Video Article

A Single-fly Assay for Foraging Behavior in *Drosophila*

Orel A. Zaninovich[1], Susy M. Kim[1], Cory R. Root[1,2], David S. Green[1,3], Kang I. Ko[1,4], Jing W. Wang[1]

[1]Neurobiology Section, Division of Biological Sciences, University of California-San Diego

[2]Department of Neuroscience, Columbia University

[3]Dart NeuroScience

[4]School of Dental Medicine, University of Pennsylvania

URL: http://www.jove.com/video/50801
DOI: doi:10.3791/50801

Keywords: Neuroscience, Issue 81, *Drosophila*, olfaction, neuromodulation, chemotaxis, hunger, nervous system, behavioral sciences

Date Published: 11/4/2013

Citation: Zaninovich, O.A., Kim, S.M., Root, C.R., Green, D.S., Ko, K.I., Wang, J.W. A Single-fly Assay for Foraging Behavior in *Drosophila*. *J. Vis. Exp.* (81), e50801, doi:10.3791/50801 (2013).

Abstract

For many animals, hunger promotes changes in the olfactory system in a manner that facilitates the search for appropriate food sources. In this video article, we describe an automated assay to measure the effect of hunger or satiety on olfactory dependent food search behavior in the adult fruit fly *Drosophila melanogaster*. In a light-tight box illuminated by red light that is invisible to fruit flies, a camera linked to custom data acquisition software monitors the position of six flies simultaneously. Each fly is confined to walk in individual arenas containing a food odor at the center. The testing arenas rest on a porous floor that functions to prevent odor accumulation. Latency to locate the odor source, a metric that reflects olfactory sensitivity under different physiological states, is determined by software analysis. Here, we discuss the critical mechanics of running this behavioral paradigm and cover specific issues regarding fly loading, odor contamination, assay temperature, data quality, and statistical analysis.

Video Link

The video component of this article can be found at http://www.jove.com/video/50801/

Introduction

States of hunger promote two types of appetitive behaviors: food search and food consumption[1]. This simple behavioral assay is useful for the study of chemotactic behaviors associated with foraging[2,3]. Specifically, it tracks fly position, walking speed and latency to locating a food odor target. Latency of food finding serves as metric for measuring changes in the sensitivity of the fly's odor detection system downstream of changes in its internal appetitive state. A manual version of this assay was previously used to show GABA-B receptor signaling is important for odor localization behavior in adult flies[3]. The current automated version of the assay was instrumental in the study of how short neuropeptide-F (sNPF) signaling reshapes the olfactory map in *Drosophila* and influences appetitive behaviors[2].

Testing is done in a dark, temperature and humidity controlled room. Digital video cameras set above the clear acrylic testing plates track flies backlit by 660-nm LED illumination. Information from the camera is processed in real time by a computer stationed next to the testing area. We use data acquisition software to record and save the coordinates of fly positions during the testing period.

In this paradigm, the subject is released into an arena that contains a food odor at the center; the odor object creates a food odor gradient within the arena that induces food search behavior in the fly. A similar odor search protocol has been applied toward the study of chemosensation in single *Drosophila* larvae[7]. While other behavioral assays such as the four-field olfactometer[4,5] or the t-maze[6] evaluate odor aversion or attraction behaviors, this paradigm is best suited to evaluate olfactory sensitivity and chemotaxis behaviors.

Several key advantages accompany this assay. First, it permits rapid acquisition of large data sets, because data collection and analysis are mostly automated. Second, this assay isolates and measures the behavior of single flies, thus eliminating social olfactory cues that may influence their behaviors. Third, the simplicity of the protocol and simple experimental design make the assay efficient and easy to teach others.

In addition, this assay may be used to further probe neural circuits underlying food search behavior by combining it with the extensive genetic toolkit available to *Drosophila melanogaster*[8]. Targeted expression of transgenes that silence or excite neurons can be achieved with tools such as the GAL4-UAS system as well as the UAS-shibire^{ts1}, UAS-tetanus-toxin, and UAS-TrpA1(B) transgenes[9-12].

