Video Article

High-resolution Measurement of Odor-Driven Behavior in *Drosophila* Larvae

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Abstract

Olfactory responses in *Drosophila* larvae have been traditionally studied in Petri dishes comprising a single peripheral odor source. In this behavioral paradigm, the experimenter usually assumes that the rapid diffusion of odorant molecules from the source leads to the creation of a stable gradient in the dish. To establish a quantitative correlation between sensory inputs and behavioral responses, it is necessary to achieve a more thorough characterization of the odorant stimulus conditions. In this video article, we describe a new method allowing the construction of odorant gradients with stable and controllable geometries. We briefly illustrate how these gradients can be used to screen for olfactory defects (full and partial anosmia) and to study more subtle features of chemotaxis behavior.

Protocol

1. Equipment and Reagents

Odor dilutions:

- Paraffin oil (Sigma, CAS number: 8012-95-1)
- Odors of the highest purity available which elicits attraction in *Drosophila* larvae¹. In this video article, animals were tested with isoamyl acetate (Sigma, CAS number: 123-92-2).
- 1.5ml glass vials with Teflon cap (Agilent Technologies)
- Digital measuring scale for precise dilution by weight

Assay arena set-up:

- Agarose (Promega)
- Lids of 96-well Microplate (BD Falcon, catalogue number: 353071). Our behavioral arena consists of three stacked lids.
- Single-channel 1-20 μ l pipetter or multi-channel pipetter to load odorant droplet(s) into the top lid of the stack. To set up the multiple source assay, we use 5-50 μ l Pipet-Lite multi-channel pipetter from Rainin.

Preparation of *Drosophila* larvae for behavioral tests:

- 15% (w/w) sucrose solution
- Regular Petri dishes (BD Falcon) to wash and store larvae
- Paint-brush (4-5mm bristle length) to manipulate and transfer larvae

Recording of larval motion:

- Small pins with a flat head for transferring larvae into arena
- Forceps or paintbrush to remove larvae after recording
- Illuminated stage with a homogeneous non-heating light source, "Slim Edge" Light Pad manufactured by Logan Electric
- Cabinet to surround the behavioral arena and control light conditions
- Video tracking system for recording of animal movement. We are using the Ethovision software (Noldus, The Netherlands).
- CCD camera and frame grabber video card (EureCard Piccolo Basic) purchased with Ethovision software
- Thermometer and humidity recorder to monitor the atmospheric conditions during the behavioral tests (optional)
- Matlab (The Mathworks) for customized data analysis (optional)

2. Behavioral arena

The behavioral arena consists of three stacked 96-well dish lids. The bottom lid is used to isolate the rest of the system from the light pad and reduce convection in the arena. The second lid, covered with 25ml of a 3% agarose gel, serves as a stage for the larvae to walk on. The top lid holds the odor droplets which remain suspended by surface tension.

Odor droplets are pipetted directly into the wells of the 96-well dish lid. A single or multiple odor sources can be used to generate gradients with distinct, verifiable profiles². To test for anosmia and chemotaxis defects in larvae, we propose to use the following two assays.

2.1. Single odor source assay: a single odor droplet laid in the well #E7 (standard 96-well plate reference system).

- Stimulus: 10 μ l of odor diluted in paraffin oil
- Initial conditions: a single larva is introduced under the odor source (or in its close neighborhood).

- Basic observations: For attractive stimuli (e.g. isoamyl acetate), wildtype larvae accumulate underneath the source. Anosmic animals or animals subjected to olfactory defects quickly wander away from the droplet.
- Duration of a trial: 3 min. The motion of a given animal is recorded until the trial time has elapsed or as soon as the animal contacts the wall of the plate.
- Possible metrics for behavioral quantification: percentage of time spent by an animal in a small circular zone centered on the odor source; mean distance to the odor source; time course of distance to odor source, etc.

This assay efficiently tests for basic anosmia in mutant larvae. It can also be used to reliably determine the detection threshold for particular odors².

2.2. Multiple odor source assay

Several odor droplets are laid in the well of the top lid. In the present video article, we set up gradients along the central row E. A total of six droplets were introduced in wells #E2, #E4, #E6, #E8, #E10 and #E12 (standard 96-well plate reference system). In contrast with the Petri dish and single odor source assays, the multiple odor source assay allows qualitative control of the gradient geometry established along the length and width of the arena. This assay was used to characterize the sensory mechanisms allowing *Drosophila* larvae to chemotax².

