g glycerol. Dissolve in water, and make up to 100 mL with water. Store at room temperature.
4. Nematode growth media (NGM) plates: Prepare agar, peptone, 5 mg/mL cholesterol in ethanol, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M KH_2PO_4, and 1 M K_2HPO_4. To make 5 mg/mL cholesterol, add 2.5 g of cholesterol to 500 mL of ethanol, followed by overnight incubation at 37 °C. Prepare 1 M KPO_4 buffer (pH 6.0) by mixing 13.2 mL of 1 M KH_2PO_4 with 86.8 mL of 1 M K_2HPO_4 in a separate sterile beaker, and use fresh within 1–2 days. To make 1 L of NGM, add 1 mL of 5 mg/mL cholesterol in ethanol, 3 g NaCl, 22 g agar, and 2.5 g peptone to a 2 L Erlenmeyer flask. Add 975 mL water and stir well. Cover flask with aluminum foil, and autoclave for 50 min. Cool flask to 52 °C with stirring. Add 1 mL of 1 M $CaCl_2$ and 1 mL of 1 M $MgSO_4$ and mix well. Add 25 mL of 1 M KPO_4 (pH 6.0) to the flask. Using sterile procedures and a peristaltic pump, dispense 24 mL of the NGM solution into

a 90 mm diameter Petri plate, and 10 mL into a 55 mm diameter Petri plate [4]. Make NGM plates in large quantities and store in the cold room at 4 °C.

5. OP50 culture: From a single colony, grow a culture of the *E. coli* strain OP50 in 100 mL LB media at room temperature for 3–4 days. Measure OD 600 and use at a density range of OD 600 = 0.4–0.7. Store the bottle at 4 °C for up to 2 weeks.
6. Platinum wire pick: Prepare platinum wire 0.255 mm in diameter (Tritech Research) and Borosilicate Glass Pasteur Pipette 5.75 in. in length (Fisherbrand) or equivalent. Cut 2–3 cm of platinum wire and insert one end into the narrow end of a glass Pasteur pipette, and flame the glass to melt it around the platinum wire. Flatten a few mm at the free end by pressing the wire between pliers, leaving a free flat wire tip.
7. Glass capillary: 10 μL Glass microcapillary pipettes (Kimble) or equivalent.
8. Filtered plastic tips: 1–200 μL Graduated filter tips (USA Scientific, INC) or equivalent.
9. Unfiltered plastic tips: 1–200 μL Natural beveled tips (USA Scientific, INC) or equivalent.
10. Tubing: Rubber tubing about 40–60 cm in length and 1.5 mm in inner diameter.

3 Methods

3.1 Pheromone Drop Test (Off Food)

1. Four days prior to the assay, pick three animals in the L4 larval stage onto a new NGM plate (40 mm diameter) that was previously seeded with 100–200 μL *E. coli* OP50 culture. Seed plates 1–3 days before adding worms. The progeny of these animals will be used for the drop test.
2. Grow the animals for 4 days at 20 °C (*see* **Note 1**).
3. On the day of the assay, take out unseeded NGM plates (90 mm diameter) from 4 °C. Dry at room temperature for 1–2 h with the lid open to remove excessive moisture. Assays will be performed on these plates (*see* **Note 2**).
4. Remove the animal growth plates from the 20 °C incubator. The majority of the animals should have reached the young adult stage.
5. Transfer 30 young adult animals to a new unseeded NGM plate (55 mm diameter) at room temperature with the platinum wire, transferring as little food as possible (*see* **Note 3**).
6. Let the animals crawl away from any food that was transferred with them.

