Video Article

A *Caenorhabditis elegans* Nutritional-status Based Copper Aversion Assay

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Abstract

To ensure survival, organisms must be capable of avoiding unfavorable habitats while ensuring a consistent food source. *Caenorhabditis elegans* alter their locomotory patterns upon detection of diverse environmental stimuli and can modulate their suite of behavioral responses in response to starvation conditions. Nematodes typically exhibit a decreased aversive response when removed from a food source for over 30 min. Observation of behavioral changes in response to a changing nutritional status can provide insight into the mechanisms that regulate the transition from a well-fed to starved state.

We have developed an assay that measures a nematode's ability to cross an aversive barrier (*i.e.* copper) then reach a food source over a prolonged period of time. This protocol builds upon previous work by integrating multiple variables in a manner that allows for continued data collection as the organisms shift towards an increasingly starved condition. Moreover, this assay permits an increased sample size so that larger populations of nematodes can be simultaneously evaluated.

Organisms defective for the ability to detect or respond to copper immediately cross the chemical barrier, while wild type nematodes are initially repelled. As wild type worms are increasingly starved, they begin to cross the barrier and reach the food source. We designed this assay to evaluate a mutant that is incapable of responding to diverse environmental cues, including food sensation or detection of aversive chemicals. When evaluated via this protocol, the defective organisms immediately crossed the barrier, but were also incapable of detecting a food source. Hence, these mutants repeatedly cross the chemical barrier despite temporarily reaching a food source. This assay can straightforwardly test populations of worms to evaluate potential pathway defects related to aversion and starvation.

Video Link

The video component of this article can be found at https://www.jove.com/video/55939/

Introduction

Caenorhabditis elegans has been used as a model for the study of neurobiology for decades due to the relative ease in analyzing the circuitry of a nervous system composed of only 302 neurons[1]. Provided that the organism is reliant on responding to environmental cues, much of the nervous system is dedicated to regulating the integration of environmental signals[2]. Despite the simplicity of its nervous system, *C. elegans* can detect and respond to diverse environmental signals including repellents[3], attractants[4], temperature[5], and even humidity[6]. A failure to properly integrate environmental signals has been linked to a number of behavioral disorders and neurodegenerative conditions in mammalian model systems[7,8,9]. With a range of available neural disease models[10] in *C. elegans* and the development of nematode pharmaceutical screens[11], this organism has proven to be a useful system for the study of neurobiology. Given the availability of a mapped nematode connectome[1] and mutations to almost every gene in the nematode genome[12], our understanding of the nematode nervous system, and by extension our own, is partially limited by the design of creatively appropriate assays.

A number of chemotaxis assays have been developed over the past 40 years to evaluate nematode responsiveness to diverse aversive stimuli[3,4,13,14,15]. Initial experimentation involved the introduction of an acute environmental stimulus while a single worm roamed on an agar plate[3,14,16]. Immediate changes to locomotory responses were recorded. For example, the volatile odorant octanol can be applied to a hair and wafted in front of a nematode's nose to stimulate the initiation of backwards locomotion in wild type worms[17]. More complex assays have also been developed to incorporate multiple variables as a means of assessing behavioral choice[18]. A variation of this assay entails the use of a copper solution to create an aversive midline barrier[4]. An attractant, namely diacetyl, was placed on one side of the chemical barrier with worms transferred away from the diacetyl source. Worms defective for copper aversive responses immediately crossed the barrier to reach the diacetyl, while wild type worms were initially repulsed by the barrier. Responses were scored when worms first approached the copper barrier without long term observations.

When worms are evaluated after undergoing starvation conditions, their sensitivity to environmental stimuli is decreased[19]. When the aversive chemical octanol is wafted in front of the nematode nose, wild type organisms stimulate backwards movement within 3 - 5 s when on food. After these organisms have been removed from food for 10 min, they exhibit a delayed response of 8 - 10 s[20]. Thus, with increased starvation,