Protocol

1. Fly Collection and Starvation

1. Rear the experimental flies under controlled temperature and humidity conditions (*e.g.* 21 °C, 50-60% relative humidity) on a 12-hr light/dark cycle.

2. Collect female flies on the day of eclosion and place them, along with 4-5 males, into new food vials (maximum 30 per vial). Age flies 2-5 days.
3. Prepare the chambers for fly starvation.
    1. Push a single tissue (4.8 x 8.4 inch) down to the bottom of an empty plastic vial. Completely soak the tissue with distilled water. Use an object to push down on the tissue and gently squeeze out excess water.
    2. Invert the vial to discard the extra water. There should be enough water to keep the flies hydrated and the starvation chamber moist, but not enough to drown the flies.
4. Transfer the flies from the food vial into a starvation chamber and plug the vial about 18-24 hr before the experiment begins. Store the vials under controlled temperature and humidity conditions overnight until the experiment begins the next day.

2. Preparation of the Food Odor

1. Prepare a 1% agarose solution by adding 0.1 g of low melting temperature agarose to 10 ml of distilled water in a glass flask. Heat the agarose solution in a microwave just until it begins to boil but well before it boils over.
    1. Stop the microwave and swirl the flask once. Repeat this step twice more until the agarose has completely dissolved. Keep the agarose solution in a liquid state by keeping the flask warm on a hotplate set at 50 °C.
2. Add 990 µl of the 1% agarose solution and 10 µl of apple cider vinegar to a 1.5 ml Eppendorf tube to make a 1% apple cider vinegar solution. Vortex the solution until mixed and place in a dry bath incubator set to 50 °C.

3. Testing Room and Behavior Chamber Setup

1. Set the testing room to the desired conditions (*e.g.* temperature and humidity).
2. Turn on the LED panel (660 nm).
3. Rinse the sieves and testing plates with hot water and heat them in a drying oven until all moisture has evaporated. Cool the sieves and plates down to the testing room temperature before beginning experiments.
4. Position a shallow dish on top of the diffuser plate and fill it with water to increase the local humidity and to mask the water in the agarose droplet.
5. Position the sieves over the water dish.

4. Fly Loading into the Testing Plates

Diagrams with specifications for testing plates can be found in the Supplemental Files section. The testing plate is made of clear acrylic and consists of 6 testing arenas. A simple slider contains holding chambers that permit fly loading, temporary containment, and simultaneous release of 6 flies into their respective chambers at the start of the experiment. Cross-hairs etched at the center of each arena in the plate indicate where odorants should be pipetted.

1. Insert the sliders into the acrylic testing plate.
2. Gently slide the aspirator into the vial past the cotton plug and allow about 6 flies to walk into the aspirator.it is critical to be as gentle as possible in handling them. One may take advantage of phototactic fly behavior to induce flies to crawl towards the aspirator by pointing the vial opening towards a dim light source. If necessary, one may also apply gentle suction to aspirate approximately 6 female flies.
3. Insert the tip of the aspirator into the first hole of the testing plate. Allow a single fly to pass into the holding cell and gently advance the slider forward to load a fly into the next hole. Continue until flies occupy all 6 holding cells of the plate.
4. Pipette 5 µl of 1% apple cider vinegar agarose solution directly onto the center of the cross-hairs on the inside face of the testing plate.

5. Positioning the Testing Plate

1. To center the testing plate, open the file named "Positioning Tool.vi." “LabVIEW VI for Positioning Tool. vi can be found in the Supplemental Files section. Run the file by clicking on the white arrow in the upper-left corner of the screen.
2. Place the testing plate on top of the sieve such that the arena opening faces the sieve floor and the odor target is on the ceiling of the plate. Align the crosshairs etched into back of the testing plate with the cross hairs on the monitor screen.
3. When alignment has been completed, abort execution by clicking on the red dot located near the upper-left corner of the monitor.

