Video Article

Measuring Physiological Responses of *Drosophila* Sensory Neurons to Lipid Pheromones Using Live Calcium Imaging

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Abstract

Unlike mammals, insects such as *Drosophila* have multiple taste organs. The chemosensory neurons on the legs, proboscis, wings and ovipositor of *Drosophila* express gustatory receptors^{1,2}, ion channels³⁻⁶, and ionotropic receptors⁷ that are involved in the detection of volatile and non-volatile sensory cues. These neurons directly contact tastants such as food, noxious substances and pheromones and therefore influence many complex behaviors such as feeding, egg-laying and mating. Electrode recordings and calcium imaging have been widely used in insects to quantify the neuronal responses evoked by these tastants. However, electrophysiology requires specialized equipment and obtaining measurements from a single taste sensillum can be technically challenging depending on the cell-type, size, and position. In addition, single neuron resolution in *Drosophila* can be difficult to achieve since taste sensilla house more than one type of chemosensory neuron. The live calcium imaging method described here allows responses of single gustatory neurons in live flies to be measured. This method is especially suitable for imaging neuronal responses to lipid pheromones and other ligand types that have low solubility in water-based solvents.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53392/>

Introduction

Animals rely on olfactory and gustatory information to mediate decisions essential for survival and reproduction. Understanding how chemosensory cues are detected and processed by the nervous system requires identification of the sensory receptor(s) and the corresponding chemical ligands. *Drosophila* detect a staggering variety of volatile and non-volatile compounds and are an excellent model in which to study the physiological mechanisms underlying chemosensation. While the olfactory organs perceive volatile molecules, the gustatory organs are specialized to detect low volatility compounds. Here, we present a method to directly measure neuronal responses from the taste organs of *Drosophila melanogaster* to low-volatility, lipophilic ligands.

Gustatory organs of the fly include the forelegs, proboscis, and wings. Distributed on the surface of the taste organs are hair-like structures known as sensilla that respond to sugars, bitters, salts, water and pheromones⁸. The sensilla have been classified morphologically into taste bristles and taste pegs⁹. There are about 31 taste bristles on the labellum that are classified into the long (l-type), short (s-type) and intermediate (i-type) morphologies. The 'l' and 's' sensilla house 4 sensory neurons that respond physiologically to sugar (the S cell), low salt (the L1 cell), high salt and bitter compounds (the L2 cell) and water (the W cell)^{10,11}. The 'i' sensilla house 2 sensory neurons, one of which responds both to low salt and sugar, while the other responds to high salt¹². There are approximately 41 taste sensilla in males and 26 sensilla in females distributed on each of the forelegs. For both males and females, there are 21 sensilla on the midleg, and about 22 sensilla on the hindleg¹³. The gustatory neurons enclosed by the taste sensilla on the legs are also classified into L1, L2, W and S types.

One standard method for measuring electrical activity from single neurons uses extracellular or intracellular electrodes to record ion flow. Electrode measurements allow neuronal function to be studied in non-model organisms such as *Drosophila* species, moths, and bees which lack extensive genetic tools for neural labeling. However, while electrophysiological methods have been routinely used to measure activity from *Drosophila* taste sensilla^{4,13,14}, applying this approach presents several technical challenges. First, taste bristles need to be identified based on morphology and spatial location. Upon identification, electrophysiological measurements can be hindered by the small size of the sensilla, limited accessibility due to position, and difficulty in applying controlled volumes of a chemical stimulus to a taste bristle. Also, stimulation of sensilla may generate signals from more than one neuronal type¹⁵. Second, detection of electrical signals can be confounded by background noise resulting from mechanical vibrations and noise from electronic equipment. Third, the use of a sharpened electrode can damage the fly preparation, if used

incorrectly¹⁴. Finally, assembling an electrophysiology rig requires specialized electronic components for stimulus delivery, signal recording, and data analysis and can be costly.

