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In Vivo Calcium Imaging of Taste-Induced Neural Responses in Adult *Drosophila*

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Abstract

For nearly two decades, *in vivo* calcium imaging has been an effective method for measuring cellular responses to taste stimuli in the fruit fly model organism, *Drosophila melanogaster*. A key strength of this methodology is its ability to record taste-induced neural responses in awake animals without the need for anesthesia. This approach employs binary expression systems (e.g., Gal4-UAS) to express the calcium indicator GCaMP in specific neurons of interest. This protocol describes a procedure in which flies expressing GCaMP are mounted with the labellum securely positioned, enabling fluorescence in the brain to be recorded at millisecond resolution under a confocal microscope while a solution is applied to the labellum, stimulating all labellar taste sensilla. The examples provided focus on calcium responses in primary gustatory receptor neurons of *D. melanogaster*. However, this approach can be adapted to record from other neurons of interest within the brain of *Drosophilids* or other insect species. This imaging method enables researchers to simultaneously record collective calcium responses from groups of gustatory neurons across the labellum, complementing electrophysiological tip recordings that quantify action potentials from individual neurons. The *in vivo* calcium imaging technique outlined here has been instrumental in uncovering molecular and cellular mechanisms of chemosensation, identifying unique temporal response patterns in primary taste neurons, investigating mechanisms of gustatory modulation, and exploring taste processing in downstream circuits.

Introduction

The fruit fly, *Drosophila melanogaster,* is celebrated for the powerful genetic research tools available in this model organism. These tools provide the ability to readily manipulate specific genes in targeted cells, making it ideal for exploring fundamental neural circuits such as vision and chemosensation[1,2,3]. Gustation, through contact chemosensation, is a key neural pathway that regulates behaviors involved in feeding, mating, reproduction, and

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ultimately, the survival and fitness of animals[4,5,6,7,8,9]. Understanding how this important chemosensory information is encoded and transmitted requires describing the activity of the neurons in the circuits that are activated by taste stimuli.

In *D. melanogaster*, external gustatory receptor neurons (GRNs) are located on the forelegs, proboscis, and wings[10,11]. The labellum, at the end of the proboscis, contains hair-like structures called sensilla that can be mapped by their morphology based on size: long (L-type), intermediate (I-type), and short (S-type)[10]. Most of the GRNs are concentrated on this sensory organ, with each sensilla containing 2-4 different types of GRNs so that each taste modality is spread across the labellum[12,13,14,15]. While electrophysiological tip recordings can be used to quantify action potentials coming from GRNs in a single sensilla[16], *in vivo,* calcium imaging can be used to isolate the activity of a specific type of GRN across the full labellum[14,17]. This same calcium imaging technique can also be used to study neural responses in downstream taste circuits[18,19,20]. Calcium imaging requires binary expression systems, such as Gal4-UAS[21,22,23], and crossing a driver line containing cell-specific transcriptional activators to an effector line to get expression of a GCaMP in neurons of interest. When intracellular calcium levels rise, these genetically-encoded calcium indicators increase in fluorescence intensity so that the level of fluorescence is correlated with changes in neuronal activity[24,25].

Here, a method for using calcium imaging to observe neural responses to taste stimuli *in vivo* is described. The overall goal of this method is to stimulate only the labellar GRNs to quantify taste-induced neural responses in the brains of awake flies. Examples are provided for using this method to record responses in the primary GRNs of the labellum in *D. melanogaster*, and the benefits and challenges of using this approach are discussed. This preparation was developed to allow experimenters the ability to apply a tastant solution to an immobilized fly labellum while under a confocal microscope to record neural responses when the entire sensory organ is immersed in a solution, which occurs in natural settings. The *in vivo* calcium imaging approach described here can be used to uncover novel tastant-receptor interactions[8,14,26,27], temporal details of GRN responses[27,28], molecular mechanisms of GRN modulation[29,30], and taste processing in downstream circuits[8,18,19,20,28,31].

Protocol

The details of the reagents and the equipment used in this study are listed in the **Table of Materials**.

1. Preparation of adult hemolymph-like (AHL) solution

1. Prepare a stock solution containing 108 mM of NaCl, 5 mM of KCl, 4 mM of $NaHCO_3$, 1 mM of NaH_2PO_4, 5 mM of HEPES, and 15 mM of ribose.