6. Record the Fly Position During the Experiment

1. To track and record the coordinates of the fly during each food search trial, open the acquisition software file "Fly Tracking--Six Zones.vi." LabVIEW VIs for “Fly Tracking--Six Zones.vi” can be found in the Supplemental Files section. Run the file by clicking on the white arrow in the upper-left corner of the monitor.
2. Assign the file a name and click on "OK."
3. Advance the sliders in the testing chambers to release the flies into the testing arenas. Be careful not to move the testing chambers as this will lead to improper alignment with analysis software coordinates.
4. Click on "Start" (recording begins) and ensure that the only source of light in the testing chamber is the 660 nm LED panel.
5. When the trial is finished, remove the sieve and behavior chamber. Lift the testing plate from the sieve and remove the flies by submerging the plate in ice. Gently clean the plate with hot water and remove any agarose debris. Place the testing plates in a drying oven to remove moisture.

6. Ventilate the testing area by turning on a small fan for approximately 2 min. Turn the fan off and load the next group of flies into the next testing plate.

7. Data Analysis Using Custom Software

"Data Analysis for Fly Tracking—Six Zones" can be found in the Supplemental Files section. During data acquisition, the acquisition software records individual fly position coordinates for each time point in a text file. A single digital camera positioned above the testing plates acquires images at a frame rate of 0.5 Hz. The analysis software program "Data Analysis for Fly Tracking-Six Zones" extracts information from that text file to a) calculate the average speed, b) determine the time point at which a fly successfully located the odor source, and c) build graphical windows that allow the user to view: fly location, distance of the fly from the odor source over time and average fly speed over time. It also formats the data for easy export into a spreadsheet program. In this macro, food search latency is defined as the time point at which flies spend at least 5 sec within a 5 mm radius of the center of the arena.

1. Open the analysis software file "Data Analysis for Fly Tracking--Six Zones". Under the "Windows" tab, click on "Create New Table." Repeat this step until six tables have been created.
2. Under the "Macros" tab, click on "Foodfinding." A Main Panel should appear with the following options: Open Raw Data File for Layout; Open Raw Data File for Data File; Fly Location; Distance; Speed; Layout; FormatDataFile.
3. To view raw data without appending values to a text file, click on "Open Raw Data File for Layout." Locate and select the experimental data file in the browser window that appears. Click on "Open."
4. Click on "Fly Location" to view each fly's location in each of the six arenas (six X-Y plots depicting each fly's position over time should appear on the screen).
5. Click on "Distance" to view each fly's distance from the odor source (six plots depicting the fly's distance from the odor source over time should appear on the screen). The horizontal line at y = 5 mm indicates the threshold at which the fly is considered to be located at the food source.
6. Click on "Speed" to view each fly's average speed during the trial (six plots depicting the fly's speed over time should appear on the screen).
7. Click on "Layout" to display a layout with all the fly location, distance, and speed graphs in addition to the average speed (during the first 50 sec) and latency of finding the odor source for each fly **(Figure 1**). To properly view the layout, it may be necessary to adjust the margins. To do this, first click on the layout window. Under the "File" tab, click on "Page Setup for Layout." Reset the margins to 0.2 inches and click on "OK." Immediately left of each location plot is a small table with the headings "Speed" and "Latency." The values entered under each heading indicate the average speed in mm/sec and food search latency in seconds. Blank entries under Latency indicate the fly failed to locate the odor source. Food search latency is defined as the time point at which the flies spent at least 5 sec within a 5 mm radius from the center of the chamber.
8. To print a layout, click on the layout area (updates layout to current file). Click on "File" and then click on "Print Layout."
9. To view the next file, simply click on "Open Raw Data File for Layout." Click on the next raw data file you wish to view and click on "OK." Click on the Layout window to update the window with the new data file.
10. The settings may be saved in an experiment file for later use.