In *D. melanogaster*, the availability of genetically-encoded tools has facilitated the development of imaging techniques that allow the responses of small populations of neurons to be studied. One such approach is the use of the CaLexA reporter¹⁶. In this method, gene sequences encoding the LexA-VP16 transcription factor and a calcium sensitive protein NFAT are fused together. Calcium activation of the protein phosphatase calcineurin catalyzes the de-phosphorylation of NFAT and, in turn, facilitates its import into the nucleus. Inside the nucleus, the LexA domain binds to a LexAop-DNA binding motif which directs the expression of a green fluorescent protein (GFP) reporter, thus allowing sustained identification of functionally activated neurons. The approach has been used successfully to measure the response of specific olfactory glomeruli in the antennal lobe following exposure of live flies to an odorant¹⁶. Recently, physiological responses of IR52c gustatory receptor neurons were measured from live flies using CaLexA⁷. However, for this study, it was necessary to age flies for up to 6 weeks to enhance GFP transcription. While immunostaining with an anti-GFP antibody can be used to boost the CaLexA signal, this method would require tissue fixation, thus precluding possibility for live-cell imaging.

The genetically encoded calcium indicator protein GCaMP has also been extensively used to study neuronal responses in a number of species¹⁷. The protein GCaMP fluoresces with low intensity prior to neuronal stimulation. Application of a stimulus triggers an action potential in the neuron, resulting in the influx of Ca^{2+} . GCaMP, bound to Ca^{2+} , undergoes a conformational change, causing it to fluoresce with brighter intensity (**Figure 1**). A recently developed single-neuron calcium imaging approach was used to identify glucose as the ligand for the foreleg specific Gr61a gustatory neurons¹⁸. In this study, dissected forelegs from transgenic *Drosophila* expressing GCaMP in Gr61a gustatory neurons were covered with a layer of agarose prior to imaging. However, the use of dissected tissue can cause eventual rundown of GCaMP expression, thus limiting measurement time and detection sensitivity. In addition, agarose can limit sensitivity due to high background levels of fluorescence and its light scattering properties.

To address some of these drawbacks, we describe the use of GCaMP-mediated calcium imaging to record physiological responses from foreleg and proboscis gustatory neurons of intact animals. We show the physiological responses of Gr68a and ppk23 neurons expressing genetically encoded GCaMP5G¹⁹ to a *Drosophila* lipid pheromone, (3R, 11Z, 19Z)-3-acetoxy-11,19-octacosadien-1-ol (CH503)^{20,21}. Neuronal responses are measured by quantifying the increase in fluorescence of the GCaMP5G signal during pheromone stimulation. In this protocol, neurons are imaged for a total duration of 120 sec, which is sufficient to differentiate patterns of neuronal activation in individual cells.

Protocol

1. Sample Preparation

- Cross the *Gal4*^{3,22} driver line to *UAS-GCaMP5G*¹⁹ flies ($w^{1118}; P\{20XUAS-IVS-GCaMP5G\}attP40$). Allow the cross to grow at 25 °C.
Note: Generating flies with multiple copies of the *Gal4* or *UAS-GCaMP* transgenes can help to enhance the fluorescent signal intensity. An earlier version of the *UAS-GCaMP* transgene (*UAS-GCaMP3*) showed very weak fluorescence intensity when expressed under the control of the *Gr68a-Gal4* driver.
 - Collect and separate by sex newly eclosed adult flies and raise at 25 °C.
Note: The baseline level of GCaMP5G fluorescence is optimal in flies aged between 14-28 days. Isolating flies by sex precludes the possibility of social interactions that may influence the neural responses.
- Anesthetize a fly under a mild stream of CO_2 .
- Attach the fly to a glass coverslip by first placing a small drop of nail polish in the center of a 0.17 mm coverslip. Gently place a fly on its side on the drop of clear nail polish and ensure that the entire drop is covered by the fly.
- Perform steps 1.5 and 1.6 under a dissecting stereomicroscope, used at 8X magnification.
- Use a wet paintbrush to extend a foreleg of the fly. Secure the foreleg in position by placing two thin strips of tape over the first and fifth tarsal segments (**Figure 2A**). This will expose tarsal segments T2-T4 for imaging (**Figure 2B**).
- To image neurons on the proboscis, place a thin strip of tape over the rostrum of the proboscis to expose the labellum (**Figure 2C, D**).
- Draw a hydrophobic barrier around the fly with a PAP pen to prevent solution from flowing over the coverslip surface. Make measurements within 30 min of mounting.

