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Calcium Imaging of Pheromone Responses in the Insect Antennal Lobe

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Abstract

Calcium imaging is a powerful technique that permits the visual monitoring of neural responses to pheromones and other odors in large ensembles of neurons. Here, we describe a method that permits the monitoring of *Drosophila* antennal lobe responses to odors using the genetically encoded calcium monitor GCaMP.

Keywords

Drosophila melanogaster; Antennal lobe; *cis*-Vaccenyl acetate (cVA); GCaMP; Olfactory system; Calcium imaging; Pheromone

1 Introduction

Elucidating how neural circuits process pheromone information is crucial towards understanding the role of chemical communication in the social interactions between conspecific insects. While electrophysiological methods permit monitoring of neural activity with unparalleled resolution, they are labor intensive and not amenable to monitoring large groups of specific neuronal types. Continuous technical improvements of genetically expressed calcium sensors and other activity reporters in recent years [1–3], however, bypass some of these problems. A genetic approach allows one to easily target calcium sensors to specific populations of neurons [4, 5], thus making calcium imaging a useful approach for monitoring pheromone responses at different levels of the fly brain.

Two-photon calcium imaging of odor responses in the antennal lobe is a useful technique in the study of how odor information is processed at this second relay in the insect nervous system [4]. In calcium imaging studies of antennal lobe odor processing in *Drosophila melanogaster*, a genetically encoded calcium indicator such as GCaMP [2] is typically expressed in either the odorant receptor neurons or the projection neurons of this circuit [6]. Other methods for monitoring neural activity such as synaptopHluorin have also been successfully used in this preparation [3, 7]. In this technique, the fly brain is dissected out from the rest of the head capsule with the antennae and the antennal nerve connections to the antennal lobe still intact. Highly volatile and relatively inexpensive chemical odorants are typically delivered to the intact antennae via odor streams redirected through bottles containing odorant [6–9] (Fig. 1). However, many important insect pheromones such as 11-*cis* vaccenyl acetate (cVA) exhibit lower volatility than many chemical odorants due to

relatively high molecular weights (>300 g/mol). In addition, these lipids must be purified and therefore can be expensive reagents. Standard odor delivery techniques, therefore, must be modified to accommodate these limitations. We describe here a simple system for delivering odor streams of pheromone using minute quantities of the pheromone cVA.

2 Materials

2.1 Antennal Lobe Preparation Materials

1. Adult hemolymph-like (AHL) saline: 108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM trehalose, 10 mM sucrose, 5 mM HEPES, pH 7.5, 265 mOsm. Weigh and transfer to a 1-L graduated flask:

6.312 g NaCl.

0.373 g KCl.

0.337 g NaHCO₃.

0.120 g NaH₂PO₄.

1.892 g Trehalose·2H₂O.

3.423 g Sucrose.

1.192 g HEPES.

Add 8.2 mL of 1 M MgCl₂. Add deionized and ultrafiltered (D.I.U.F.) water (Fisher W2-20) to a volume of 990 mL. Mix and adjust pH with 1 M NaOH. Transfer to a 1 L volumetric flask and make up to 1 L with water. Filter sterilize 500 mL to get calcium-free AHL saline. Add 1 mL of 1 M CaCl₂ to the remaining solution and filter sterilize to get AHL saline with calcium. Store at 4 °C (*see* Note 1).

2. 2.5 % Agarose solution: Weigh out 0.5 g low melting point agarose (Type VII-A; Sigma A0701) and dilute with 20 mL AHL saline containing 2 mM Ca²⁺. Heat agarose solution in microwave just until agarose is completely dissolved. Keep the solution warm and in a liquid state on a hot plate set to 37 °C.
3. Two #55 fine forceps (Dumont).
4. Scalpel.
5. Tungsten wire pins: California Fine Wire Company, Grover Beach, CA 93433.
Tungsten 99.95 %, diameter = 0.001 in.
Tungsten 99.95 %, diameter = 0.0005 in.
6. Dissection dish with Sylgard layer: Prepare Sylgard according to instructions. Fill small petri dish (Falcon, 35 × 10 mm) with approximately 3–5 mm layer of Sylgard and let it cure for 24–48 h until hardened.

¹For best results, saline should be made fresh every week. A freezing-point osmometer can be used to measure the osmolarity of the AHL saline, which should be about 265 mOsm for adult *Drosophila melanogaster* [10].

7. Glass slide ($25 \times 75 \times 1$ mm) with small square (~ 5 mm $\times$ 5 mm $\times$ 2 mm) of Sylgard placed near one of the edges.
8. Coverslip 11×22 mm, 0.13–0.17 mm thick (Thomas Scientific).

2.2 Odor Delivery System

The odor delivery system described here permits quantitative control of odor stimulation by diluting saturated vapor concentration up to a 1:1,000 ratio (Fig. 1). Two independent humidified airstreams are generated. One airstream enters the solenoid valve system, which can be switched from an empty vial (air) to the pheromone odor cartridge (Fig. 2). The constant airflow before, during, and after the 2-s odor stimulation ensures minimum mechanical change for odor stimulation. The other airstream is used to dilute the saturated vapor. The sum of flow rates for the two airstreams is kept constant at 1,000 mL/min. When a new odor is used in the system all tubing and odor vials downstream of the solenoid valves must be replaced to eliminate any potential contamination from previously used odors.