- Stimulus: 10µl odor droplets are laid in alternating wells along a row. This arrangement leads to the creation of a gradient centered selected row. To establish a homogenous gradient along the width of the arena, several rows can be loaded with multiple sources.
- Initial conditions: a single larva is introduced under the odor source between wells #E3 and #E4.
- Basic phenotypes: anosmic larvae wander at random from the starting point. For attractive stimuli (e.g. isoamyl acetate), animals which are able to smell navigate along the direction of increasing odor concentration. The accuracy with which an animal ascends the odor trail is correlated with its ability to sense and process the odorant stimulus. Tortuous paths are often caused by sensory defects. Once an animal has reached the point of highest concentration in the trial, it remains in the vicinity of this position.
- Duration of a trial: 3 min maximum (longer tracking durations tend to introduce noise as larvae lose interest and eventually abandon the gradient). For the gradients illustrated in this video article, the motion of an individual larva was recorded until the animal reached the neighborhood of the highest concentration source (well #E12). The recording was stopped as soon as the animal reached the target area or contacted any wall of the arena.
- Possible metrics for behavioral quantification: percentage of time spent by an animal under the odorant line (blue rectangle); mean heading with respect to the local direction of the odor gradient; a combined chemotaxis score measuring the global tendency of an animal to follow the odorant line².

3. Gradient profile verification

Before testing larvae in a particular gradient, it is important to determine its stability over time. On this basis, the experimenter can determine an adequate duration for each trial and the number of larvae that can be tracked sequentially in the same arena. To test the stability of the gradient, we have developed a method based on IR spectroscopy². This method is beyond the scope of this protocol and will not be elaborated further.

4. Protocol for the preparation of an experiment

4.1. Preparation of agarose plate and odor dilutions:

1. Pour 25ml of 3% agarose onto the flat top of a 96-well dish lid; allow the agarose to cool down and solidify (~ 30 min).
Important: Ensure that the 96-well lids are on a flat surface before pouring the agarose as the presence of a slope on the solidified agarose gel may affect the behavior of the larvae. When pouring the gel, remove any bubbles that form on the surface.
2. Dilute odors to desired concentrations in paraffin oil using a digital scale to accurately measure the amounts of paraffin oil and odor in each dilution.

Notes: When low odor concentrations are used, it is preferable to prepare a set of source concentrations based on serial dilutions. Start, for instance, with a 1.0M solution and perform a 1:2 dilution to obtain a solution 0.5M. By proceeding recurrently, one obtains a dilution series following the geometric progression 1.0M, 0.5M, 0.25M, 0.12M, 0.06M, 0.03M, 0.015M.

Important: Since many organic odors react with plastic, odor dilutions must be stored in glass vials with Teflon caps. Ideally, odors should be prepared fresh before each experiment to reduce fluctuations in actual odor concentration across experiments. This is especially true when using highly volatile chemicals.

4.2. Larvae preparation:

1. Prepare 15% sucrose solution in distilled water.
Notes: A 15% sucrose solution is denser than larvae and will cause them to be buoyed to the surface of the solution. However, undissolved fly food chunks have a greater density than the solution and rapidly sink to the bottom. Thus, using this sucrose solution provides an efficient means of separating larvae from the food. Sucrose solution is an excellent medium for biological contaminants, if the solution becomes cloudy it can be filter sterilized to remove the contamination.
2. Obtain a vial with 6-day-old larvae or larvae at the desired developmental stage.
Notes: All our experiments are carried out with 6-day old larvae (middle of third instar developmental stage); larvae at this developmental stage are large enough for ease of detection with our tracking software, but also highly active.
3. Pour sucrose into the food vial containing the larvae.
4. Using a spatula, break up and dissolve the fly food gently to release the larvae. Released larvae will float up to the surface.
5. Allow a few minutes for the fly food chunks to sink to the bottom of the vial; then, pour the sucrose and larvae into a Petri dish. If pre-selection of the larvae is necessary, animals can be collected by pouring the sucrose through a filter. Larvae can then be transferred to a dish for selection with a paintbrush.
6. Leave the larvae for approximately 30 minutes in the sucrose solution before proceeding with behavioral tests to allow the animals to habituate to the sucrose solution. As larvae do not appear to feed on sucrose, the animals will experience a state of starvation which improves the chemotaxis performance.
7. The lid of the Petri dish can be filled with water (or sucrose) and used as a receptacle for discarded animals after behavioral tests.

5. Protocol to perform behavioral recordings

5.1. Arena set-up:

1. Apply odor droplets to the underside (welled side) of a fresh 96-well dish lid.
Important: To avoid contamination from different odors and odor concentrations, a fresh lid must be used for each loading set-up. None of the lids containing odor sources are recycled after the experiment. Depending on the odor concentration range used, odorant gradients have been found to be stable only for 10 to 20 minutes in a closed system. It is therefore important to minimize the time between gradient set-up and the actual beginning of the behavioral test.
2. Invert the lid and stack it above a second lid coated with the 3% agarose layer.
Important: Invert the lid carefully to prevent odor droplets from spreading to adjacent wells.
3. Upon inversion of the top lid on the agarose surface, allow the odor to diffuse for 30 seconds before introducing the larva.

