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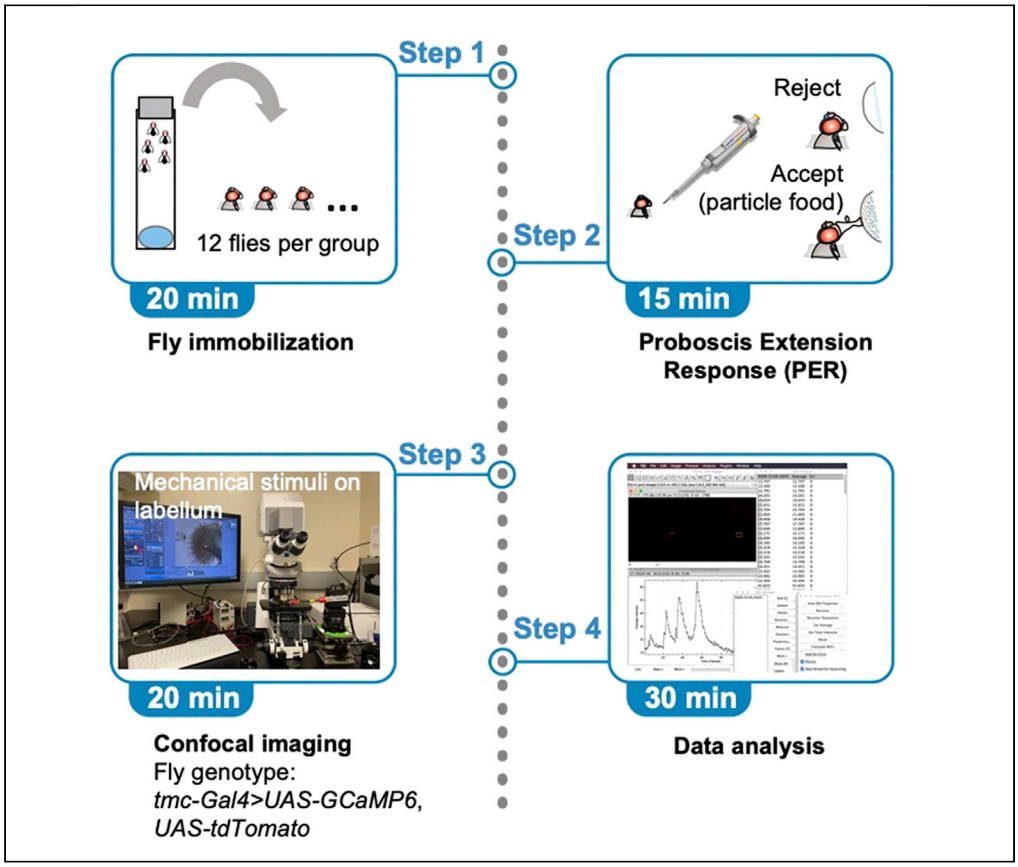
Drosophila proboscis extension response and GCaMP imaging for assaying food appeal based on grittiness



Fruit flies sense the features of food that are driven by particle size, including smoothness versus grittiness, by deflection of sensilla decorating the labellum, and md-L neurons. We describe adaptation of the *Drosophila* proboscis extension response assay, including steps to perform the taste tests and score behavioral responses, to determine preferences to foods with different sized particles. We also describe calcium imaging in GCaMP-expressing flies to assess the responses of md-L neurons to different levels of taste sensilla deflection.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Behavioral assay to score a fly's preference for smooth versus gritty food

Precise indentation of the fly labellum to mimic taste hair deflection by gritty food

Ca^{2+} imaging to assess responses of md-L neurons to deflection of taste hairs

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Drosophila proboscis extension response and GCaMP imaging for assaying food appeal based on grittiness

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SUMMARY

Fruit flies sense the features of food that are driven by particle size, including smoothness versus grittiness, by deflection of sensilla decorating the labellum, and md-L neurons. We describe adaptation of the *Drosophila* proboscis extension response assay, including steps to perform the taste tests and score behavioral responses, to determine preferences to foods with different sized particles. We also describe calcium imaging in GCaMP-expressing flies to assess the responses of md-L neurons to different levels of taste sensilla deflection.
For complete details on the use and execution of this protocol, please refer to Li and Montell. (2021).

BEFORE YOU BEGIN

Stock food preparation

Timing: 10–30 min

1. Prepare stock sucrose solution, 500 mM.
   a. Dissolve 34.2 g sucrose in 200 mL Milli-Q water, filtered with a 0.22 μm membrane.
   b. Store in 1.7 mL tubes at −20°C. The frozen samples can be used for at least one year.
2. Prewash silica particle powders.
   a. Place ~0.2 g silica powder in a 1.7 mL tube, and add 1 mL Milli-Q water.
   b. Gently rock the tube for 10 min on a 3D rocker, briefly spin the tube in a microcentrifuge, and remove the clear supernatant. Repeat 3 times.
   c. Dehydrate the particles in the tube with the lid open in a desiccator at room temperature for 2–3 days.

Alternatives: Milli-Q water can be replaced by standard distilled water.

Starve flies for the proboscis extension response (PER) assays

Timing: 5 min for transferring flies

Timing: 2–3 h for starvation

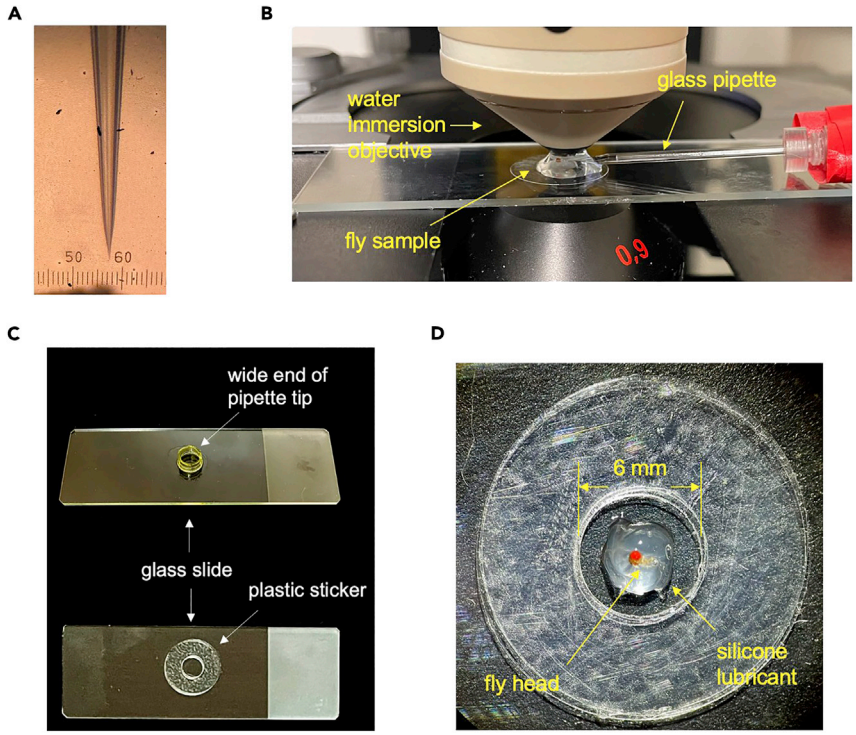

Figure 1. Preparation for performing calcium imaging on a fly head

(A) Pulled glass pipette tip. The space between the vertical lines is 10 μm, and 100 μm between 50 and 60.