1. 2 port bottle cap (SV-435, Western Analytical, CA).
2. Flangeless Fitting for 1/8" OD tubing, P-300× ETFE ferrule (Western Analytical).
3. Delrin Nut, P-305 flangeless nut (Western Analytical).
4. 1/8" OD $\times$ 1/16" ID Teflon Tubing from bottlecap to odor puffer (PTFE2062, Western Analytical).
5. 1/8" OD $\times$ 0.08" ID Teflon Tubing from bottlecap to manifold (FEP2080, Western Analytical).
6. Custom manifold (*see* Note 2).
7. Odor puffer: 7-Port PTFE Manifold with 6 1/4–28 ports and 1 1/4" NPT port (MA-014, Western Analytical; *see* Note 3).
8. Pipe Hex Nipple, 1/2" MNPT $\times$ 1/2" MNPT (PN-008, Western Analytical) with steel mesh placed at both openings (Fig. 2).
9. Stimulator box (Grass Instruments).
10. Custom eight-channel solenoid valve controller system.
11. *cis*-vaccenyl acetate (neat, Cayman).
12. Whatman filter paper #8.
13. Pasteur glass pipette (5 3/4") truncated at both ends and fire polished such that ~1 cm of the tip and 1 1/2" of the barrel remain.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

²The custom manifold with eight odor ports is under the control of the custom solenoid valve controller. Switches on the solenoid valve controller determine which of the eight odor ports is open at the time of odor stimulation.

³The custom-made odor puffer in our lab contains nine ports. A 7-port version is available commercially from Western Analytical.

3.1 Antennal Lobe Dissection

1. Anesthetize fly on ice or with CO₂ (*see* Note 4).
2. Decapitate fly with scalpel.
3. Transfer head with paintbrush or forceps and place with anterior side facing upwards on the dissection dish with antennae positioned in front.
4. Pin down proboscis with large tungsten pin (dia. 0.001 in.).
5. Cover head with cold calcium-free saline (*see* Note 5).
6. Remove air bubbles by gently coaxing them away from the surface of the head with the sides of forceps. This prevents the brain from floating upwards towards the surface of the saline during the dissection.
7. With a pair of forceps in either hand, begin by inserting the tips of both forceps under one corner of the cuticle plate between the eyes. Hold the cuticle firmly with one set of forceps and use the other pair to tear away pieces of the cuticle and air sacs away from the brain (*see* Note 6). Leaving the proboscis and antennae intact, continue peeling off the outer cuticle and compound eyes. The brain itself can be clearly distinguished from the outside cuticle by its opaque and white appearance.
8. Flip the head and reattach the large pin through the proboscis such that its posterior aspect now faces upwards. Remove the back panel of cuticle attached to posterior side of the brain.
9. Position the dissection dish so that the proboscis points away from you. Gently pull the brain back towards yourself. Observe the white connective tissue and nerves lying in between the brain and proboscis. Begin gently clearing away this material by pulling it away with your forceps. Utmost caution, however, must be taken to avoid severing the nerves connecting the antennae to the brain.
10. Stop once the nerves connecting the antennae to the brain become visible. These should be located within a short distance on either side of the brain's midline and must be intact in order for information about odors detected by the antennae to reach the antennal lobe in your experiments.
11. A slender pair of motor nerves may also be visible even closer to the brain's midline than the antennal nerves. These may be severed at this time with your forceps to reduce spontaneous proboscis muscle contractions.
12. Remove the pin holding down the proboscis on the Sylgard plate. Gently suction up and transfer the brain with a glass pipette to a slide with square piece of Sylgard. The brain should rest on the Sylgard in a drop of saline (*see* Note 7).

⁴Antennal lobe preps from younger flies are more robust and easier to dissect out than older flies. Flies, however, should be old enough to permit sufficient levels of GCaMP to accumulate in the glomeruli. Therefore, flies approximately 2–3 days old are ideal.

⁵Dissection with saline containing calcium may lead to reduced odor responses. Cold calcium-free saline serves to preserve the integrity of the fly brain and minimize the loss of synaptic strength.

⁶Using two sets of forceps in this manner—one to hold the cuticle, and the other to tear away pieces of it—minimizes damage to the brain by reducing the exertion of tensile forces on the tissue.

13. Arrange the brain such that its anterior aspect is facing upwards and the antennae are pointing out towards the edge of glass slide (*see* Note 8).
14. Replace the calcium-free saline with regular saline containing 2 mM calcium.
15. Draw up agarose solution in glass pipette. Make a thin ring of agarose around brain prep to create a well. Then cover brain with agarose. Mix the agarose and saline by gently pipetting the solution in and out of the glass pipette until it is uniform (*see* Note 9).
16. Place the slide in a humidified chamber [a petri plate with a wet kimwipe] to prevent dehydration. Allow the agarose approximately 20 min or more to solidify (*see* Note 10).
17. When ready, use a scalpel to cut away chunks of agarose from areas immediately adjacent to the antennae and proboscis while leaving the brain intact and submerged in agarose. Next using a scalpel, remove the layer of agarose lying on top of the proboscis (Fig. 1). Next, insert forceps into agarose above antennae and gently peel agarose layer away from the antennae. Do not disturb either the antennal nerves or the brain itself (*see* Note 11). Use a kimwipe to dry off the antennae and rub off any bits of agarose that may still be clinging to the cuticle.
18. Drop coverslip on top of the prep.