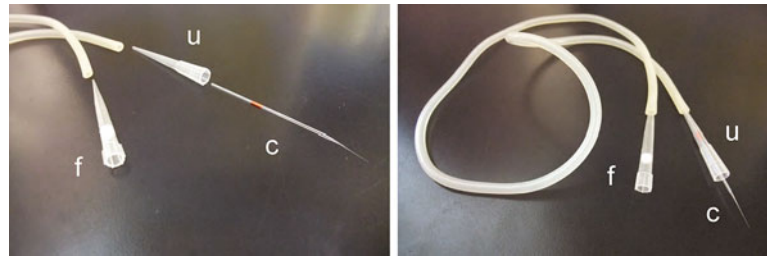

Fig. 1 The pheromone delivery system. The filtered tip (f) connects the *upper end* of the tubing to the mouth and prevents saliva from going into the tubing. The unfiltered tip (u) connects the *lower end* of the tubing to the drawn-out glass microcapillary that contains pheromones diluted in M13 buffer (c)

7. Transfer animals to a second unseeded NGM assay plate with the platinum wire, using no food. Animals should be food-free (*see* **Note 3**).
8. Wait for 30–60 min to precondition the worms.
9. During preconditioning, make a serial dilution of ascaroside in M13 buffer in Eppendorf tubes (*see* **Note 4**).
10. Pull the glass capillary by hand on a flame and break it to reduce the diameter of the tip.
11. Set up the pheromone delivery system using a pulled glass capillary, a rubber tube, an unfiltered tip, and a filtered tip (*see* Fig. 1). Push the broad end of the capillary into the unfiltered tip firmly to seal the joint.
12. Place the narrow end of the capillary into the ascaroside solution. The ascaroside solution will enter the glass capillary by capillary action.
13. Apply a droplet of ascaroside by mouth pressure through the filtered tip to a forward moving worm. Score the positive and negative responses. A long reversal is defined as a backward movement that equals or is larger than the half-length of the worm, initiated within 4 s after the stimulus touches the worm. Most long reversals begin within a second (*see* Fig. 2).
14. Repeat **step 13** for additional worms. Moving from one side of the plate to the other side and testing only 20 worms per plate help prevent testing the same animal twice.
15. The fraction reversing is (number of animals that make a long reversal)/(number of total animals tested). A fraction reversing of 1 represents complete avoidance and 0 represents no response (*see* **Note 5**).
16. The same assay is performed with a drop of M13 buffer alone on a similar number of animals. The fraction reversing is obtained as above. The fraction reversing of wild-type N2 hermaphrodite animals to buffer is typically about 0.1–0.2.