(B) An example of the setup for performing calcium imaging.

(C) Glass slide with the wide end of a 200 μL pipette tip (top) and a glass slide with two layers of round punched stickers (bottom).

(D) Image of the plastic stickers, grease, and a fly head loaded in the center of the well.

3. Flip 3–5 days-old flies into a vial (30–50 flies per vial) containing a wet tissue (e.g., Kimwipe) in the bottom.
   a. Starve the flies (typically 2–3 h) before testing.

   Note: Longer starvation times increase the motivation to feed, thereby reducing the influence of particle size on food appeal.

   ⚠ CRITICAL: Following eclosion, maintain the age-matched flies in fresh food vials (see key resources table).

Prepare the glass tip to mechanically stimulate the labellum for the calcium imaging experiments

Timing: 10 min

4. Pull glass needles to create tips that will be used to push the cuticle of the labellum, or to deflect a taste hair. The needle should have an initial diameter of 1–3 μm, and a short (~3 mm) taper (Figures 1A and 1B).
   a. To achieve the correct diameter and taper using a Sutter Instruments P-97 puller, set the program on the puller as follows: melting temperature of the glass needle plus 10°C (ramp + 10°C), pull force (pull 1), velocity of the pull (velocity 20), time setting for cooling (time 250).
   b. Gently tap the tip in a vertical position on a piece of paper to break and widen the tip, achieving an opening of ~20–40 μm.

Note: The needles can be used on more than one fly. However, prepare one per fly so there are extra in case they break.

Note: See Figures S5D–S5H (Li and Montell, 2021) for deflection of the cuticle by a glass tip.

Prepare chambers for the calcium imaging experiments

Timing: 30 min

5. Prepare a circular plastic chamber by cutting ~3 mm from the wide end of a 200 μL pipette tip (Figures 1C and 1D).

   Note: Prepare 5–10 chambers so that extras are available if a chamber breaks or is contaminated with debris.

6. Place the 3 mm wide end of the tip on a glass slide with wet nail polish to immobilize the chamber. Let the nail polish dry.

   Alternatives: Stick two layers of circular plastic stickers on a glass slide to make a small well (Figures 1C and 1D).

1× PBS preparation

Timing: 10 min

7. Add 5 mL 10× PBS (Thermo Scientific™ Phosphate-buffered saline; PBS, 10×, pH 7.4) to 45 mL Milli-Q water to make a 1× PBS solution. Store at room temperature for up to 4 weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sucrose	Sigma-Aldrich	Cat # S0389
Monodisperse silica microspheres, diameter 9.2 μm	Cospheric, Co.	Cat # SiO2MS-2.0 9.2 μm
10× PBS	Fisher Scientific	Cat # AAJ62036K2
Agar	Genesee Scientific	Cat # 66-104
Dry yeast	MP Biomedicals	Cat # ICN90331280
Soy flour	Genesee Scientific	Cat # 62-115
Cornmeal	LabScientific	Cat # NC0535320
Corn syrup	Genesee Scientific	Cat # 62-109
Ethanol	Gold Shield	Cat # 1016-4233
Tegosept	Genesee Scientific	Cat # 20-259
Propionic acid	Acros Organics	Cat # 200001-092
Phosphoric acid	Sigma-Aldrich	Cat # 438081-500MI
Experimental models: Organisms/strains		
Male and female *Drosophila melanogaster* (3–5 days old) w^{1118}	Bloomington Drosophila Stock Center	RRID:BDSC_3605FBst0003605 Cat # BL5905
Male and female *Drosophila melanogaster* (3–5 days old) *tmc-Gal4*	(Zhang et al., 2016)	RRID:BDSC_66557 Cat # BL66557
Male and female *Drosophila melanogaster* (3–5 days old) *UAS-GCaMP6f*	Bloomington Drosophila Stock Center	Cat # BL42747 Other GCaMP fly lines can also be used.

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Male and female *Drosophila melanogaster* (3–5 days old) *UAS-tdTomato*	Bloomington Drosophila Stock Center	Cat # BL36327
Software and algorithms		
ZEN SP5	ZEISS	N/A
Fiji	(Schneider et al., 2012)	https://imagej.net/software/fiji/downloads
Prism 8	GraphPad	https://www.graphpad.com/guides/prism/8/user-guide/index.htm
Linlab 2 software	Scientifica	https://www.scientifica.uk.com/products/scientifica-linlab-2
Other		
Motorized micromanipulator	Scientifica PatchStar	N/A
Microcentrifuge Model 5418	Eppendorf	Cat # 05-401-201
Milli-Q Reference Ultrapure water purification system	MilliporeSigma	N/A
Glass capillaries	World Precision Instruments	Cat # 1B150F-3
Silicone lubricant	Dow Corning	Cat # DC976
Bel-Art™ SP Scienceware™ Space Saver Vacuum Desiccator	Fisher Scientific	Cat # S43284
Parafilm	Fisher Scientific	Cat # S37440
Rubber tubing (soft)	6 mm ID, 8 mm OD.	Ordered on Amazon. Use any brand of soft rubber tubing with the appropriate size to connect to a 1 mL pipette tip to make the air duct for aspirating flies.
Cotton wool	Genesee	Cat # 51-101
Vannas Spring Scissors	Fine Science Tools	Cat # 15000-04
Forceps	Fine Science Tools	Cat # 11295-10
W Plan-Apochromat 20× 1.0 NA water-immersion objective	ZEISS	Cat # 421452-9800-000
Upright confocal microscope	Zeiss LSM700	N/A
3D rocker	Benchmark Scientific	B3D 1308
Self-adhesive hole reinforcement stickers, 1/4″ diameter hole punch reinforcements	Every	N/A
Gardens nail enamel, clear water base	Honeybee	N/A
Micropipette puller	Sutter Instruments	Cat # P-97
Pipette tips 10 µL	Olympus Reach Tip	Cat # 24-121RL
Pipette tips 200 µL	Olympus Reach Tip	Cat # 24-150RL
Pipette tips 1000 µL	Olympus Reach Tip	Cat # 24-165RL
Kimwipes	Fisher Scientific	Cat # 06666A
Centrifuge tubes 1.7 mL	Thomas Scientific	Cat # 1159M35
Olympus 0.6 mL microtubes	Genesee	Cat # 22-272