8. Export Data from the Data Analysis Software to a Spreadsheet Program

1. To export speed and latency data for each file, click on "Open Raw Data File for Data File." Select an experimental data file and click on "Open." A new browser window will appear.
2. In the new browser window, right-click on "New" and then click on "Text Document." Name the new text document. Select the newly named text file and click on "Open." This stores data from the raw data file into the text file.
3. To export data from another file, click on "Open Raw Data File for Data File." Click on another file and click on "Open." Select the text file from step 8.2). Continue this process for the remaining data files you wish to export.
4. Once all the desired data files have been added to the text file, click on "Format Data File." Select the Text Document used to store data from the previous steps and click on "Open" (a new window will automatically open).
5. Create a new Text Document in the window, assign the file a name, and click on "Save." This creates a text file that contains the filename, average speed, and latency for each fly and can be imported into a spreadsheet program.
6. Cumulative plots are constructed from data on the total number of flies reaching the food odor target as a function of time **(Figure 3**).
7. Experimental data sets are analyzed for statistical significance using a z-test for proportions.

Representative Results

The data analysis software and the layout, an example of which can be seen in **Figure 1**, are used to evaluate each fly's performance during its 10 min trial according to a set of analysis criteria. The following criteria are used to determine whether data from each fly will be used for data analysis and are designed to eliminate those flies that are unable to perform the food search task due to injury, illness, stress, or lack of motivation.

Flies that are inactive for more than 300 sec are considered 'inactive' and are rejected from the data set unless they a) already have succeeded in locating the food source or b) exhibit an average speed >10 mm/sec for at least 100 sec following the inactive period **(Figures 2a, 2b, 2c**).

Healthy flies exhibit robust search behavior immediately upon release from their holding chambers. Thus, to select only those flies that exhibit healthy speeds during the early stages of active odor search, only flies that move within a certain range of speeds for the first 50 sec of the trial are accepted for data analysis. This criteria is based on our observations that a) as flies near the odor source, their speeds decrease and b) few flies reach the odor target within the first 50 sec of the assay. The speed criteria are determined by evaluating the average speeds of at least 100 control flies at a given experimental temperature. The upper/lower speed limits are set by the average speed +/- standard deviation, respectively.

For example, at 21 °C, only flies that move between 3.5-10.5 mm/sec in the first 50 sec of the trial are used for data analysis. Exceptions to this rule are made for flies that successfully located the odor source within the first 50 sec and are thus slower than the lower speed limit.

Flies that do not move through all four quadrants in the arena and head straight for the food source after the trial begins are rejected **(Figure 2d**).

Flies that weave towards and away from the food source within a 10 mm radius for a minimum of 50 sec are considered to have successfully found the food source. The plot depicting distance of the fly from the odor source over time can be used to evaluate this rare case. This is the only instance of a successful search that is not automatically detected by the current data analysis macro and must be detected manually **(Figure 2e**)

Arenas with visible artifacts in fly position trace are rejected. Artifacts can be created by any event where the data acquisition software detects an object other than the fly. They often appear as long, straight lines that span across the arena or radiate from its center **(Figure 2f**).

In **Figure 3**, adult flies starved 18-24 hr exhibit a higher olfactory sensitivity to food related odors than their fed counterparts[1]. A graphical plot of the cumulative percentage of flies that successfully locate a food odor source shows 30% of all starved flies succeed within a 10 min window; in contrast, only 7% of all fed flies do so (**Figure 3**). This heightened olfactory behavioral response has been previously shown to require intact antennae[1]. Failure to observe a clear difference between fed and starved control flies in this assay can be resolved by examining environmental rearing and testing conditions.

One useful strategy for troubleshooting testing conditions is to examine whether flies are attracted to additional cues other than the odor target by measuring fly attraction to the odor vehicle, agarose. Starved flies should exhibit a significantly greater attraction to vinegar than to the agarose vehicle alone (**Figure 4b**). **Figure 4a** exhibits results from a food search experiment that was performed at 32 °C with an environmental humidity of 35% using wild type flies. In this data set, no significant difference between fly attraction to vinegar and the agarose control was detected. This is likely due to an increasing attraction to the water found in the agarose droplet under warmer testing temperatures. By increasing testing humidity to 50-60%, we were able to correct for this behavioral shift and restore the significant difference between attraction to vinegar and the agarose vehicle (**Figure 4b**, * denotes p-value <0.05).