   NOTE: Ribose is used as a non-energetic sugar to maintain osmolarity without altering nutrient levels in the brain.

2. Adjust the pH of this solution to 7.5 before filtering and storing at 4 °C. Check the osmolarity of the AHL after adjusting the pH to ensure consistency across preparations.

3. Prepare separate stocks of 1 M of $CaCl_2$ and 1 M of $MgCl_2$, filter, and store at room temperature.

4. To prepare an aliquot of the main AHL stock, add a small volume of the calcium and magnesium to get

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final concentrations of 2 mM of calcium and 8.2 mM of magnesium. This AHL can be stored at 4 °C and used for up to one month, using small aliquots brought to room temperature for experiments.

2. Mounting flies on the imaging chamber

1. Prior to mounting flies, sharpen the tip of a dental waxer into a small, pointed cup using a sharpening stone (**Figure 1D**). Attach the sharpened tip to the waxer and turn it on to preheat. Heat settings will depend on the type and length of the tip: a minimum temperature that allows the wax to stay melted upon contact is needed (50.5 °C works in this example).
   NOTE: A wire can be wrapped around the end of the dental waxer to affix a sharpened metal tip as an alternative.
2. Gently anesthetize 1-5 flies (following institutionally approved protocols). Minimize anesthesia exposure time, as extended periods of exposure to CO_2 or cold can impact behavior. For CO_2, use a fly pad that provides a continuous, even flow of 99.9% CO_2 at a rate of 5 L/min under a dissection microscope.
3. Use dissection scissors to remove portions of the legs by cutting the middle and hind legs at the femoral/tibial joint and the forelegs at the trochanter. Use blunt forceps to help manipulate the fly. Trimming the tarsi will prevent tarsal sensation and kicking of the coverslip or taste stimulator.
4. Pick up the fly by the wings using blunt forceps to position the fly so the head is above the targeted cervix slot of the imaging chamber, but the body is below. It is helpful to start the fly fully to the right or left. Using the blunt side of the scissors and blunt forceps, gently push the head and thorax simultaneously into the slot.
5. Once securely inside the slot, push the fly to the back of the slot and gently reposition it so that the fly faces the front of the chamber. Avoid rotating the head too far out of alignment with the thorax.
6. Repeat for as many flies as needed (this imaging chamber can mount up to 5 flies).
7. Gather a small droplet of nail polish on the end of a toothpick and apply a thin coat to secure the head of the fly to the imaging chamber.
   NOTE: the precise area to apply the nail polish depends on what part of the brain is being imaged. If imaging the SEZ (as shown here) or other inferior medial regions, nail polish can be applied generously to the top of the fly head, but to image superior medial regions, nail polish can be applied minimally to the top of the head and added laterally near the eyes to leave this area clear for dissection. This protocol is optimized for taste stimulation only without olfaction, but if nail polish volatiles are a concern, use wax or UV glue as alternative methods for securing the fly head.

3. Waxing the proboscis in an extended position

1. Pick up the waxer with one hand and gather a small droplet of wax on the tip.
2. On the other hand, use semi-sharp forceps to grab one maxillary palp and gently pull out and hold the proboscis in full extension.
   NOTE: Be careful to grip only the maxillary palp as pinching the cuticle on the proboscis increases the potential for damage. Do not proceed if the proboscis is pinched or the proboscis cuticle is punctured. A small fly

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pooter may be used as an alternative to forceps to pull out the proboscis using suction while applying the wax.

3. Touch the tip of the waxer to the chamber near the base of the proboscis until the wax starts to flow, then move to make contact with the base of the proboscis. Wax halfway down the shaft, but avoid touching the labellar sensillae with the wax or waxer. The wax on this side will hold the proboscis in place. Do not proceed if the sensillae are touched by the wax or waxer at any time.
4. Apply wax using the method above for the other side, making a continuous bridge of wax over the proboscis.
5. Fully extend the proboscis as straight as possible. If needed, moving the proboscis can be done by reheating the wax and gently pushing the proboscis into the desired position.
6. Repeat for other flies to be mounted in the same chamber.
7. Turn off CO_2 or remove flies from ice anesthesia. Place mounted flies into a humidity chamber for 60 min to recover (clean, empty pipette tip box with wet, lint-free wipes).