MATERIALS AND EQUIPMENT

Fly food recipe		
Reagent	Final concentration	Amount
Agar	~8.3 g/L	380 g
Dry yeast	~16.5 g/L	758 g
Soy flour	~9.6 g/L	442 g
Cornmeal	~70.0 g/L	3,218 g
Corn syrup	~69.0 g/L	3,173 g
Ethanol	~1.0%	480 mL
Tegosept	~3.1 g/L	144 g
Propionic acid *	~0.47%	216 mL
Phosphoric acid *	~0.037%	17 mL

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
H_2O	N/A	45 L
Total	N/A	~46 L

The fly food can be stored for 2–3 days at room temperature, or up to 10 days at 10°C.

⚠ CRITICAL: *Pure Propionic acid and phosphoric acid are corrosive to the skin and eyes.

STEP-BY-STEP METHOD DETAILS

Part 1: Proboscis extension response assay

Prepare fresh particle food

Timing: 5 min

This section describes the preparation of the particle food solution for the following behavioral tests.

1. Prepare a 50 mM sucrose solution from a 500 mM sucrose stock solution by adding 950 µL water to 50 µL 500 mM sucrose solution.
2. Weigh the silica microsphere powder (~2–4 mg).
3. Transfer the particles to a 0.6 mL microfuge tube.
4. Add 50 mM sucrose solution (20–40 µL depending on exactly how much silica is weighed out) to make a 10% w/v particle solution.
5. Mix by pipetting with a 10 µL pipette tip for 5 s.

Note: Seal the tube with parafilm, store at room temperature, and use within 3 days of preparation.

Trapping flies in a pipette tip

Timing: 20–30 min

This section describes how to prepare flies for the taste assays.

The PER assays are performed after immobilizing the flies in a pipette tip as described below and demonstrated in Methods video S1. The 20–30 min is the time required to immobilize ~24 flies (~12 flies/group). The PER assays are performed after the fly preparations are done. Repeat steps 7-12 as needed depending on how many flies are to be tested. Beginners may take ~2 min to prepare one fly. After practice, this typically requires only 30–60 s per preparation.

6. Attach a 1 mL pipette tip to soft rubber tubing with the narrow side pointing out.
   a. Trim the narrow side of the 1 mL tip slightly with a razor blade to allow an individual fly to be introduced (Figure 2A; Methods video S1).
   b. Attach another 1 mL pipette to the other side with the wide end sticking out. This latter end is used to mouth aspirate (Figure 2A).
7. Aspirate a fly into the 1 mL pipette tip (Figure 2A).
8. Attach a 200 µL tip to the end and expel the fly into the 200 µL pipette tip.
   a. Gently shake the pipette tip downward while blowing out the air to trap the fly in the end of the tip with the fly head towards the open end (Figure 2B).

Note: The head should be slightly loose.

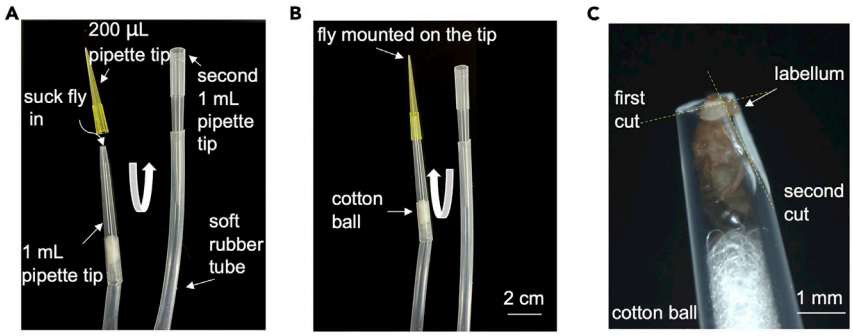

Figure 2. Introducing a fly into a pipette tip for performing PER assays

(A) Apparatus for aspirating a fly into a 1 mL pipette tip.

(B) Aspirate the fly into a 200 mL pipette tip.

(C) A fly immobilized for performing a PER assay.

9. Using a razor blade, make a horizontal cut to remove any extra tip material above the fly head (first cut, Figure 2C).
10. Draw the fly back slightly from the end of the 200 µL by gentle inhalation, before introducing a second cut at an angle (Figure 2C),
11. Expel air to push the fly head a little out over the open end of the tip so that the labellum only is exposed.
    a. Remove the 200 µL tip from the apparatus.

Note: The legs should be hidden inside of the tip.

12. Insert a very small cotton ball with a sharp wooden stick under the fly to immobilize it (Figure 2C).

Note: It may be necessary to try a second or third fly to obtain a fly with the proper orientation. Do not jam the fly too hard at the end of the 200 µL tip.

Note: Place each individual fly preparation on a wet paper towel to keep the fly hydrated if you do not use the fly immediately.

Note: If the flies are properly fixed, they will survive overnight in a humidified chamber (a tip box containing wet paper, e.g., a Kimwipe). However, it is best to use the flies right away.

Alternatives: The sharp wooden stick (step 12) can be replaced with a 10 µL pipette tip overlaid with the top half of a second 10 µL pipette tip, which can reach the inside tip of the 200 µL pipette.

⚠ CRITICAL: To avoid damaging the fly labellum, be careful to inhale gently to withdraw the fly slightly from the end of the 200 µL pipette (step 7).

Proboscis extension response assay

Timing: 15 min (for step 13–18)

This section describes how to perform the taste tests and score the behavioral responses.

13. Saturate the fly with water before performing a PER assay with sugar/particle food.
    a. To test if each fly is water saturated, offer each fly a drop of water at the end of a 10 µL pipette tip.

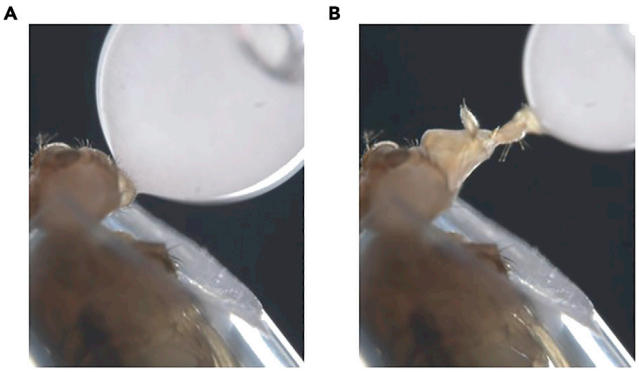

Figure 3. Images of fly responses to particle food
(A) Particle food contacting the labellum.
(B) Fly extending its proboscis to ingest particle food.