3.2 Antennal Lobe Stimulation and Imaging

1. Pipette 1 μ L of cVA on a small slip of filter paper and place in glass pipette. Push filter paper towards the tip and then add small wisps of cotton to prevent it from falling back out. Secure the pipette to the pheromone tubing system.
2. Position slide in the imaging microscope setup. Position the tip of the glass pipette delivering the pheromone such that it is resting on the slide or the Sylgard slab approximately 5 mm away from the antennae. Position the odor puffer as close to the antennae as possible without disturbing the glass pipette.
3. After locating the prep with a low magnification objective (e.g., 4 $\times$), add a drop of water on top of the coverslip. Add a second drop to the tip of a higher magnification water immersion objective (e.g., 40 $\times$). Position the high magnification objective until the water droplets touch before adjusting the focus to locate the brain.

⁷Brain tissue is highly charged and tends to stick to the side of a glass pipette. Prevent the loss of your tissue by first suctioning up a small volume of saline into your glass pipette. Expel a tiny volume of the saline before suctioning up the brain. The brain should hang just at the tip of the pipette in a drop of saline.

⁸Orient the fly brain such that the antennae are fairly close to the edge of the slide. This later ensures that the antennae may be positioned as close to the odor puffer as possible.

⁹Be sure that the saline around the brain and the agarose are thoroughly mixed. Otherwise, the agarose may fail to solidify around the antennae, making its complete removal messy and difficult in a later step.

¹⁰To determine whether agarose has sufficiently dried, one can make a small incision into the agarose with the scalpel. If it is properly set, saline should not appear to seep out from the gel.

¹¹Use a Kimwipe to dry off the antennae and rub off any bits of agarose that may still be clinging to the cuticle.

4. Starting at a zero setting, increase the laser power slowly just until brain structures expressing GCaMP are visible. Focus and zoom in on the antennal lobe (*see* Note 12). To maximize the dynamic range of the signal and to minimize phototoxicity, adjust the laser power to set basal fluorescence to about 10–15 % of the full dynamic range of the light detector.
5. Begin stimulation and image acquisition. Stimulate for 2 s with at least 2-min interval between odor presentations to allow any desensitized odor receptors to recover and to permit odors to clear from the fly's headspace. For antennal lobe imaging, we typically acquire images at ~4 frames per second (256 ms per frame) with a resolution of 128×128 pixels ($0.75 \times 0.75 \mu\text{m}$ for each pixel; Fig. 3). After all odor stimulations are finished, acquire a high-resolution image stack of the antennal lobe at 512×512 pixels (1–2 μm step in *z*-axis) for glomerular identification.

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¹²If the image of the antennal lobe is not clear, there may be too much agarose in between the brain and the objective. Strive to make the layer of agarose a little thinner to improve image quality.

Fig. 1. Schematic diagram depicting *Drosophila* antennal lobe preparation and odor delivery system. **(a)** Close-up view of antennal lobe prep. Note that the antennae are exposed to the open air while the rest of the brain is submerged in agarose. A rectangular coverslip is placed on top of this prep and lies between the objective and the brain. **(b)** Teflon odor puffer carrying humidified airstream. The total airflow is kept constant at 1,000 mL/min. **(c)** Truncated glass Pasteur pipette containing a small slip of filter paper with 1 µL of cVA. The solenoid valve controller diverts a small percentage of the total airflow through the glass pipette containing cVA upon receipt of a signal from the stimulator box. **(d)** Vacuum fittings for odor delivery system consist of inert tubing, a flangeless ferrule, and a flangeless nut. As the nut is screwed into threaded fittings (1/4-28) in the bottle cap, the ferrule seals the vacuum line at the juncture

Fig. 2. Schematic diagram depicts the flow of air from the (b) manifold/solenoid valve controller, (c) glass vials containing odor samples, and (d) the odor puffer. A small fraction of the total airstream flows into the manifold through a side port (a). The bulk of the total airstream flows through the central port in the odor puffer (d). Upon receipt of a signal from the stimulator box, the solenoid valve controller (b) switches off the flow of air through valve 1/ bottle 1 which is empty and switches airflow into selected valves (2–8) or through the glass pipette containing cVA. Air flows from selected open valves in the manifold (b) into bottles containing odor (c). Air containing odor is then sent into the odor puffer and delivered to the antennoal lobe prep

Fig. 3.
Anterior view of antennal lobe from a female fly bearing the transgenes GH146-Gal4 and UAS-GCaMP3. Grayscale (*left*) and pseudocolored (*right*) images depict odor-evoked activity in DA1 projection neurons in response to delivery of cVA at 2 % SV (saturated vapor pressure)