4. Dissection to reveal the brain region of interest

1. Remove the flies from the humidity chamber. Flies must be clearly alive, actively moving their abdomen, legs, and antennae. Set up the confocal or two-photon microscope prior to dissection.
2. Turn the waxer on to repair potential breaks in the wax during the dissection and prepare room temperature AHL.
3. Using very sharp forceps, pinch off both antennae, then pinch the cuticle to provide a hole for inserting one side of the sharp forceps. Run the forceps under the cuticle to remove it from the region covering the brain area of interest. **Figure 1F** indicates an X over areas of the cuticle to target with the forceps for removal.
4. Wash the exposed brain in AHL by generously applying AHL (~100 µL) to the head and then removing all but a thin layer of AHL to prevent the brain from drying out.
   NOTE: If the wax breaks at any point and needs to be repaired, briefly remove all AHL from around the head before reheating the wax to resecure the proboscis in the extended position.
5. Using sharp forceps, remove air sacs and any large debris covering the brain. Avoid penetrating the brain by keeping the tips of the forceps visible.
6. Wash with AHL ~3 times to remove all small debris.
7. Ensure the brain region of interest is clearly visible. To specifically image the subesophageal zone (SEZ) as in this example, cut the esophagus at the base near the proboscis and near the point where it passes through the brain by pinching with very sharp forceps and removing this piece to expose the SEZ.
   NOTE: Flies cannot ingest solutions after the esophagus is removed, and no pharyngeal GRN activation can occur.
8. Under the dissection microscope, position the 10 mm x 20 mm coverslip into the angled slot of the imaging chamber. Ensure it rests at the base of the proboscis without breaking the wax. The tip of the labellum must not touch the coverslip.

5. Imaging and taste stimulation

1. Turn on the confocal or two-photon microscope and be ready for image capturing. Set up a micromanipulator

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with a capillary tube that is positioned to deliver the tastants to the fly while they are on the microscope stage.
NOTE: Two-photon imaging can capture fluorescence from neurons that are deeper in the brain tissue.

2. Load ~2 µL of water (or another negative control) into the capillary tube of the
stimulator on the microscope stage.

3. Find the dissected fly and focus on the labellum using 10x air immersion brightfield. Align the capillary with the labellum under this view. Additional cameras pointed at the fly's labellum can be included to view the alignment of the capillary with the fly from multiple angles.
NOTE: Ensure that all labellar sensilla are being stimulated with the solution during alignment; if the position of the stimulator or labellum is not perpendicular enough for this, adjust accordingly. Capillaries can be pulled and filed down with a sharpening stone to provide a tighter fit around the labellum.

4. Leave the capillary positioned directly in front of the labellum, close but not touching.

5. Move the stage so that the brain region of interest is centered and switch to a higher magnification, water immersion objective (40x used in this example).

6. Add approximately 200 µL of AHL on top of the brain to make contact with the objective for immersion. Remove any bubbles.

7. Orient the focus using brightfield to carefully move in the z-plane to find the edge of the cuticle that was removed and center the brain region of interest.

8. Switch to 488 nm laser power to find GCaMP expression in the area of interest.
NOTE: Depending on the driver line and the version of GCaMP used, some initial optimization may be needed to amplify the signal-to-noise for individual preparations. Co-expressing RFP can be helpful for neurons with low baseline GCaMP fluorescence.

9. Prepare a timelapse image collection. The speed will depend on the specific microscope and GCaMP signal, but capturing at least one image every ~100 ms is optimal.
NOTE: Capturing a single Z-plane of fluorescence over time will optimize the capture speed to provide detailed calcium kinetics. Image stacks taken at multiple Z planes at each timepoint may slow capture rates, but will record responses across neurites that are at different depths in the tissue.

10. After collecting at least 5 s of baseline fluorescence, manually move the stimulator so that the capillary covers the labellum for a specific amount of time (5 s in this example), then remove the stimulus and capture for as long as desired.

11. Remove the AHL and return to 10x brightfield to ensure the coverslip, stimulator, and labellum are still in the same position.
NOTE: If the stimulator is not aligned well with the labellum or is rotated too far, the labellum can be moved, or the capillary can hit the imaging chamber, potentially breaking the wax and creating a leak from the AHL.