Note: Wild-type and w^{1118} flies usually do not drink upon water application. ~10% of the flies drink water ≤5 s and then do not drink the second offering of water.

Note: Flies that drink water >5 s are discarded (typically <1% of the flies tested).

14. Mix the particle food in the 200 μL microfuge tube by pipetting for 3 s.
15. Withdraw 2 μL of the particle food into a 10 μL pipette tip. Pipette the particle food in and out of the tip 2–3 times to mix the contents.
16. Make contact between the labellum and the drop at the end of the tip (Figure 3A). The contact time is ~0.2–0.6 s. The liquid drop should be quickly withdrawn from the fly to allow full extension of the proboscis (Figure 3B).
17. As a control, perform PER assays using 50 mM sucrose alone with a parallel group of flies.
18. Test all the flies with 500 mM sucrose alone. Flies that do not respond should be discarded.
19. Score the responses as follows:
    a. 1—The fly fully extends its proboscis and consumes food for ≥1 s (Figure 3; can see the esophagus moving).
    b. 0.5—The fly extends its proboscis and consumes food for <1 s.
    c. 0—The fly fails to extend its proboscis.
20. Each trial (n=1) includes ≥ 12 flies.

Note: Based on the effect size comparing control flies and *tmem63* mutants (Li and Montell, 2021), ≥8 flies are required. For comparing other mutants with control flies, we recommend ≥ 12 flies in case the effect size is smaller.

21. The PER index is calculated as:
    a. PER = (sum of the scores)/(number of flies tested)

Repeat the experiments 5 times on different days.

Note: See below for more information on how to calculate and display the PER data (expected outcomes; Figure 9, Table 1).

Part 2: Calcium imaging of md-L neurons

When the fly proboscis contacts liquid food, ~3–4 L-type hairs per labium are deflected. The bending angles of the hairs cause curvature at the base of multiple sensilla and induce mechanical stimulation of md-L neurons. The angle of deflection of the hairs by food mixed with particles is larger compared with clear food. In this assay, we push the cuticle to deflect multiple hairs with

various degrees of intensity to mimic the force on the labellum conferred by the grittiness in the food. Refer to Li and Montell (2021).

Genetic crosses

Timing: 2 weeks (for step 22)

This section describes the genetic crosses to obtain flies for performing calcium imaging on md-L neurons.

22. Cross *tmc-Gal4* flies with the *UAS-GCaMP6f,UAS-tdTomato* line or alternatives (see below).
23. Collect 20–30 F1 flies (*UAS-GCaMP6f,UAS-tdTomato/+;tmc-Gal4/+*).
24. To make sure that the flies are healthy and are a consistent age, 1 day before dissecting the heads, transfer 3–5 day-old flies to a fresh vial.

Note: It is acceptable to use older flies, as long as the ages are consistent among the genotypes.

Note: *UAS-GCaMP6f,UAS-tdTomato/+;tmc-Gal4/+* flies express the fluorescence proteins tdTomato, which is red, and the GCaMP6f, which is green, in the md-L neuron located in the middle of each labellum.

Alternatives: In this example, the *UAS-GCaMP6f* and *UAS-tdTomato* transgenes are recombined onto a single chromosome. Other versions of GCaMP (including GCaMP7 and GCaMP8) can be used. For the baseline control, other fluorescent proteins (such as *UAS-mCherry*) can be used.

Fly head dissection and specimen preparation

Timing: 10 min (for step 25)

This section describes the procedures for dissecting and preparing fly heads for calcium imaging.

25. Obtain the chambers previously prepared and silicone lubricant. Place a drop of silicone lubricant in the middle of the chamber (see above: "prepare chambers for the calcium imaging experiments."
26. Briefly anesthetize the flies by injecting CO_2 into the vial or by incubating the vial on ice for 2–3 min.
27. Withdraw the fly by grabbing its wings with forceps and place it in a dissection well containing 1× PBS.
28. Dissect the head away from the body by cutting the neck with fine scissors.
29. Incubate the isolated head for 5–8 min in PBS until the proboscis fully extends and the labellum is in a closed state (Figure 4A).
30. Transfer the fly head from dissection well to the chamber. Immobilize the fly head in the silicone in the chamber.
31. Orient the head on one side with one eye facing up.
32. Pick the fly head with labellum in a closed state using forceps (Figure 4A).
    a. Hold the head carefully with one side facing up.
    b. Attach one side of the labellum to the grease so that the labellum sticks to the grease.

Note: If the proboscis is contracted, then hold the head and pull back slightly to fully extend the proboscis, and attach the head on the grease so that one side of the labellum remains flat

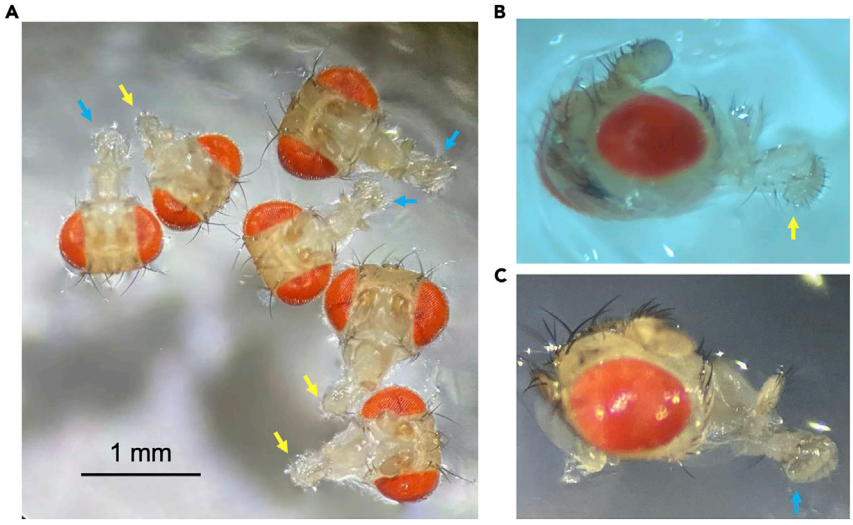

Figure 4. Images of dissected fly heads

(A) Dissected fly heads floating and incubating in 1× PBS. The blue arrows point to labella in an open state, while the yellow arrows point to labella in a closed state.

(B) An example of a fly with the labellum in a flat and unfolded position (yellow arrow).