5.2. Loading larvae:

1. Lift the top lid containing the odorant droplets just enough to allow loading of the larva.
2. Transfer a larva from the sucrose solution to the agarose layer. The starting position of the animal depends on the assay being used:
For the **single odor source assay**, larvae are released under the odorant droplet.
For the **multiple odor source assay**, larvae are released between wells #E3 and #E4.
Important: Orient the newly introduced animals in a consistent manner. We orient our animals in the direction of the gradient, ensuring that the animal body and head face the highest concentration. Animals introduced in an orientation opposite to that of the gradient initiate a searching behavior. In our view, this initial search period represents an unnecessary source of noise. Controlling the head orientation reduces the number of animals which contact the arena wall during their exploratory behavior. This practice does not prime the direction of motion of the animal or significantly increase the number of anosmic animals chemotaxing along the odorant line by chance (i.e. false-positive in smell tests).
3. Replace the top lid and start the recording promptly.
Notes: Healthy larvae start crawling immediately upon their introduction in the arena and may cover a distance of 1cm within the first 10 seconds. If the experimenter is slow at initiating the recording, initial datapoints will be lost. For this reason we recommend that the experimenter becomes well acquainted with the software commands to initiate recording before attempting to run an experiment.
4. Allow the larva to behave for the duration of the recording (typically 3 minutes).
Notes: Recordings are terminated before the end of the 3 min if the larva hits the arena wall or reaches a target area (such as in the gradient assay).
5. At the end of the recording, promptly lift the lid and remove the larva with forceps or a paintbrush. Place the tracked animals in the Petri dish lid containing water (or sucrose).
Important: Recorded animals are not reused. The water dish is merely a convenient receptacle to separate recorded and un-recorded animals while running the experiments.
6. If tracking multiple animals under an arena set-up, promptly load the next larva and start the recording as shown above.
Important: The number of trials performed in the same arena set-up should be decided on the basis of the gradient stability which should be verified before performing any behavioral experiment².
7. Once all the larvae to be tested under the arena set-up have been tracked and removed from the arena, discard the top lid and agarose layer. The intermediate lid (i.e. the agarose lid) can be recycled.
Note: We advise Ethovision users to choose the 'background subtraction' detection method.

6. Sample experiments demonstrated in the video article

6.1. Single odor source assay with 1.0M of isoamyl acetate.

We blindly tested ten Or83b^{-/-} mutants and ten wild type (W1118) larvae to each of which a number had been assigned. The tracking experimenter did not receive any information about the genotype of each animal. After each recording, a predictive phenotype was assigned to each animal tested: smellers, if accumulation was observed under the source for the entire trial; non-smellers, if the path quickly left the neighborhood of the odor source. After having recorded the behavior of 20 animals, paths were grouped according to phenotype. The result of this grouping was then compared with the actual genotype of the larvae: all assignments made were found to be correct.

6.2. Multiple odor source assay with exponential and linear gradients of isoamyl acetate.

In this experiment, we compared the performances of wild type larvae chemotaxing along a linear and an exponential gradient. Chemotactic behaviors were recorded for two gradients set up with the same highest concentration of 0.5M. As explained in the article, the odor sources of the exponential gradient ranged between 0.015 and 0.5M. The sources of the linear gradient ranged between 0.25 and 0.5M (arithmetic progression with common difference 0.05M).

The linear gradient represented a more challenging chemotaxis environment as the local difference in concentration was constant and relatively small, and the concentration range narrow (0.25M). In contrast, the concentration range of the exponential gradient was larger with increasing local differences in concentration along the trail.

Fifteen larvae were recorded for each of the two gradient geometries and the paths superimposed in one graph. A visual inspection of the two graphs shows that wild type larvae were able to reliably ascend the gradient for both geometries but that the amount of meandering was greater for the linear gradient condition. This suggests that the signal present along the gradient length is more reliably detected for an exponential shape than for a linear one.

6.3. Multiple odor source assay with isoamyl acetate gradient in a "roof" geometry.

Here we verify that larvae introduced in an odor gradient are sensitive to local changes in odor concentration rather than just the presence or absence of odor. An isoamyl gradient was established by the application of odor droplets with the following concentrations: 0.06, 0.12, 0.25, 0.5, 0.25, 0.12M. The ensuing gradient geometry resembled an asymmetrical "roof" peaking at 0.5M.

Larvae were released between the 0.06M and 0.12M odor droplets. Our hypothesis was that if the larvae were merely computing the presence of odor they would follow the entire gradient without a particular preference for the concentration maximum. In contrast, if larvae are sensitive to local changes in concentration they would accumulate underneath the 0.5M droplet. Fifteen larvae were recorded, and it was found that following a short exploratory period all larvae accumulated underneath the higher concentration well. This suggests that local changes in odor concentration are computed and inform the chemotactic behavior of *Drosophila* larvae.

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