12. Remove the imaging chamber. Use a lint-free wipe to remove the first solution and flush the pipette with water. Then, pipette ~2 µL of the next tastant into the capillary tube.

13. Move the imaging chamber back to the stage and repeat steps 5.4-5.12 for this solution.
NOTE: The high surface tension of most tastants presents residual chemicals from staying on the fly

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labellum. However, if tastants are highly saturated or viscous, water can be moved over the labellum several times to wash the sensory organ before the next stimulus.

14. Repeat for as many solutions as desired for this fly.
15. Return to step 3 to prepare the next fly for imaging.

6. Image analysis

1. Open the image stack to be analyzed in image processing software. If necessary, perform a background subtraction using a region outside of the GCaMP signal.
2. Select a tight region of interest (ROI) around the projections to be quantified using the freehand or shape tool and ensure the selection is applied to all images in the stack.
3. Generate a list of the fluorescence readings over time for this ROI and export it to a spreadsheet for further processing. Use the **plot Z-axis profile** in ImageJ (FIJI) or a similar function in the image processing software of choice.
4. In the spreadsheet, select 10 consecutive timepoints during the baseline recording in step 5.10 as a representative baseline. Calculate the mean and standard deviation.
5. To get ΔF/F as a percentage, calculate ((F - mean baseline F)/baseline mean F)*100) for each timepoint. To get ΔF/F as a z-score, calculate ((F - mean baseline F)/ standard deviation baseline F) for each timepoint.
6. To calculate the peak change in fluorescence during stimulation, select 3 consecutive points with the highest fluorescence and calculate the mean.
7. Repeat for each image stack across stimuli and flies.

NOTE: Adapt these steps as needed based on the specific microscope, signal quality, and preferred image analysis software.

Representative Results

Figure 1 provides details of the imaging chamber (**Figure 1A,B**) and waxer tip (**Figure 1D**) used in this preparation. **Figure 1** also illustrates the main steps of the procedure for mounting flies (**Figure 1C**), waxing the proboscis into place (**Figure 1E**), dissecting over the brain region of interest (**Figure 1F**), and stimulating the labellum with a tastant while recording fluorescence in the brain (**Figure 1G**). To quantify taste-induced responses in primary gustatory receptor neurons (GRNs) *of Drosophila melanogaster* flies with *Gr64f-Gal4* driving expression of *UAS-GCaMP6f* were produced to get the calcium indicator genetically expressed in all sugar-sensing "sweet" GRNs of the labellum[14,27,30,32,33,34,35]. For these experiments, a confocal microscope with the following components was used: an upright fluorescent microscope with 40 fps sCMOS camera, 10x and 40x objectives, spinning disk confocal, dichroic 488 emitters, and 488 nm solid-state lasers. The 40x objective was immersed in AHL and centered on the SEZ brain region to locate the baseline GCaMP signal in the axon terminals of these labellar GRNs (**Figure 2A**). A fluorescence image was captured every 100 ms during baseline (no stimulation), during 5 s of taste stimulation (stimulator moved over the labellum), and after stimulation until the fluorescence returned to baseline (**Figure 2A,B**). Water was used as a negative control, and 1 M sucrose was used as a positive control. The relative change in fluorescence was calculated as ΔF/F (z-score) for 13 flies and plotted over time to show the kinetics of the calcium responses during taste stimulation (**Figure 2B**). The peak ΔF/F (z-score) was plotted and used for statistical

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comparisons to indicate that the sucrose response in these cells is significantly higher than in water (**Figure 2C**). This technique captures that "sweet" GRNs have a strong peak upon sucrose onset that remains high with some decay over the stimulation period.

For comparison, this protocol was repeated in flies with a different driver, *Gr66a-Gal4*, expressing *UAS-GCaMP6f* specifically in all "bitter" GRNs on the labellum[14,17,28,34,36]. Similarly, the axon terminals of these GRNs were located in the SEZ: notice the projection pattern is distinct from the sugar-sensing GRNs (**Figure 2D**). The fluorescence was captured and analyzed as before, except for 100 mM caffeine, which was used as a positive control. The curve averaged from 11 flies shows a strong peak with the onset of caffeine stimulation, but there is also a small "off" response with stimulus removal that is known to occur with certain bitter stimuli[28] (**Figure 2E**). This method allows for both "on" and "off" responses to be quantified to characterize the temporal patterns of taste-induced responses[27,28]. Here, only the "on" peaks were quantified to indicate the response to caffeine is significantly stronger than water (**Figure 2F**). The experiments in **Figure 2** are highly reproducible and can be used to ensure the protocol is working properly.