(C) An example of a fly head positioned on silicone lubricant with the proboscis extended and with the labellum curled up (blue arrow).

and unfolded (Figure 4B), and adjust the grease beneath the sample to make the labellum horizontal.

33. Add a drop of water (~60 μL) to the chamber.

Note: Keep the labellum flat and stretched in a horizontal position to help visualize the position of the labellum and sensillum as you apply force. If the labellum curls up (Figure 4C), use a new preparation.

Note: After sticking the extended proboscis on the silicone lubricant, remove any bubbles around the labellum, which would interfere with the imaging.

⚠ CRITICAL: Be careful not to damage the labellum when removing the head with the forceps and scissors.

Live confocal microscopy

Timing: 10–20 min per sample (for step 34)

This section describes the confocal microscopy setup, and the methods to record the responses of md-L neurons due to mechanical stimuli.

34. For imaging experiments, use a laser scanning confocal microscope and perform the imaging at room temperature (~23°C).

Note: We used a Zeiss LSM 700 upright confocal microscope with a w Plan-Apochromat 20×/1.0 water objective and 1.9× optical zoom. Set the pinhole to 2.07 Airy Units = 4.1 μm section and scan at 614.4 ms per frame in line at 8 bits (Figure 5).

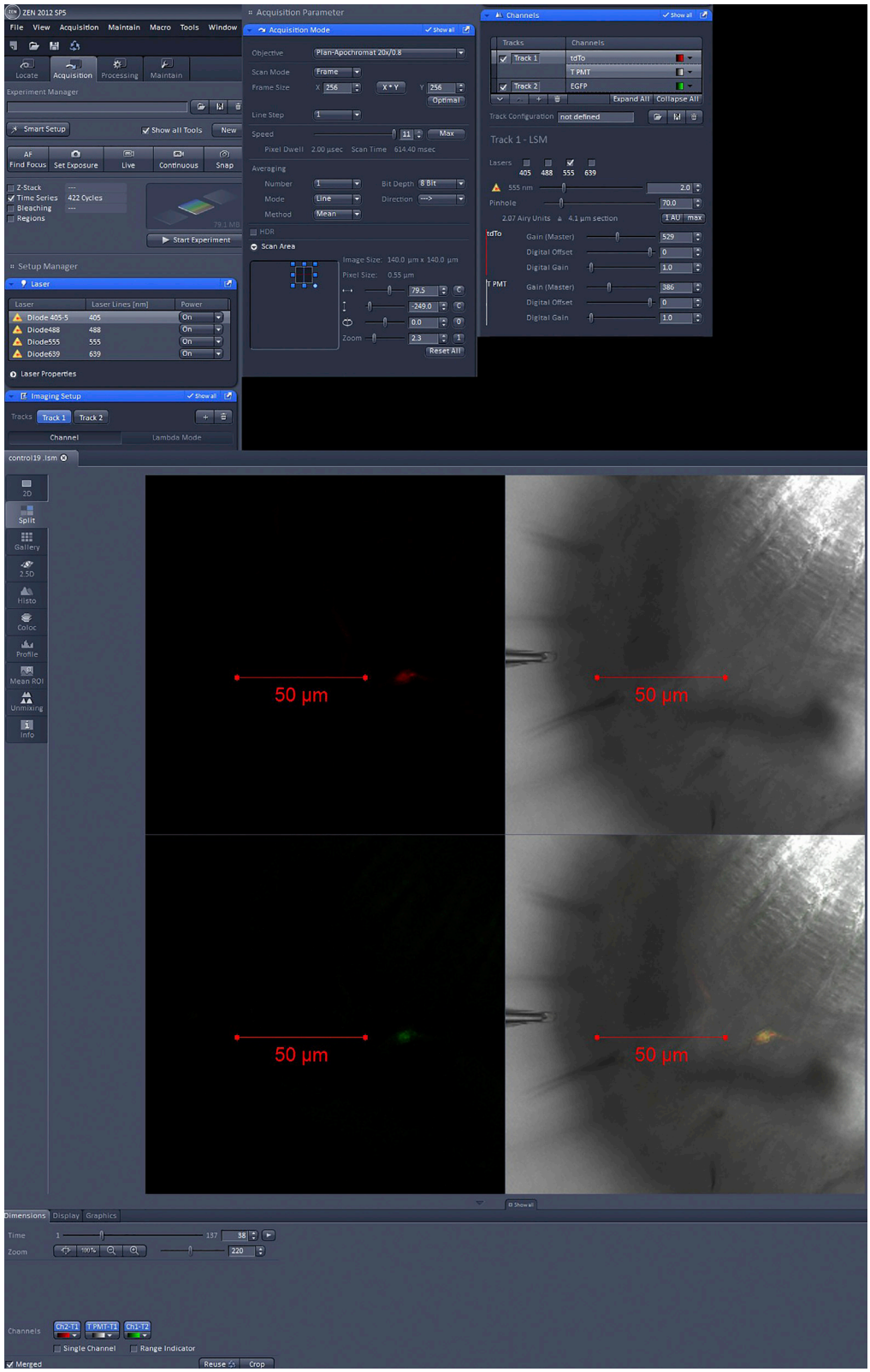

Figure 5. Panel settings on the Zeiss LSM 700 for live image recordings

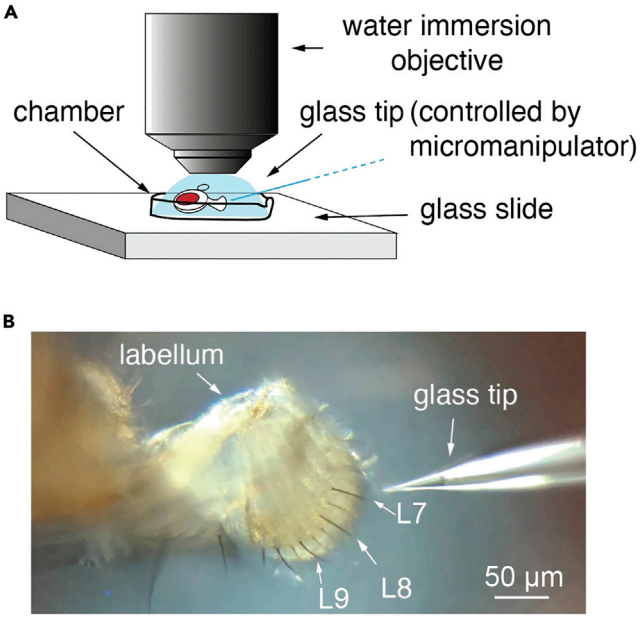

Figure 6. Preparation for performing calcium imaging on md-L neurons

(A) The setup for applying mechanical force to the labellum. The glass pipette tip is controlled by a motorized micromanipulator. The GCaMP6f responses are monitored using a 20× water immersion objective.