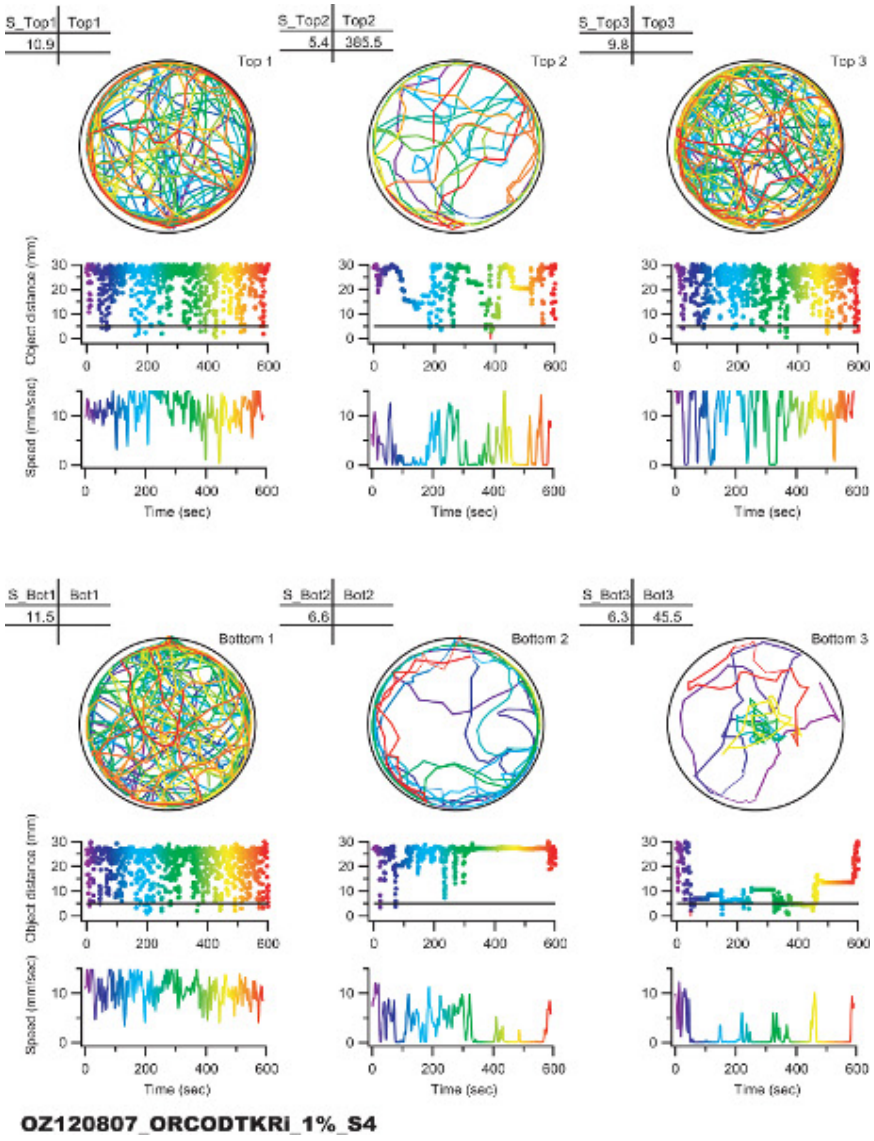

Figure 1. A typical analysis software data layout illustrates fly position over time, fly distance from odor source over time, and fly speed over time. The 2 column table in the upper left corner of each arena displays average speed (mm/sec) during the first 50 sec (column 1) and the latency of food finding in sec (column 2). In addition, the name of the opened text file is appended to the lower left corner (illustrated as, "OZ120807_ORCODTKRi_1%_S4"). Click here to view larger figure.