nematodes display a decreased aversive response to harmful environmental signals as the search for food becomes more essential to survival. Conversely, nematodes that over-express neuropeptide receptor 9 (*npr-9)*, do not respond to octanol on or off food and exhibit an inability to respond to a number of aversive stimuli[21]. These *npr-9(GF)* organisms also do not modulate their reversal frequency in the presence of food, but can reverse in response to harsh touch stimulations indicating that they are capable of backwards locomotion[21]. We have also evaluated *npr-9(LF)* mutants given that they exhibit an abnormally decreased reversal frequency off food yet can modulate their behavior in the presence of food[21]. Coupling the nutritional state of the worm with the introduction of acute external stimuli has aided in elucidating the mechanisms by which a food-related pathway can broadly modulate sensory signaling pathways[22,23]. The presence of food in the nematode environment has also been used to evaluate ethanol withdrawal responses[24]. In this experiment, worms were incubated in varying concentrations of ethanol and then were placed on an agar plate with a patch of food known as a "food-race assay". The food patch was placed on one edge of the plate while the nematodes were placed away from the food source. Ethanol withdrawal was evaluated by measuring the duration of time required for worms to reach the patch of food.

This nutritional-based copper aversion assay builds upon the food-race assay to integrate additional environmental variables, namely food and copper, while assessing behavioral changes over time. This is an adaptation of a commonly use protocol throughout the *C. elegans* community[4]. This protocol has been used to evaluate aversive responses and the detection of food over a four-hour period[21]. Since worm's exhibit starvation behaviors after 30 min of food deprivation[25], we are also able to evaluate how changes to nutritional status can influence environmental responses. The conditions of this assay measure how experimental organisms change responsiveness to aversive stimuli over time, hence this evaluates behavioral changes as organisms progress towards a starved state (and continued measurements of prolonged starvation). Since the *npr-9(GF)* animals do not alter their behavior in response to food or many aversive cues, we sought to identify if these behavioral deficits would persist in the context of starvation. Ultimately, this assay design has been formulated to specifically evaluate the *npr-9(GF)* mutants but can be further adapted to also characterize novel strains.

Protocol

1. Preparation of Experimental Organisms

1. **Pick 10 L4 staged nematodes per strain 24 h prior to commencing the assay to ensure that organisms are young adults when tested. For each mutant or control nematode tested, pick 10 L4s (10 for the control and 10 for the assay).**
    1. Maintain L4 organisms using standard methods[26,27] for 24 h on standard agar plates seeded with OP50 *Escherichia coli*. If organisms are lost during the subsequent wash steps, compensate by increasing the starting sample size (*i.e.* pick 20 worms rather than 10). Note: Behavior is an innately variable phenotype. Perform the method in triplicate for each strain on three separate days. Include additional control strains and conditions for novel strains, as highlighted in the discussion section.

2. **Immediately prior to assay, transfer experimental organisms to an agar plate with no bacteria and allow nematodes to move freely for 1 min to remove excess bacteria.**
    1. If experimental organisms experienced contamination conditions during the 24 h prior to the assay, discard them.

3. Pipette 1 mL of M9 onto the bacteria-free plate to wash worms into a microcentrifuge tube.
4. Centrifuge at 3,000 x g for 1 min. Worms should form a pellet at the bottom of the tube. Aspirate M9 solution without disrupting the worm pellet. Add 1 mL of M9 to the worm pellet, invert tube to mix worms with the solution.
5. **Repeat steps 1.4 three more times.**
    1. If excess bacteria was initially transferred with the worms, repeat for a total of 5 times. No bacteria should be transferred to the copper food race plates. If food is transferred to the assay plate, it will interfere with accurate data collection.

6. After the final wash, aspirate supernatant until 100 µL of M9 and the worm pellet remains.
Caution: Starvation related behaviors become evident after 30 min. Consequently worms should be immediately transferred from solution once the wash steps have been completed.

2. Preparation of Assay Plates

1. **Prepare standard NGM agar plates two days prior to the assay.**
    1. If plates are kept in a humid environment, make agar plates 3 days prior to assay. Alternatively, remove the lid of the plate for 3 - 6 h to ensure proper dryness (if in a sterile environment).

2. With a thick permanent marker, make a line on the underside of the plate along the outer edge and another to form a mid-line barrier (**Figure 1**). The mid-line barrier should be equidistant from each edge of the plate. Use a ruler to ensure precise measurements. These lines will serve as a guide when transferring bacteria and the copper solution. Provided that *E. coli* is transferred before the copper solution, these lines will serve as indicators.
3. **Seed plate with 50 µL of OP50 *E. coli* on only one side of the copper barrier to create a uniform lawn (Figure 2). Bacterial concentration should remain consistent across assays; however, little variability has been observed in response to mild differences in concentration.**
    1. Use the marked lines on the underside of the plate to ensure the bacteria do not come into contact with the copper solution. Provided that the copper solution lines the edge of the plate and forms a mid-line barrier, transfer the bacteria so that the copper solution will not come into contact with the food source.