(B) A glass tip pointing at the labellum. The L7, L8 and L9 sensilla are indicated.

35. Set the ZEN SP5 software to acquire the images at 256 × 256 pixels. With these settings, the pixel sizes are 0.67 µm × 0.67 µm and the image sizes are 171.8 µm × 171.8 µm.
    a. Use the 488 and 555 nm laser excitation lines to image EGFP and tdTomato, respectively.
    b. Acquire the bright-field image using the tdTomato channel (Figure 5). Set the time series to record 3 images on the three channels at 614.4 ms intervals.

Note: To obtain the highest spatial resolution with the highest temporal resolution (fastest scanning speed), we chose 256 × 256 pixels, and limited the scanning area to the region containing only the labellum and the extruded hairs. The soma of the md-L neuron will cover ~200 pixels with the LSM700 set at a scanning frequency of 614.4 ms. For other confocal microscopes, adjust the image pixels and the scanning frequency to so that it is in the range of dozens to hundreds of ms.

36. Place the slide with the plastic chamber containing the head on the microscope stage under the 20× water immersion objective (Figure 6A).
37. Place the end of the glass tip on the cuticle between the L8 and S6 sensilla (Figure 6B; S6 not visible in this image. Adjust focus to visualize S6).
38. Begin recording the GCaMP6f, tdTomato fluorescence signals, and the bright-field at the same time.
39. To provide a baseline, record ≥5 frames before introducing the mechanical stimulus.
40. Using the glass tip that is touching the cuticle, apply mechanical force by controlling the step distances in one direction (5–40 µm) with a motorized micromanipulator and Linlab 2 software.
    a. Push the glass tip on the labellum in the proximal direction (Figure 7B).
    b. The duration of each stimulus is 2 s, which is the total time for stepping in and out.
    c. The step distances are measured using video recordings of the bright-field images and Zeiss confocal software, or Fiji software.
41. Save the videos using the .lsm format.

Note: The L8 and S6 sensilla are in the middle distal region of the labellum (see troubleshooting section; Figure 11). L8 is on the top layer of the labellum, while S6 is on the bottom

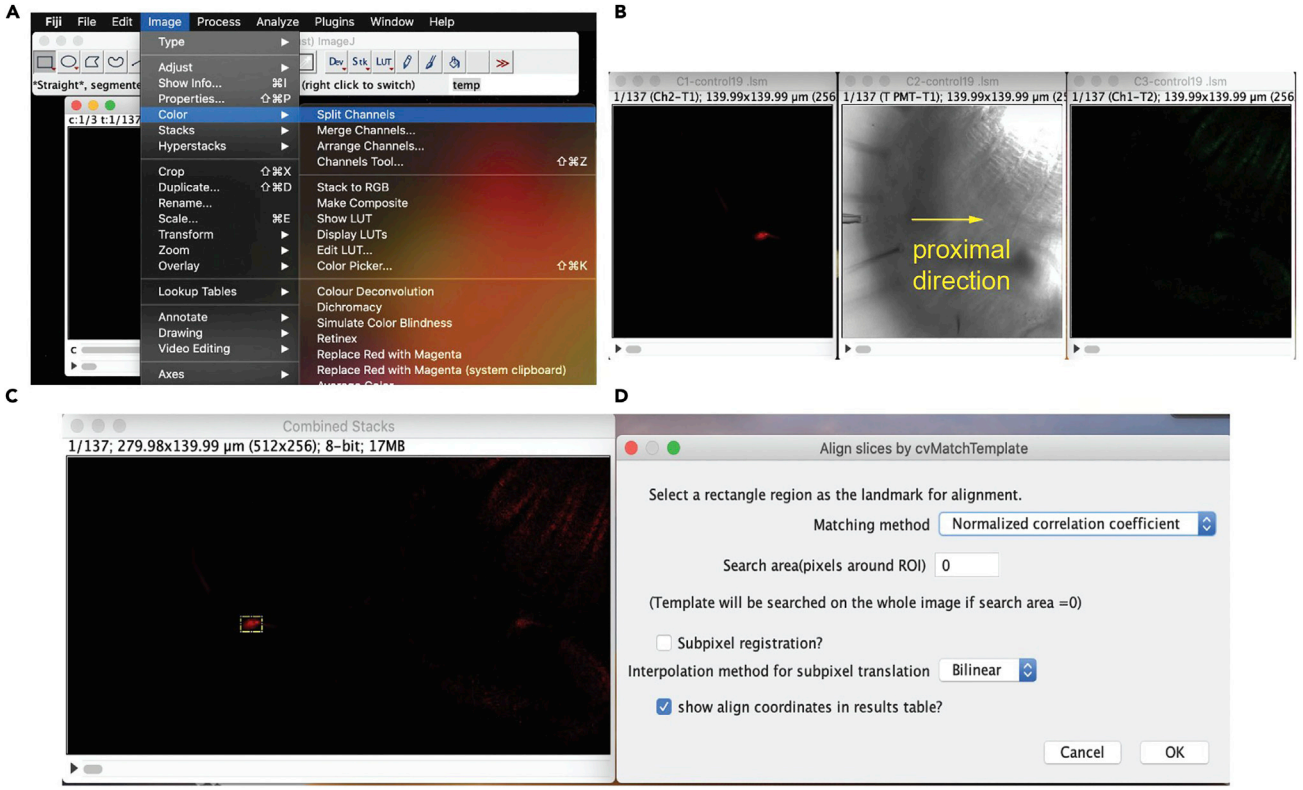

Figure 7. Image alignment

Align the soma of the md-L neuron in the calcium imaging videos using the template matching plugin in Fiji.

(A) Fiji control panel and selection of the Split Channel menu option.

(B) Split the videos into the tdTomato (left), bright field (middle) and EGFP (right) channels.

(C) Combine the split tdTomato and EGFP image stacks in the same frame and select the ROI using the tdTomato signal as the landmark for the alignment, ROI is selected as a rectangular yellow box shown in the figure.

(D) Align slices dialog box.

layer. After placing the glass tip on the cuticle between L8 and S6, and before applying mechanical stimuli, re-adjust the focus on the md-L neuron cell soma and start recording. Because the soma of the md-L neuron soma is located near the middle of the labellum, the glass tip can still be seen on the screen.

Note: Set the XYZ parameters on the Linlab 2 software (5–40 µm for one direction and 0 for the other two directions) to control the motion distance in each direction.

Optional: The indentation distances on the cuticle (10–40 µm; see Figure 10A), which are produced by the motion of the glass tip, are measured with software on the confocal microscope (Figure 7).