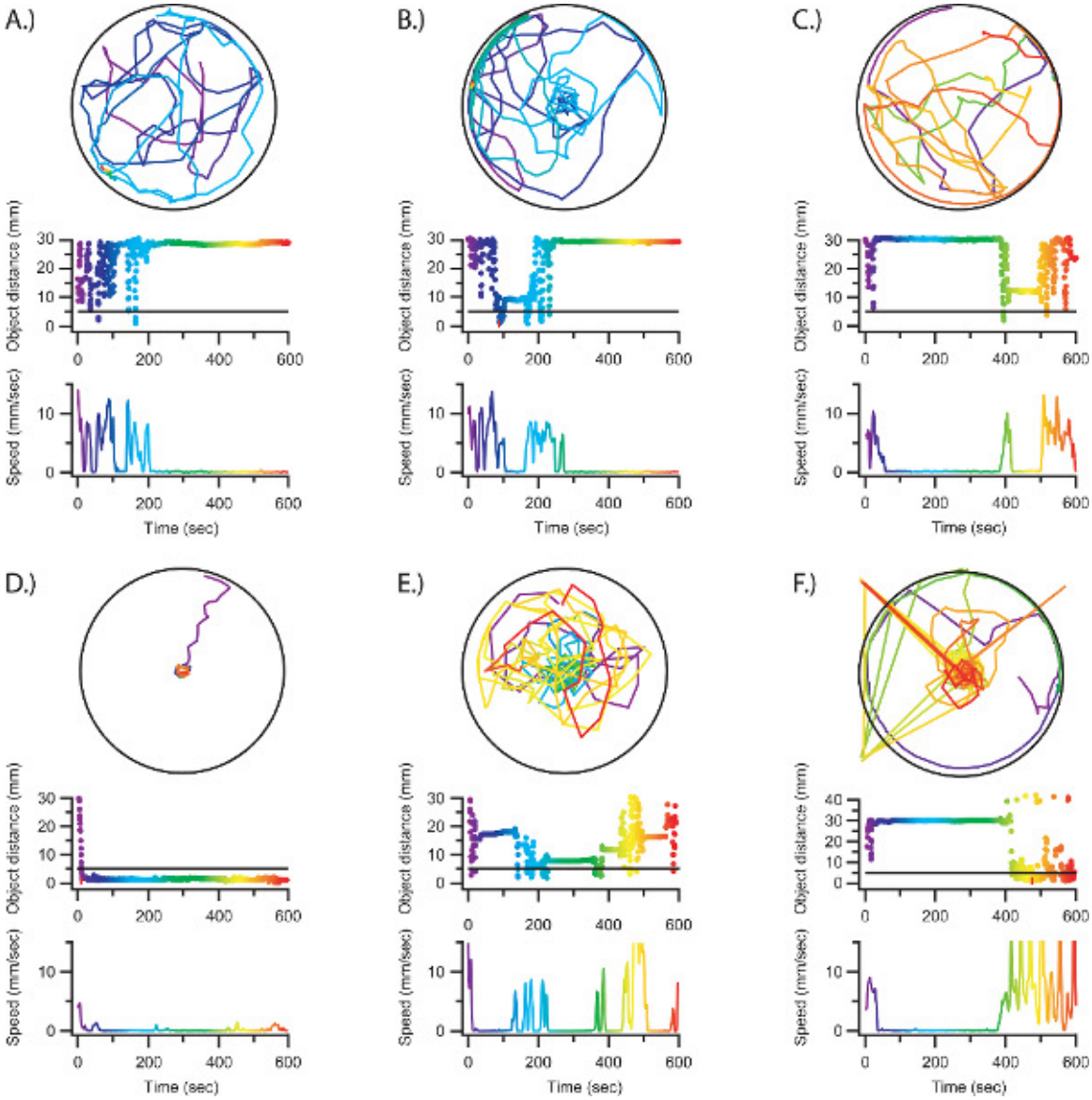

Figure 2. Examples of different types of traces addressed in Analysis criteria. A.) Fly that has been inactive for 300+ sec is rejected. **B**.) Fly that has been inactive after successfully locating food is accepted. **C**.) Fly that has been inactive for 300+ sec but shows robust activity for at least 100 sec after inactive period is accepted. **D**.) Fly that heads straight for the food source after release is rejected. **E**.) Flies that weave towards and away from the food source within a 10 mm radius for a minimum of 50 sec are considered to have successfully found the food source and are accepted. **F**.) Arenas with visible artifacts in the layout are rejected. Click here to view larger figure.