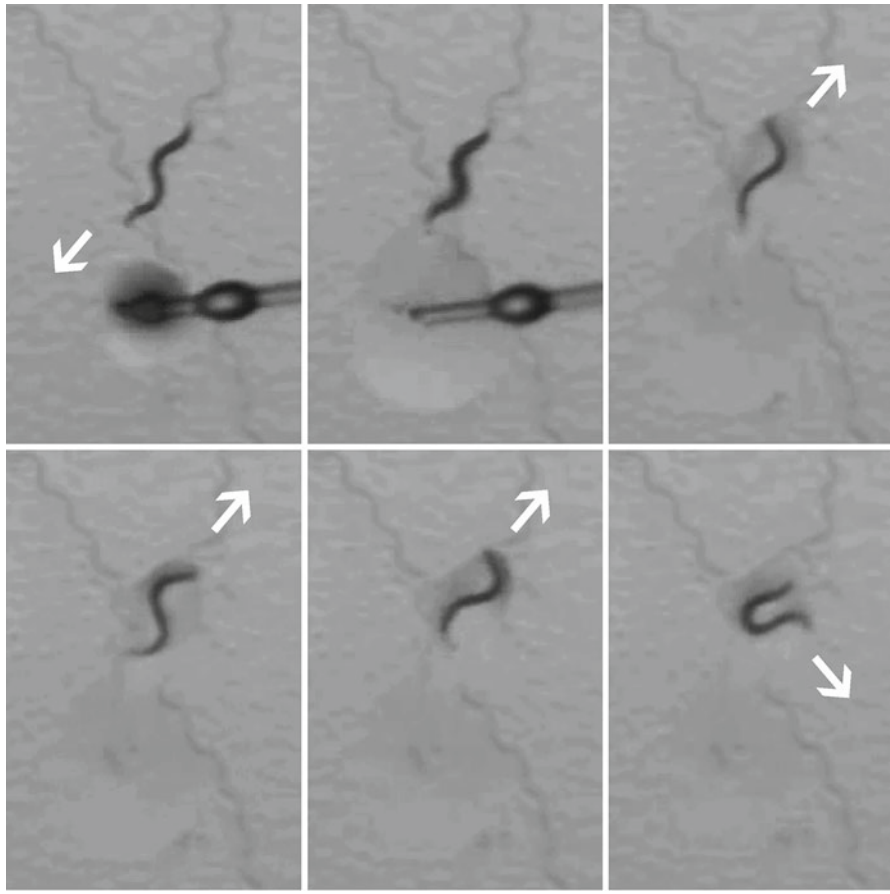

Fig. 2 The long reversal response of *C. elegans* in the pheromone drop test. Time runs from *left* to *right* in the *top row* and then the *bottom row*; total time elapsed is about 5 s. *Top left panel*: 10 nM of ascr #3 (C9) is applied to the side of a wild-type N2 hermaphrodite worm moving forward in the direction of the *arrow* (toward *lower left*). As the stimulus touches the worm (*second panel*), the animal initiates a long reversal toward the *upper right* (*arrows*, *third to fifth panels*). *Bottom right panel*: Animal ends the long reversal with a sharp turn, and changes direction of movement toward the *lower right* (*arrow*)

17. The avoidance index is the effect size of (fraction reversing to pheromone) – (fraction reversing to buffer alone). An avoidance index of 1 represents complete avoidance, but is rarely observed because of background response to buffer. Typical repulsion value ranges from 0.3 to 0.7. A neutral response is zero. An attraction response is represented as a negative number, but cannot easily be tested in the absence of food due to the low basal reversal rate to buffer.
18. As a positive control, you may perform the same assay with high-osmolarity glycerol solution in the capillary (1 M). The fraction reversing of wild-type N2 hermaphrodite animals is about 0.9. Therefore, the avoidance index of glycerol (with subtraction of the buffer-alone response) in wild-type N2 hermaphrodites is about 0.7–0.8.

3.2 Pheromone Drop Test (on Food)

Acute pheromone avoidance is enhanced 10–100-fold by the presence of bacterial food. For example, wild-type N2 hermaphrodite worms avoid ascr #3 (C9) at 100 nM or higher concentrations of food, and 1 nM or higher concentration on food. The basal avoidance response to buffer alone is also generally increased.

1. Four days prior to the assay, pick three animals in the L4 larval stage onto a new NGM plate (40 mm diameter) that was previously seeded with 100–200 μL *E. coli* OP50 culture. Seed plates 1–3 days before adding worms. The progeny of these animals will be used for the drop test.
2. Grow the animals for 4 days at 20 °C (*see* **Note 1**).
3. The day before the assay, take out unseeded NGM plates from 4 °C. Dry at room temperature for 1–2 h with the lid open to remove excessive moisture. Assays will be performed on these plates.
4. Pour about 6 mL OP50 culture in LB media onto each dried NGM plate, swirl, and remove excessive media. This will allow you to obtain a homogenous bacterial lawn.
5. Incubate the seeded assay plates with the lid on at 37 °C for 14–18 h.
6. On the day of the assay, remove the assay plates from the 37 °C incubator and incubate at room temperature for 1–2 h to equilibrate. Remove moisture from the lid of the plate.
7. Remove the animal growth plates from the 20 °C incubator. The majority of the animals should have reached the young adult stage.
8. Transfer 30 young adult animals to the assay plate with the platinum wire, using minimal food.
9. Wait for 30–60 min to precondition the worms.
10. During preconditioning, make a serial dilution of ascaroside in M13 buffer in Eppendorf tubes (*see* **Note 4**).
11. Pull the glass capillary by hand on a flame and break it to reduce the diameter of the tip.
12. Set up the pheromone delivery system using a pulled glass capillary, a rubber tube, an unfiltered tip, and a filtered tip (*see* Fig. 1). Push the broad end of the capillary into the unfiltered tip firmly to seal the joint.
13. Place the narrow end of the capillary into the ascaroside solution. The ascaroside solution will enter the glass capillary by capillary action.
14. Apply a droplet of ascaroside by mouth pressure through the filtered tip to a forward moving worm. Score the positive and negative responses. A long reversal is defined as a backward movement that equals or is larger than the half-length of the