Image processing

Timing: 20–30 min

This section describes the processing of calcium imaging videos of md-L neurons using Fiji software, and the analysis of the neuronal responses to obtain curves showing the Ca^{2+} dynamics.

42. Drag the .lsm file to the Fiji control panel to load the file, and select: Image>Color>Split channels (Figure 7A).

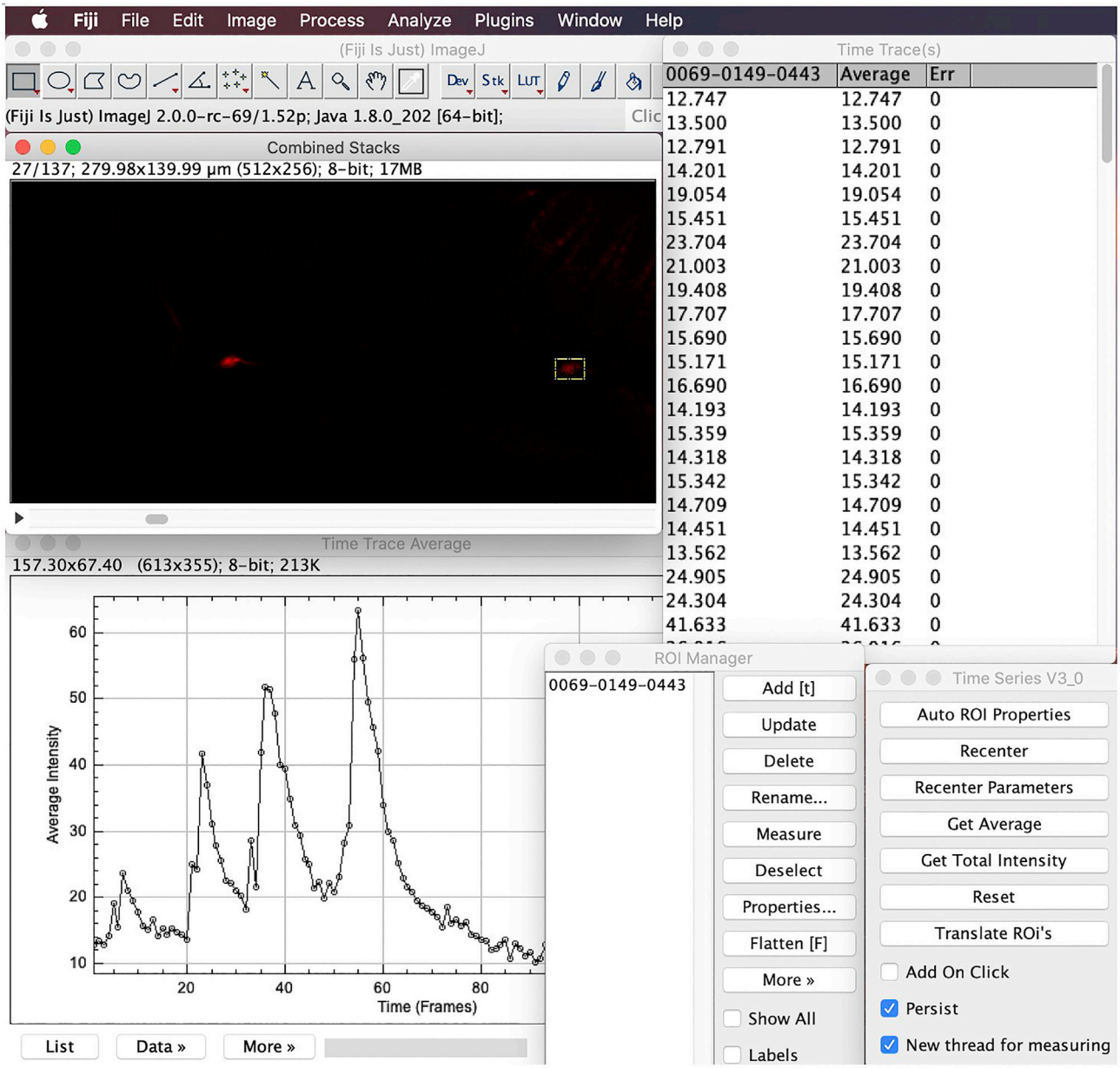

Figure 8. Data analysis

Calculate the average EGFP fluorescence signal in the selected ROI using the time series analyzer plugin in Fiji.

43. Combine the tdTomato and EGFP channel files into one video by selecting: Image>Stacks>Tools>Combine (Figure 7B).
44. Align the positions of the md-L neuron in the video using the template matching plugin of Fiji.
    a. To do this, select the tdTomato signal in the soma of the md-L neuron as the region of interest (ROI; Figure 7C).
    b. Then select Plugins>Template Matching>Align slices in stacks (Figure 7D).
45. Obtain the fluorescence intensity in separate time series stacks for both the tdTomato and EGFP signals in the soma of the md-L neuron. Select Plugins>Time series analyzer V3 (Figure 8).
46. Select a rectangular region including the md neuron soma, and add it to the ROI manager.

47. Click "Get Average" (Figure 8) to obtain the average signal intensity of the series of images in the stack. Use the same ROI to analyze the tdTomato and EGFP signals.
48. Based on the bright field images showing the pushing of the labellum by the glass tip, the peak intensity of the GCaMP fluorescence is shown at the time point when each mechanical stimulus is applied on the labellum.
49. Select the bright field images at the base and at each peak stimulus (Figure 10A). Use the Image>Lookup tables>Thermal to display the heatmap of the GCaMP and the tdTomato images (Figure 10B).
50. Calculate the changes in fluorescence using the following formula:

$$\frac{\Delta R}{R0} = (R - R0) \Big/ R0$$

$$R0 = \frac{F0(GFP)}{F0(tdTomato)}$$

The GCaMP fluorescence (F) is normalized to the tdTomato fluorescence:

$$R = F(GFP)/F(tdTomato)$$

The base GCaMP and tdTomato fluorescence (F_0) are obtained from the average of the 5 frames before the stimulus is applied.

An average response curve ± SEM is shown in Figure 10C.

EXPECTED OUTCOMES

Wild-type or w^{1118} flies starved for 2 h exhibit a modest PER score in response to 50 mM sucrose (0.35 ± 0.04 from 5 trials). The PER score using 50 mM sucrose containing 9.2 μM particles increases significantly (0.77 ± 0.04) (Figure 9, Table 1). For more data, refer to Li and Montell (2021).

Mechanical stimulation (10–40 μm steps) of the cuticle on the labellum should induce an intensity-dependent increase $[Ca^{2+}]_i$ in the soma of the md-L neuron (Figure 10).