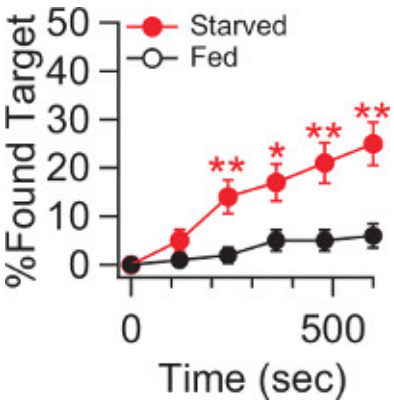

Figure 3. Graphical plot showing a cumulative percentage of fed and starved flies that find odor source over time using latency of food finding. (n = 88-96 flies; * denotes p-value <0.05, **denotes p-value < 0.01).

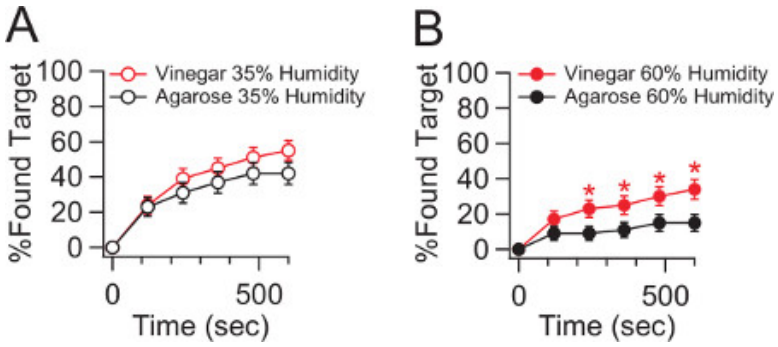

Figure 4. Troubleshooting testing conditions A) Graphical plot showing cumulative percentage of flies that find 1% vinegar or agarose vehicle over time. No significant difference was detected in fly attraction to either odor target when testing conditions were at 32 °C and 35% humidity (n = 62-94 flies). **B**) Graphical plot showing cumulative percentage of flies that find 1% vinegar or agarose vehicle over time. Testing conditions were at 32 °C and 50-60% humidity. Under these conditions, flies are significantly more attracted to 1% vinegar than to the agarose vehicle (n = 55-71 flies; * denotes p-value < 0.05).

Discussion

In this protocol, we describe a step-by-step procedure for the food search behavior assay. In addition to food related odors, it may also be adapted for the study of the fly's ability to locate other odor objects. For example, it may be applied toward the study of mate localization behavior in male flies[3] .There are several additional considerations for this protocol that we will mention here regarding this procedure:

First, the rearing temperature determines how long experimental flies should be aged before testing. It is recommended that a range of ages be examined to determine the most appropriate age for the experiment. For example, in our experience, when reared at 21 °C, differences between fed and starved fly responses are most robust after they have been aged 4-5 days.

Second, the LEDs illuminate a glass diffuser plate which serves to create constant, even illumination beneath the acrylic chambers. Sufficiently even, constant, and bright illumination is critical for automated tracking of fly movement. Uneven lighting or flickering light sources may lead to errors in automated tracking of the fly that either result in intermittent inability to detect the fly's position or cause the software to mistake the light artifacts as the fly. We've found both a commercially available LED backlight or a custom built LED array work equally well in meeting the lighting needs for this assay.

Third, if the software mistakenly detects small changes in lighting or agarose as extra objects, the object detection settings in the "Fly Tracking-Six Zones" acquisition software may be adjusted for threshold as well as object size. Adjusting the detection settings ensures that only one object is detected in each arena. To view the number of objects being tracked in each arena, click the "Threshold" tab of the "Fly Tracking-Six Zones" acquisition software. If more than one object is being tracked, one may adjust the Min Size, Max Size, Min Threshold, or Max Threshold until the detected artifact disappears.