2. Preparation of Stimulus Solution

- Dilute lipophilic ligands (such as CH503, used in this study) in a PBST solution: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 0.1% Triton X-100 (v/v); pH 7.4.
 - Make a 1 mg/ml stock solution of the ligand using PBST. Prepare all subsequent dilutions of the stock solution using PBST.
Note: Depending on the solubility of the ligand of interest, different solvents may be needed to dissolve the ligand. The solubility of CH503 in various solvents is described in **Table 1**. Unlike other solvents, PBST did not produce an increase in fluorescence.

3. Stimulation of Gustatory Neurons and Image Acquisition

- Using a 10 μl pipettor, place 10 μl of PBST onto the tarsal segments.
Note: This step moistens the leg and prevents the tarsal segments from drifting.
- Use an optical system that has a camera of sufficient speed and sensitivity to achieve good fluorescence signal at high time resolution.
Note: For this study we used a spinning disk confocal microscope and a sCMOS camera (see **Materials List**).
- Choose the specific tarsal segment and neurons to be imaged. Acquire three pre-stimulation confocal Z-stacks.

1. Ideally, use acquisition settings that are fast enough to allow maximal time resolution while still capturing the entire cell volume. Example acquisition settings are: 200 msec exposure, binning 2 x 2 and 6 x 0.5 μm^2 optical sections, captured every 2 sec.
Note: Immobilized legs have been imaged successfully for up to 10 min, at 30 sec intervals, with no discernible bleaching of the GCaMP signal. For longer recording times, ensure that the legs are held very firmly in place on the coverslip.
2. Optimize laser power for highest signal-to-noise achievable with minimal photobleaching during the entire imaging regime. For the experiments described here, use a 491 nm diode laser (100 mW) at 30% transmission for imaging both Gr68a and ppk23 gustatory neurons on the legs and proboscis. Use 2 x 2 binning to allow for shorter exposure times while still achieving sufficient spatial resolution.
Note: The laser power settings were optimized to allow detection of fluorescent change ranging from a 1.2- to 4.5-fold increase.
4. Pipette 10 μl of the stimulus solution onto the tarsal segments. For control experiments, add another 10 μl of PBST. Immediately acquire 117 post-stimulation images.
Note: The number of post-stimulation images acquired is based on the amount of time in which maximal change in fluorescence was reached (approximately 2 min).
 1. CRITICAL STEP: Whilst pipetting solution on the preparation, do not to touch the fly with the pipette tip since this will cause the foreleg to move out of position.
Note: Alternatively, use a micromanipulator to accurately position a glass capillary containing stimulus close to the tarsal segment to be imaged. Deliver controlled volumes of stimulus using an intracellular microinjection dispenser according to manufacturer's instructions.

4. Image Processing and Analysis: Quantification of the Response

1. Open a series of confocal Z-stacks using Fiji (<http://fiji.sc/Fiji>)²³ or ImageJ (<http://imagej.nih.gov/ij/>)²⁴ image analysis and processing software.
2. Make a sum-intensity projection of all six Z slices for each of the 120 time points.
 1. Select the "Stack" option under "Image". Further, select the "Z projection" option and enter "1" as the start slice and "4" as stop slice. For projection type, select "sum slices" from the pull down menu, and choose "All time frames."
3. Define the region of interest (ROI) by using the freehand selection tool to draw a boundary around a cell body or neuronal projection. Click on the "Analyze" label and select the "ROI manager" option under "Tools".
4. Quantify the fluorescence of the ROI by selecting the "Set measurements" option, under the "Analyze" menu and checking the boxes for integrated density, area, mean and maximum gray value and label. Then, select the "More" option under the ROI manager menu, and further select the "Multi measure" option.
Note: This will generate a popup box with values for each of the selected parameters.
5. Check the maximum grey values to ensure that none of the pixels have reached saturation (e.g., 65,535 for a 16-bit camera).
NOTE: For the experiments shown in this paper, responses were measured either from cell bodies or neuronal projections, depending on where the maximal increase in $\Delta F/F$ was observed.
6. Calculate the maximum relative change in fluorescence ($\Delta F/F$) at time point t is as follows:

$$\frac{\Delta F}{F} = \frac{F(\text{cell})_t - F(\text{cell})_{t=0}}{F(\text{cell})_{t=0}}$$
 1. Subtract background fluorescence from each $F(\text{cell})$ value. To quantify background fluorescence, measure the integrated density of a ROI of equivalent size and shape from an area of the foreleg (or proboscis) devoid of detectable neurons.
 2. To quantify the post-stimulation fluorescence, $F(\text{cell})_t$, measure the integrated density of the ROI of the cell body or process at time point " t " after stimulation.
 3. To quantify the pre-stimulation fluorescence, $F(\text{cell})_{t=0}$, analyze pre-stimulus images of the cell body or process in the same way as the post-stimulation signal. Use the mean integrated density of 3 images to determine $F(\text{cell})_{t=0}$.

Representative Results

The GCaMP calcium indicator was genetically expressed using *Gr68a-Gal4* or *ppk23-Gal4* drivers. Distinct populations of foreleg neurons and non-neural support cells are labeled by each driver (**Figure 3A-C**). Ligand-specific responses to the lipophilic pheromone CH503 were observed in *Gr68a-Gal4* and *ppk23-Gal4* cells expressing GCaMP (**Figure 3D**). The relative change in fluorescence intensity of the GCaMP5G signal ($\Delta F/F$) and increased with higher concentrations of CH503 (**Figure 3E**). Interestingly, the 5 ng dose of CH503 may suppress the neural response.

Gr68a and ppk23 neurons exhibited different response patterns. Gr68a neurons showed a tonic pattern of neuronal response (**Figure 3F**), whereas ppk23 neurons showed a phasic, oscillatory response (**Figure 3G**). Responses from ppk23 neurons in the proboscis were also measured using this preparation and exhibited a combination of phasic and oscillatory responses (**Figure 3H**).

Figure 1: Basic principle of the GCaMP calcium imaging technique. GCaMP5G is genetically expressed in *Gal4*-labeled cells and fluoresces with low intensity prior to neuronal stimulation. Application of a stimulus triggers an action potential in the neuron and causes Ca^{2+} influx. GCaMP5G, once bound to Ca^{2+} , undergoes a conformational change, resulting in increased fluorescence (ΔF), which changes over time. The baseline fluorescence (F) refers to fluorescent signal intensity prior to stimulation.

Figure 2: Mounting a live fly for imaging of the foreleg or proboscis neurons. (A) Mounting the foreleg: a 3.2X image of a live male fly, showing the forelegs fastened to the coverslip with tape. (B) A 40X image of the fly, showing each of the tarsal segments (T1-5) and the placement of tape above the first tarsal segment and on the fifth tarsal segments. (C) Mounting the proboscis: a piece of tape is placed over the rostrum to expose the tip of the proboscis. (D) The pipette tip for stimulus addition is held slightly above the fly and does not make direct contact with the preparation.