4. Mark a second set of plates and transfer no OP50 *E. coli* to them (**Figure 2**). These plates will serve to evaluate the negative control. These plates should also have a marking on one half of the plate to denote the initial transfer origin.

5. Allow bacteria to dry then incubate plates at 37 °C overnight. Ensure that bacterial patches are not disturbed when transferring to a 37 °C incubator or room. Excessive disturbance could alter the location or shape of the food patch.

3. Chemotaxis Assay

1. Freshly prepare a 0.5 M copper (II) sulfate solution prior to assay start time. Provided that 125 µL of the solution is used per plate, scale up this volume dependent on the number of assay plates used (*e.g.* 5 assay plates, 625 µL).
2. Pipette 100 µL of the copper (II) sulfate solution on the edge of the agar to create an outer copper barrier. The marked underside of the plate should serve as a guide.
3. **Pipette 25 µL of the copper (II) sulfate solution to create a midline barrier.**
    1. Ensure that the copper (II) sulfate solution does not come into contact with the bacterial patch. Use a spotted technique as streaking may affect locomotion due to indentations/scratches on the agar.
4. Allow the copper solution to dry onto plate. Time period can vary depending on plate and lab conditions. Visually check for dryness every 5 min after transfer.
   Note: The copper solution displays a blueish tint and is easily identifiable. Use a laboratory tissue to lightly dab the solution near the edge of the plate to discern **dryness.**
5. **Pipette 20 µL of the worm pellet from the bottom of the tube onto the bacteria-free half of the assay plate.**
    1. Ensure that 10 worms are transferred to the assay plate. If extra worms were accidentally included, remove them by picking with halocarbon oil to ensure that no bacteria are added to the plate. Each assay should have a consistent number of nematodes during the assay.
       NOTE: If too few worms are transferred during assays, increasing the initial sample size will mitigate potential losses during washes and transfers.
6. Remove excess M9 from the plate with a laboratory tissue. M9 should not come into contact with the copper (II) sulfate solution.
   Caution: Ensure that worms and the agar surface remain unaffected while performing this step. If used too harshly, the laboratory tissue can create indentations to the agar surface of the plate and can remove worms. Worms accidentally removed *via* KimWipe should be discarded.
7. **Once the M9 solution has been removed and all worms have commenced non-liquid locomotory patterns, start the assay stopwatch.**
    1. Optimally, remove the M9 solution within a min. The essential parameter is the identification of sinusoidal locomotion. Worms locomote differently, *e.g.* thrashing rather than sinusoidal, when in liquid. Start the assay stop watch once each of the experimental organisms stops thrashing.
8. **Check assay plates every 30 min.**
    1. For the assay plates with bacterial patches, positively score organisms if they reach the food patch over a 4 h period. For the negative control plates, positively score organisms if they have crossed the barrier.

Representative Results

We utilized wild type (N2), *npr-9(tm1652),* and an *npr-9* overexpression strain, *i.e. npr-9(GF)* (IC836 *-npr-9::npr-9;sur-5::gfp;odr-1::rfp),* to evaluate responses to starvation and copper aversion. Wild type organisms are capable of detecting and responding to the aversive copper barrier, while *npr-9(GF)* mutants do not initiate an aversive response to the copper over the 4 h assay[21]. After 30 min of starvation, roughly 50 - 60% of wild type (N2) organisms cross the copper barrier and reach the food patch. At the 2 h mark, 75% of wild type nematodes reach the food source. By the end of the assay, 100% of N2 organisms have relocated to the food source. In contrast, the majority of the *npr-9(GF)* organisms do not modulate locomotory patterns in response to food and will continue to cross the aversive barrier even after coming into contact with a food source. The *npr-9(GF)* worms continuously fail to alter their locomotion in response to food over the 4-h assay and only 30% of the test organisms are found on the food at any given time. The *npr-9(LF)* animals do not perform as well as N2 organisms, but do modulate their locomotory patterns as starvation increases to reach the food patch (**Figure 3**). When N2 or *npr-9(LF)* organisms are evaluated via this assay without food, they rarely cross the copper barrier. The *npr-9(GF)* mutants repeatedly cross the barrier back and forth (**Figure 4**).