Fourth, fly stocks used in these experiments should be isogenized. Behavioral performances in this paradigm are highly sensitive to differences in genetic backgrounds. Mated females are used to reduce potential behavioral variability associated with mating status or sex. There is no reason to believe this assay would not be equally effective in studying the behavior of males or virgin females.

Fifth, the sieve should be suspended slightly above the light diffuser plate to prevent odor saturation of the odor gradient (the suspension is approximately 2 cm with our commercially purchased model). We use commercially available sieves to create a porous floor beneath the testing plates.

Finally, in order to produce reliable data sets, consistency is required in experimental rearing and testing conditions. Any failure to see significant differences between fed and starved responses in control flies can be resolved by checking to make sure 1) flies are reared under stable temperature and humidity conditions, 2) flies are fed with fresh food and are not reared in crowded conditions, 3) newly eclosed fly exposure to CO_2 is minimized, 4) flies experience the same length of starvation, 5) flies are tested under stable temperature and humidity conditions, and 6) the testing environment and chambers are not contaminated with odors from previous trials or experiments. In addition to the aforementioned parameters, isogenization of fly stocks is important as different genetic backgrounds can influence fly performance in this assay. Furthermore, if vinegar is used as the odor source, care must be taken to ensure that it does not lose its potency by keeping it tightly sealed and stored at 4 °C.

In its current form, this odor search assay is best suited for the study of chemotaxis and evaluating differences in olfactory sensitivities across different genotypes or experimental conditions. Chemotaxis inherently requires sensitivity to an odor, attraction to that odor, and the motivation to seek the odor target. Odor attraction or aversion per se, however, are best measured by behavioral assays that present the fly with odor choices such as the t-maze[6] or the four-field olfactometer[4,5].

Disclosures

The authors declare no competing financial interest.

Acknowledgements

This work was supported by research grants to J.W.W. from the National Institute of Health (R01DK092640) and National Science Foundation (0920668).

References

1. Dethier, V.G. *The hungry fly : a physiological study of the behavior associated with feeding*. Harvard University Press (1976).
2. Root, C.M., Ko, K.I., Jafari, A., & Wang, J.W. Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell.* **145**, 133-144, doi:10.1016/j.cell.2011.02.008 S0092-8674(11)00122-X [pii] (2011).
3. Root, C.M., *et al.* A presynaptic gain control mechanism fine-tunes olfactory behavior. *Neuron.* **59**, 311-321, doi:S0896-6273(08)00572-2 [pii] 10.1016/j.neuron.2008.07.003 (2008).
4. Semmelhack, J.L. & Wang, J.W. Select *Drosophila glomeruli* mediate innate olfactory attraction and aversion. *Nature.* **459**, 218-223, doi:nature07983 [pii] 10.1038/nature07983 (2009).
5. Faucher, C., Forstreuter, M., Hilker, M., & de Bruyne, M. Behavioral responses of *Drosophila* to biogenic levels of carbon dioxide depend on life-stage, sex and olfactory context. *J. Exp. Biol*. **209**, 2739-2748, doi:209/14/2739 [pii] 10.1242/jeb.02297 (2006).
6. Quinn, W.G., Harris, W.A., & Benzer, S. Conditioned behavior in *Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A.* **71**, 708-712 (1974).
7. Fishilevich E., Domingos A.I., Asahina K., Naef F., Vosshall L.B., Louis M. Chemotaxis behavior mediated by single larval olfactory neurons in *Drosophila*. *Curr. Biol*. 15, 2086-96, doi:10.1016/j.cub.2005.11.016 (2005).
8. Venken K.J., Simpson J.H., Bellen H.J. Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron*. 72, 202-30. doi: 10.1016/j.neuron.2011.09.021. (2011).
9. Hamada, F.N., *et al.* An internal thermal sensor controlling temperature preference in *Drosophila. Nature.* **454**, 217-220, doi:nature07001 [pii] 10.1038/nature07001 (2008).
10. Kitamoto, T. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* **47**, 81-92 (2001).
11. Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., & O'Kane, C.J. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron.* **14**, 341-351, doi:0896-6273(95)90290-2 [pii] (1995).
12. Brand, A.H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. **118**, 401-415 (1993).