Figure 3: Phormone-induced neural responses in GCaMP-expressing neurons. (A) Schematic of the male foreleg of *Drosophila* showing the spatial positions of *Gr68a-Gal4* labeled cells on tarsal segments T2-4. Non-neural cells (identified based on size and shape) are colored yellow. (B) Raw fluorescent image of the male foreleg showing neural and support cells labeled by the *Gr68a-Gal4* driver. Scale bar = 50 μ m. (C) Schematic of the male foreleg of *Drosophila* showing the spatial positions of *ppk23-Gal4* labeled cells on tarsal segments T2-4. (D) Response of *Gr68a*- and *ppk23*-expressing T4 neurons to CH503 (chemical structure shown above). The average $\Delta F/F$ in response to CH503 was compared to the average $\Delta F/F$ following application of PBST solvent alone; Student's t-test with unequal variance (for *Gr68a*) or Mann-Whitney test (for *ppk23*): * $p < 0.05$, *** $p < 0.001$. Error bars depict s.e.m. Statistical power = 0.93. (E) A dose-dependent change in $\Delta F/F$ is observed in *Gr68a* neurons in T4. Student's t-test with unequal variance: ** $p < 0.01$, *** $p < 0.001$. Statistical power = 0.86-0.96. (F) A color-coded time course showing the tonic response in GCaMP fluorescence following CH503 stimulation (500 ng) of *Gr68a*-expressing neurons. The positions of the neurons on the foreleg are shown in the raw fluorescent image (left). Scale bar = 10 μ m; time scale = 0-96 sec. The accompanying line graph shows the tonic response from a *Gr68a* neuron to stimulation by 500 ng of CH503. The red arrow indicates the time point at which the stimulus was added. The peak response occurs at 120 sec. (G) A color-coded time course showing an oscillatory, phasic response in GCaMP fluorescence following CH503 stimulation (500 ng) of *ppk23*-expressing neurons. The positions of the neurons on the foreleg are shown in the raw fluorescent image (left). Scale bar = 10 μ m; time scale: 0-96 sec. The accompanying line graph shows a bursting response from a *ppk23* neuron to stimulation by 500 ng of CH503. Red arrow indicates the time at which the stimulus was added. The peak response occurs at 76 sec. (H) A color-coded time course of *ppk23*-expressing neurons on the proboscis showing a late-onset tonic response from the cell body and an oscillatory response from an axonal projection following CH503 stimulation (500 ng). The positions of the cells are shown in the raw fluorescent image (left). Scale bar = 10 μ m; time scale: 0-96 sec. Reproduced with permission from Shankar, S., *et al.* The neuropeptide tachykinin is essential for phormone detection in a gustatory neural circuit. doi: 10.7554/eLife.06914 (2015). [Please click here to view a larger version of this figure.](#)

Solvent ^{1,2}	Solubility of CH ₅ O ₃ ³	Does the solvent alone trigger neuronal activity? ⁴
0.1% PBST	YES	NO
100% Ethanol	YES	YES
100% Hexane	YES	YES
10% Ethanol	NO	(not tested)
10% Hexane	NO	(not tested)
100% DMSO	YES	YES
10% DMSO	YES	YES
0.4% DMSO	YES	YES
0.2% DMSO	YES	YES

Table 1: Solubility of CH₅O₃ in various solvents.¹ DMSO: Dimethyl sulfoxide.² Ethanol, hexane, and DMSO solutions were in diluted with dH₂O (v/v).³ Solubility of CH₅O₃ in 0.2% DMSO was limited only to 250 ng/ml.⁴ Increase in ΔF/F with solvent alone was equivalent to that observed upon stimulation with pheromone.

Discussion

We describe here a method to perform live calcium imaging of *Drosophila* peripheral neurons in 2 different sensory organs. The Ca²⁺-evoked GCaMP fluorescent responses in Gr68a-neurons induced by the pheromone ligand CH₅O₃ were dose-dependent and quantitative. It was also possible to discern different neural response patterns such as phasic and tonic responses.