Figure 1: A Visual Representation of Markings to Indicate Copper Solution Placement on the Underside of a 5 cm Petri Dish. These indications are used to ensure that the sections of the plate have been appropriately measured and serve as a guideline when transferring the bacterial patch and then copper solution onto the agar surface. Please click here to view a larger version of this figure.

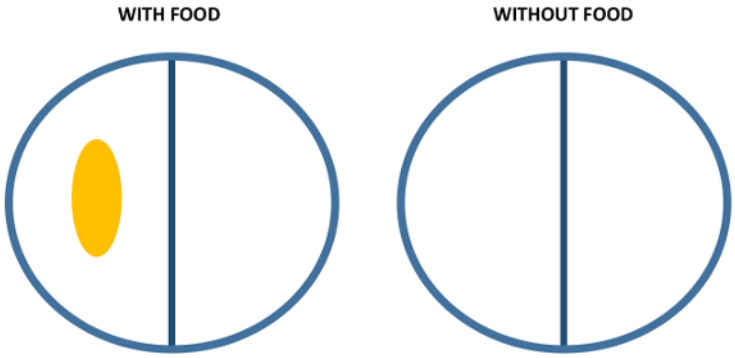

Figure 2: The 5 cm Petri Dish is Divided into Two Sections for on and off Food Assay Plates and a Bacterial Lawn is Formed in the Center of One of These Sections for the On-food Plate. The food patch should not come into contact with the test compound which lines the edges of the plate and forms a midline barrier. Please click here to view a larger version of this figure.

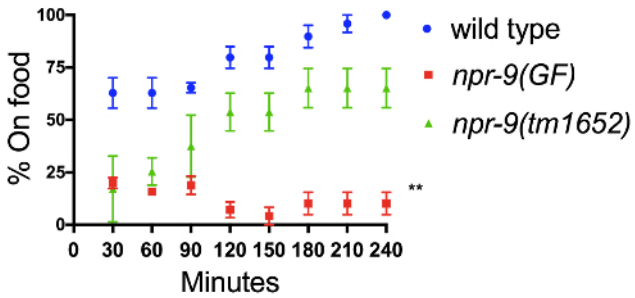

Figure 3: Percentage of N2, *npr-9(tm1652),* and *npr-9(GF)* Worms that Reach the Food Source Over a Four-hour Period in Response to the Nutritional-status Based Copper Aversion Assay. Data points are averages of at least three experiments (n > 30 worms) performed on separate days for both strains. Data are presented as mean ± standard error and analyzed with a two-way repeated measures ANOVA. ** p <0.01, significantly different from N2 animals under identical conditions. Please click here to view a larger version of this figure.

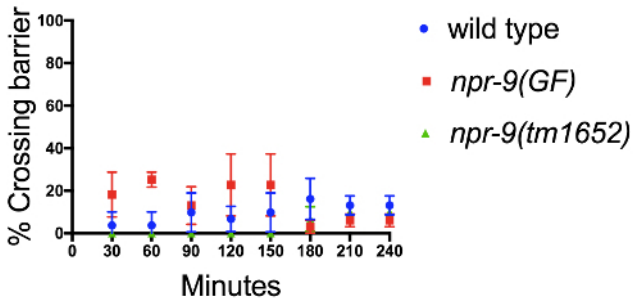

Figure 4: Percentage of N2, *npr-9(tm1652)* and *npr-9(GF)* Worms that Cross the Copper Barrier with no Food Source Over a Four-hour Assay. The aforementioned strains were evaluated for copper aversion in the absence of food. Positive responses are scored as crossing the copper barrier and remaining on the non-origin half of the plate. Data points are averages of at least three experiments (n > 30 worms) performed on separate days for both strains. Data are presented as mean ± SE and at least 10 animals were assayed in three independent experiments analyzed with a two-way repeated measures ANOVA. Please click here to view a larger version of this figure.