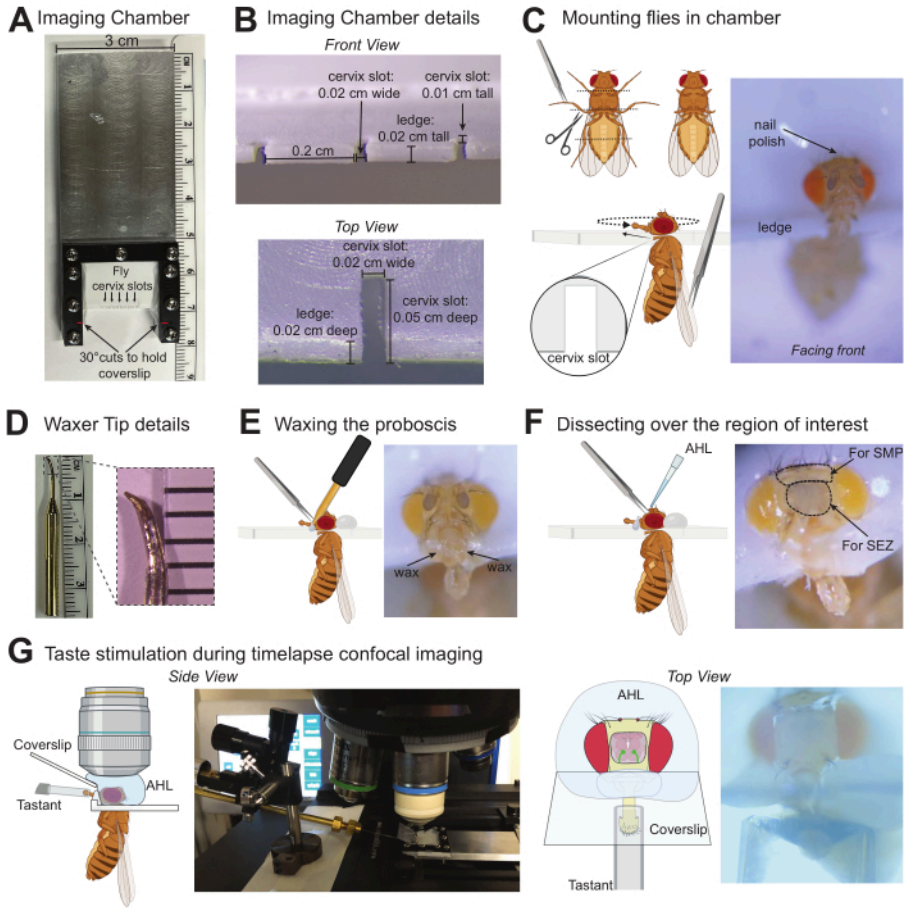

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Figure 1: Protocol illustrations for imaging taste-induced responses in the *Drosophila* brain. (**A**) Top view of the custom imaging chamber used to mount up to five flies at a time. (**B**) Details of the imaging chamber where the flies are mounted with measurements that comfortably fit the cervix of *D. melanogaster*. (**C**) Graphics indicating where to trim the tarsi (top left) and how to mount the fly into the cervix slot of the imaging chamber using forceps (bottom left). Photo of a mounted fly in the correct position in the imaging chamber (right). (**D**) Photo of the waxer tip (left), zoomed photo of the tip to indicate the approximate shape and size to target when using a sharpening stone to modify the standard tip (right). (**E**) Graphic illustration of waxing the proboscis into place using forceps (left), photo of a fly mounted with a properly waxed labellum (right). (**F**) Graphic illustration representing the dissection over the brain region of interest and application of AHL (left), photo of a fly with dotted circles around the area of the cuticle to remove when targeting the SEZ or SMP brain regions. X indicates regions of the cuticle to pinch for dissection (right). (**G**) Graphics and photos indicate the position of the mounted/dissected fly, the water immersion objective in AHL, the stimulator with a tastant over the proboscis, and the coverslip forming a barrier between these solutions. The side view zoomed out (left), and the top view was under the 10x objective (right). Please click here to view a larger version of this figure.