worm, initiated within 4 s after the stimulus touches the worm. Most long reversals begin within a second (*see* Fig. 2).

15. Repeat **step 14** for additional worms. Moving from one side of the plate to the other side and testing only 20 worms help prevent testing the same animal twice.
16. The fraction reversing is (number of animals that make a long reversal)/(number of total animals tested). A fraction reversing of 1 represents complete avoidance and 0 represents no response (*see* **Note 5**).
17. The same assay is performed with a drop of M13 buffer alone on a similar number of animals. The fraction reversing is obtained. The fraction reversing to buffer of wild-type N2 hermaphrodite animals on food is typically about 0.3.
18. The avoidance index is the effect size of (fraction reversing to pheromone) − (fraction reversing to buffer only). An avoidance index of 1 represents complete avoidance, but is rarely observed because of background response to buffer. Typical repulsion value ranges from 0.2 to 0.6. A neutral response is zero. An attraction response is represented as a negative number (*see* Subheading 3.3).
19. As a positive control, you may perform the same assay with high-osmolarity glycerol solution (1 M). The fraction reversing of wild-type N2 hermaphrodite animals is about 0.7. Therefore, the avoidance index of glycerol (with subtraction of buffer-alone response) in wild-type N2 hermaphrodite animals is about 0.4.

3.3 Pheromone Drop Test on Food for Measuring Attraction

According to the biased random walk model for *C. elegans* chemotaxis, the suppression of reversals may indicate attraction [5, 6]. Attraction to pheromones can be measured as a reduction in spontaneous reversal responses in the drop test on food, taking advantage of the increased basal reversal rate to buffer in the presence of food. When given attractive ascaroside(s) in a drop test, the worm will make fewer reversals compared to the buffer alone. This will result in a negative value of the avoidance index. For example, for wild-type male animals in the presence of food, the fraction reversing to 100 nM ascr #3 (C9) in buffer is 0.3, and the fraction reversing to buffer alone is 0.5. The resulting avoidance index is about −0.2, indicating attraction of males to 100 nM ascr #3 (C9) [3]. The maximal attraction response is defined by the response to buffer alone or another control; typical attraction values on food range from −0.1 to −0.4.

As a positive control for reversal suppression, the attractant isoamyl alcohol can be used. A 1:10,000 dilution of isoamyl alcohol suppresses the basal reversal frequency to M13 buffer on food.

Alternative assays for pheromone attraction or repulsion have been developed based on the accumulation of animals on agar impregnated with pheromones, compared to accumulation on agar alone [7, 8].

4 Notes

1. Pheromone drop test results can be sensitive to the growth conditions and the density of the worms. When strains with smaller brood size or slower growth rate are tested along with other strains, try to achieve similar population density on the day of the assay by picking more parental animals or picking a few days earlier.
2. Dryness of the plate is best if the moving worms can leave a visible track on the agar that will disappear in few minutes.
3. Using a platinum wire pick with an extensively flattened, wide end helps to scoop up worms without food. Briefly flame-sterilize the platinum wire between animals.
4. Prepare a fresh pheromone solution each day of the assay.
5. Animals can also exhibit other behavioral responses in the drop test, including short reversals, acceleration, or dwelling (short back-and-forth movements). These responses can be scored additionally and analyzed as needed.

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