Discussion

This assay design modifies the food race assay[24] to include a copper solution to create an aversive midline barrier and around the edge of the plate to prevent a loss of nematodes. Organisms are tested for their ability to cross the aversive barrier and reach a food patch over a 4 h period. In the context of *npr-9(GF)*, we have utilized this assay to evaluate how starvation conditions could affect aversive responses and the detection of food. Provided that we had previously characterized *npr-9(GF)* as defective for responsiveness to food and aversive cues, we combined multiples environmental cues with nutritional status to evaluate if starvation could modulate the defective *npr-9(GF)* behaviors. This assay relies on a worm's ability to detect and respond to chemical stimuli and food cues[3,4]. Given the diverse variables analyzed, the conditions of the experimental organisms must be meticulously controlled to eliminate confounding factors. For uncharacterized mutants, *i.e.* those with unknown aversive or food responsiveness, additional control strains would need to be utilized. Aversive defective strains, *e.g. che-2* or *odr-3*[28], and mutants that do not alter locomotory patterns off food, *e.g. tph-1*[29], should also be evaluated in parallel to ensure that the copper and starvation conditions are appropriately controlled. Moreover, to more directly highlight the contribution of starvation to the evaluated behavior, separate assay conditions can also be developed for additional controls. For example, the nematodes can be starved immediately prior to the assay and transferred to the assay plate (with food) to identify how quickly an organism in a starved condition will respond to copper and reach the food patch. Our current assay highlights aversive responses as the experimental organisms progress from a well-fed to starved state.

Our assay is a modification of the commonly used *C. elegans* chemotaxis assay[4] in which a stronger stimulus (*i.e.* high concentration) has been incorporated in order to evaluate behaviors over a more extended time period. Rather than evaluating instantaneous responses, this assay can be utilized to measure changes in aversive responses as organisms progress towards a starved state (if the necessary controls are included). Similar evaluations have been used in the absence of copper in order to evaluate ethanol withdrawal responses, *i.e.* nematodes locating food, over time[24].

As is the case with any behavioral assay in *C. elegans,* controlling for environmental variables is essential to ensuring consistently reproducible results. Organisms must all be of the same age, given that the behavioral responses can vary with age. Moreover, variability in the nutritional status of the worms can alter acclimation to starvation conditions. Therefore, the experimental worms should not undergo starvation at any point prior to this protocol. Starvation experience of the parental generation can also influence progeny behavior[30]; hence, it is advisable to ensure that the experimental organisms are well-fed for at least two generations. Moreover, the number of organisms transferred to the assay plates should remain consistent given that the local population density can influence dispersal rates[31]. Once worms have been transferred to the plates, it is critical to remove the excess M9 solution with a laboratory tissue without damaging the agar surface. Alteration to the surface may interfere with spontaneous locomotory patterns. Excess solution needs to be appropriately removed before commencing the official start of the assay to ensure that thrashing behavior, *i.e.* a nematode locomotory pattern observed in liquid, is not present. A clear indicator is to look for crawling behavior.

Assay plates must be controlled so that plate and bacterial dryness are consistent. Overly fresh plates can cause locomotory defects and can also interfere with the detection of environmental stimuli[6]. The application of the copper solutions need to be as consistent as possible so that aversive barriers are appropriately thick and they do not come into contact with the food source. Applying too little copper solution could lessen the wild type aversive response time, while too much could prove lethal to the nematodes. Aiming for a uniform thickness to the copper solution yields the best results. If indentations are created in the agar during copper solution application, the assay plate should be discarded. Worms may burrow through such holes[32], especially when faced with an aversive substance or starvation. To ensure plate uniformity amongst samples, mixed populations of worms (*i.e.* a mutant and wild type) could be measured on the same plate if one were distinctly labeled (*e.g. via* GFP).

Although this assay was performed on standard-sized agar plates, larger plates can be used (*e.g.* 100 mm diameter). In this scenario, the assay duration should be extended to 6 h to provide sufficient time for worm movement. Larger plates could allow for the testing of larger populations and could be used to investigate how population density affects aversive responses over extended periods of time. More technically advanced procedures could also be incorporated with the use of worm tracking equipment coupled to a movable stage[33]. Worm tracking would allow for more precise measurements and allow for the collection of additional variables (*e.g.* turning frequency, reversal frequency). Moreover, data collection would be continuously recorded over the 4 h period to give a more precise timeline of starvation-induced behaviors.

The assay can be easily adapted to allow for the measurement of alternative phenotypes. The age of the worm can be varied to allow for measurements on age-related starvation-induced aversive changes. Moreover, variations to the nutritional status of the experimental worms

could also allow for analysis of habituation to starvation in the context of food and chemical aversion. So long as protocols are consistent amongst comparable data sets, almost any pre-conditioning of experimental organisms could be evaluated *via* the chemotaxis assay.

Disclosures

We have nothing to disclose.

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