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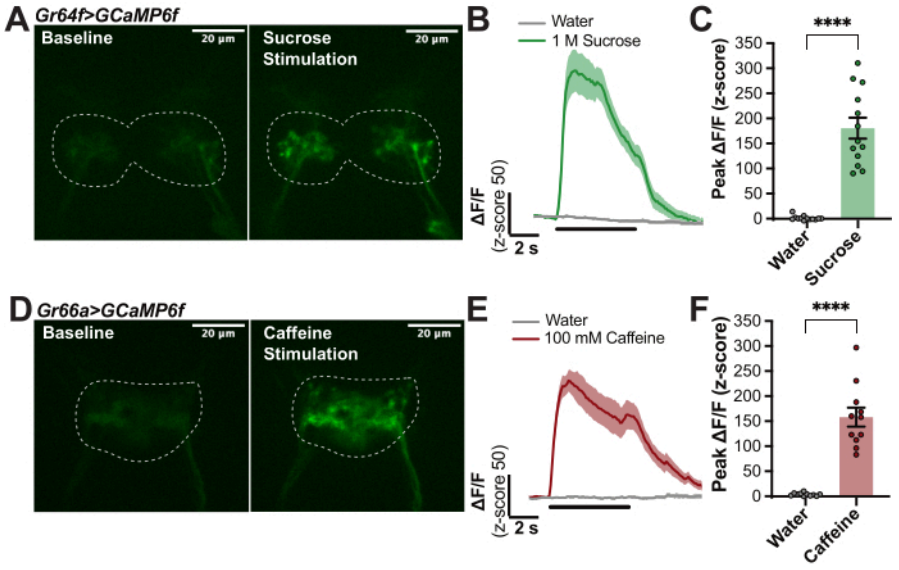

Figure 2: Example of calcium responses of labellar GRNs to taste stimuli. (**A**) Still captures from an image stack indicating the level of GCaMP fluorescence in a fly with *Gr64f>GCaMP6f* at baseline and during the peak response to 1 M sucrose, scale bar = 20 µm. The dotted lines indicate the ROI for analysis. (**B**) Calcium response curves for n = 14 flies calculated as ΔF/F (z-score) and combined for water (negative control) and 1 M sucrose (positive control) to show kinetics; the black line under curves indicates when the stimulus is over the labellum. (**C**) Peak ΔF/F (z-score) for each fly plotted for statistical comparisons. Paired t-test, ****$p < 0.0001$. (**D**) Still captures from a video indicating the level of GCaMP fluorescence in a fly with *Gr66a>GCaMP6f* at baseline and during the peak response to 100 mM caffeine, scale bar = 20 µm. The dotted lines indicate the ROI for analysis. (**E**) Calcium response curves for n = 11 flies calculated as ΔF/F (z-score) and combined for water (negative control) and 100 mM caffeine (positive control) to show kinetics: notice the small "off" response, black line under curves indicates when the stimulus is over the labellum. (**F**) Peak ΔF/F (z-score) for each fly plotted for statistical comparisons. Paired *t-test*, ****$p < 0.0001$. Please click here to view a larger version of this figure.

Discussion

One of the most challenging aspects of this protocol is the micromanipulation dexterity needed to wax the labellum and perform the targeted dissections. An additional step to secure the labellum is necessary to stimulate each sensillum evenly across this sensory organ and visualize brain regions of interest. The custom imaging chamber used here is optimized for *D. melanogaster*, but the specifications of the chamber and the waxing approach may need to be modified for other insects. This protocol can be applied to other *Drosophilids* with little modification, but other members of the Brachycera suborder, such as bees and mosquitoes, may require changes to the mounting and dissection steps to account for differences in labial palp and head morphology. Alignment of the micromanipulator for the tastant delivery can also be challenging and requires initial testing with the specific microscope stage for optimization. If the wax is broken during

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the stimulation, it can result in leaks whereby the AHL and tastant in the capillary make contact. Pulling the capillaries and filing them down with a sharpening stone to fit more closely to the labellum can help prevent the tastant and AHL from making contact. Flies with any leaks or excessive brain movement must be excluded. When possible, always include a positive control for each animal to ensure the labellum and labellar nerves are not damaged from the waxing or dissection. The "sweet" and "bitter" examples shown here are recommended as robust control experiments.