Neurons showing phasic responses are believed to allow rapid adaptation to continuous stimuli. This type of response has been associated with odor detection²⁵ and the generation of rhythmic motor activity patterns²⁶. In contrast, neural oscillations, which have been described in many sensory systems, are thought to synchronize multiple inputs onto a single postsynaptic site and to convey specific features about the stimulus²⁷. The molecular basis of oscillations associated with ppk23 neurons has not yet been reported, to our knowledge. In other types of excitable cells showing oscillatory responses, the frequency of the oscillations has been shown to be regulated by a process called calcium-induced calcium release²⁸. In this process, calcium is released from internal stores under the control of the secondary messenger inositol triphosphate, and the frequency of its release is dependent on the strength of the external stimulus. Insights into the molecular basis of tonic neuronal responses have been gained from studies on oxygen sensing receptors of *C. elegans*²⁹. A prolonged increase in the calcium levels within neurons was found to be mediated by L-type voltage gated calcium channels, ryanodine, and inositol triphosphate signaling pathways²⁹. It will be important to ascertain whether similar mechanisms play a role in the responses of ppk23 neurons.

Live calcium imaging can be adapted in several ways for the study of other populations of *Drosophila* sensory neurons or other chemical ligands. First, the responses from gustatory neurons located on the proboscis, legs, or wings can in a screen for ligands for orphan gustatory receptors. Second, modification of the solvent will allow testing neural responses to more polar molecules. Third, an onstage incubator can be coupled with the spinning disc microscope. This setup would be ideal for activating or silencing neuronal activity using temperature-sensitive transgenes like *UAS-TrpA1* or *UAS-Shibire^{ts}*, and measuring the corresponding changes in fluorescence in these neurons upon stimulation⁴. Fourth, the use of a continuous time series for image collection can facilitate detection of fast responding cells. Finally, with the addition of targeted laser illumination, this method could be expanded for use with other advanced live-cell imaging methods such as FRAP and FRET.

Several technical considerations are imperative to obtain stable images and to accurately measure the time course of physiological responses. First, the foreleg must be securely positioned since the fly exhibits vigorous movement upon addition of the solution, which can be a source of variation between preparations. Although bath application of pheromone solution allows the ligand to reach all of the gustatory sensilla on the exposed tarsal segments, some of the solution can flow towards other parts of the mounted preparation. Also, the foreleg could potentially drift away when solution is added. Second, to accurately image near-instantaneous increases in fluorescence, it is important to resume imaging immediately after addition of the stimulus since some neurons show a rapid and maximal response within 4 seconds of stimulation. Alternatively, a continuous time series can be initiated prior to adding the stimulus solution, and the precise time point at which the stimulus is administered can be marked.

Two sources of false positive responses were observed. First, the solvent itself can induce fluorescent responses even in the absence of a ligand. The PBST solvent caused such a response in some *ppk23-Gal4* labeled cells. In addition, DMSO, a commonly used solvent for polar and apolar molecules, also induced a response in some *Gr68a-Gal4* labeled neurons. It is therefore essential to determine whether the solvent itself induces a fluorescent signal and to select a solvent system with low background activity. Second, a strong phasic-type response in non-neural support cells was observed to the solvent PBST alone. The *Gr68a-Gal4* driver is expressed in both neurons and support cells that appear to surround some of the neurons and in general, are larger, amorphously shaped, and lack visible projections. It is therefore important to differentiate neural from non-neuronal responses for each *Gal4* line when choosing ROIs.

One major limitation of this method is that the same fly preparation cannot be used for successive experiments to test different concentrations or types of ligands, unless the applied stimulus is first thoroughly removed from the surface of the coverslip. In addition, this method cannot be used for high throughput imaging of several flies at once, due to the high magnification needed for visualization of *Drosophila* neurons, and the need for fast acquisition and high time resolution. Lastly, this method requires the availability of genetic tools for transgenic gene expression, thus limiting application primarily to major model organisms.

Altogether, this method is an easier alternative to cellular recordings using electrodes and does not require specialized instrumentation. In addition, unlike other genetically encoded reporters such as CaLexA, the animal is kept alive throughout imaging allowing physiological responses to be measured in real-time with high sensitivity in flies of all ages. This feature could potentially allow for screening of age-dependent responses to ligands or comparison of neural responses following changes in social conditions or reproductive state. Moreover, sustained recordings can be performed for at least 10 min without obvious photobleaching or rundown of GCaMP signal.

Disclosures

The authors declare that there are no competing financial interests.

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