The *in vivo* calcium imaging approach described here has been used to quantify taste-induced responses in primary taste neurons, higher-order neurons, and the whole SEZ in *D. melanogaster* to identify gustatory receptors and circuits[8,14,17,18,19,20,27,28,30,31,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48]. The widespread applications in this model organism are due to the readily available Gal4 and split-Gal4 drivers; thus, the need for genetically modified insects to get GCaMP expressed in specific neurons of interest is one limiting factor for this approach. Luckily, with advances in gene editing technology, this is becoming more accessible for insects beyond model organisms, and taste-induced responses using calcium imaging have recently been reported for the pest *Drosophila suzukii*[49] and for vector-carrying mosquitoes[50]. As with all calcium imaging, some initial optimization of signal-to-noise may be necessary for the targeted neurons of interest. Signals can be enhanced by using brighter versions of GCaMP and by expressing two copies of GCaMP. Co-expressing RFP in target neurons can help to visualize the target neurons at baseline and can serve as a control for brain movement in regions that have a propensity to pulsate.

This protocol is specifically designed to isolate chemosensation from the labellum by removing the tarsi and antennae, waxing over the maxillary palps, and limiting ingestion so that no pharyngeal GRNs are stimulated. However, adjustments to this protocol can be made to include chemosensation from tarsal or pharyngeal GRNs. If the tarsi are left intact, the legs can be stimulated alone or in addition to the labellum by creating a large bubble of tastant solution at the end of the capillary. There is the potential for a fly to kick and move the coverslip if the tarsi are left intact; therefore, waxing the tarsi near the base can be considered to help prevent unwanted movements. The current example includes the step of cutting the esophagus to avoid pharyngeal GRN stimulation and to better visualize labellar projections in the SEZ, but this same preparation has been previously adapted to quantify pharyngeal GRN responses by leaving the esophagus intact and imaging lateral pharyngeal projections[36]. This previous application used an appetitive sugar stimulus, which flies will freely consume to stimulate pharyngeal GRNs, but flies will not readily consume an aversive stimulus to activate bitter pharyngeal GRNs, which is one limitation of this approach. An additional limitation is that the responses of GRNs located in the wings of *Drosophila*[11] cannot be readily studied with this approach.

While the *in vivo* calcium imaging described here has become the standard method for studying higher-order taste-induced responses[8,18,19,20,28], there are currently several other approaches for quantifying primary labellar GRN responses to tastants in flies. The *in vivo* calcium imaging approach described here records GCaMP changes in the axon terminals in the brain, but an *ex vivo* approach has also been used to quantify cell body GCaMP in labellar GRNs[33]. Similarly, another mounting approach

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has been described for imaging the cell bodies of either labellar or tarsal GRNs in intact flies[51]. Electrophysiology continues to be a popular and effective technique for studying the responses of primary taste neurons in insects[13,16,32,52,53,54,55,56,57,58,59,60,64,62,63,64,65]. This does not require the need for genetically encoded calcium sensors and is a more direct quantification of neuronal activity. However, responses from only one sensilla can be recorded at a time whereas calcium imaging can record from a full population of GRNs simultaneously. The calcium imaging approach was used to discover the unique temporal dynamics of "on" and "off" responses in GRNs with certain stimuli[27,28], but a recent advancement in electrophysiological recordings from the base of taste sensilla in *D. melanogaster* now allows for "off" responses to be quantified at the level of action potentials[53]. Interestingly, the modulation of primary GRN sensitivity by hunger was detected via calcium imaging but not at the level of action potentials with electrophysiology[29], yet both electrophysiological tip recordings and calcium imaging can capture a change in GRN sensitivity with diet[30,66]. Thus, electrophysiology remains an important, complementary approach to calcium imaging for identifying taste ligands and receptors and for understanding how various factors modulate the sensitivity of primary gustatory receptor neurons.

Disclosures

The authors have no conflicts of interest and nothing to disclose.

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