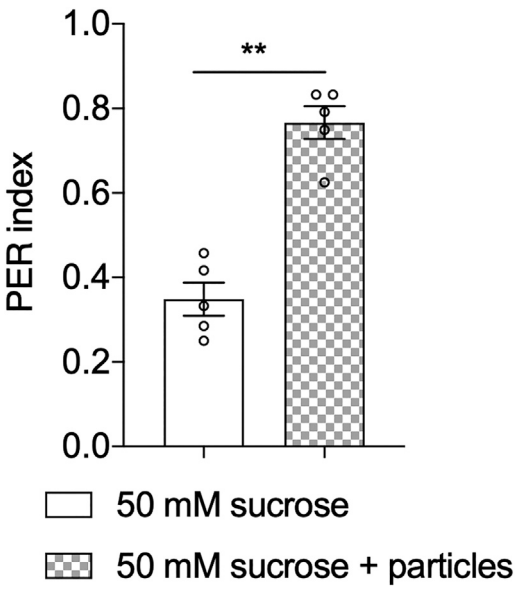

Figure 9. PER indexes of flies exposed to 50 mM sucrose alone or 50 mM sucrose containing 9.2 μm particles (10% w/v)

Means ± SEMs. **p<0.01. n=5 trials. ≥12 flies/trial. Non-parametric Mann-Whitney test between the group offered 50 mM sucrose plus particles and the control offered 50 mM sucrose only. The data points are shown in Table 1.

Table 1. Example of raw PER data for 5 groups of flies using either clear food (sucrose alone) or smooth food with 9.2 μM particles

Food type	Group	A	B	C	D	E
50 mM sucrose	Sum of the scores	5	5.5	4	3	4
	Number of flies in each group	12	12	12	12	14
	PER index	0.42	0.46	0.33	0.25	0.29
50 mM sucrose + particles	Sum of the scores	9	7.5	10	9.5	10
	Number of flies in each group	12	12	12	12	12
	PER index	0.75	0.63	0.83	0.79	0.83

The "sum of the scores" is the sum of all of the PER scores for each group.

QUANTIFICATION AND STATISTICAL ANALYSIS

The data analyses are described in the step-by-step methods. In general, the experiments should be repeated ≥5 times. More replicates are necessary to detect a smaller effect size in the presence of greater variability. The specific number of replicates will require performing power analyses: set type I error rate (α) as 0.05, power (1-β) as 0.9. If the mean is 1, and the standard deviation is 0.5, you will need 5 samples per group. The results from the PER assays and calcium imaging assays should be expressed as the mean ± SEM. We use PRISM8 to apply the distribution-free nonparametric Mann-Whitney tests because each sample is independent and the sample sizes are small, as outlined in the experimental design described in this protocol.

LIMITATIONS

PER assay

The PER assay is a quick behavioral response to evaluate how appealing a given food is to a fly. Depending on the rearing conditions and the genetic background, it may be necessary to adjust the starvation time or sucrose concentration to achieve robust PER scores.

To evaluate the impact of grittiness on food appeal, monodisperse silica microspheres have the advantage that they do not elicit a gustatory response. However, a limitation is that the silica particles (e.g., 9.2 μm in diameter) added to the 50 mM sucrose do not form a stable colloidal solution. Therefore, it is necessary to mix the particles in the solution immediately before offering the food to the flies.

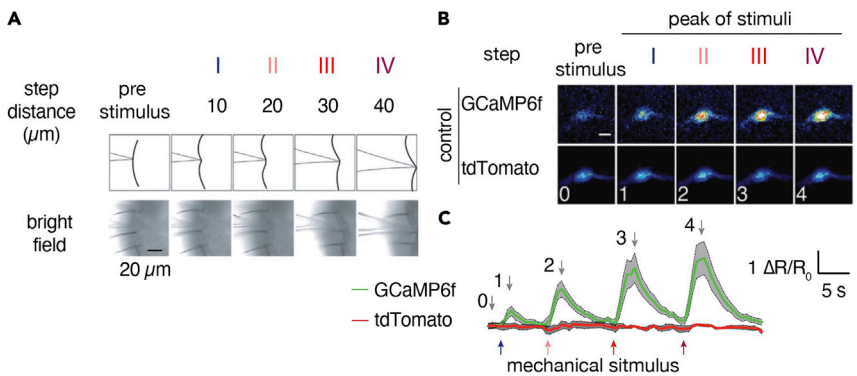

Figure 10. Calcium imaging of the md-L neuron during exposure to mechanical stimulation

(A) The cartoon (top row) depicts mechanical stimuli applied to the labellum by pushing a glass tip on the cuticle, resulting in deflections of 10–40 μm. The bottom row shows the corresponding bright field images of the labellum.

(B) Heatmap of the GCaMP responses of the md-L soma prior to the stimulus, and at the peak responses using the four levels of force shown in A. Scale bar: 20 μm.

(C) Response curves for the GCaMP6f and tdTomato fluorescence as mean ± SEM, n=5. The gray arrows indicate the peak responses shown in B. The blue, pink and purple arrows show the time that the four levels of force were applied to the cuticle as indicated in A.

Calcium imaging with GCaMP

This protocol involves applying force by indenting the cuticle to elicit a calcium response in the md-L neuron. The indentation results in deflection of the nearby sensilla. It is also feasible to deflect a single sensillum such as L7 (Li and Montell, 2021). Identifying specific sensilla requires experience. In addition, the deflection angle of the sensillum can only be measured from the two-dimensional images.

It is not feasible to use food droplets to stimulate sensilla on the labellum while imaging the md-L neuron soma, because this would require using an air lens and sealing the labellum under a cover glass.

It is critical that the labellum is not damaged prior to performing the imaging.

TROUBLESHOOTING

Problem 1

The flies do not exhibit a PER to 50 mM sucrose. Refer to part 1: proboscis extension response assay: step 16.

Potential solution

Extend the starvation time or increase the sucrose concentration. Also, check if the fly is damaged. If so, dispose of the fly and use a different one.

Problem 2

The flies drink water >5 s during the water saturation protocol. Refer to part 1: proboscis extension response assay: step 13.

Potential solution

The fly food on which the flies are reared may be too dry. This will require adding more water to the recipe or incubating the flies in a humidified chamber.

Problem 3

The labellum extends out and trembles. Refer to part 1: proboscis extension response assay: steps 11 and 12.

Potential solution

The fly may be squeezed too tightly in the pipette tip or the labellum may be damaged. If so, dispose of the fly.

Problem 4

Cannot recognize specific sensilla on the labellum. Refer to part 2: calcium imaging of md-L neuron: steps 36 and 37.

Potential solution

Orient the labellum horizontally, and adjust the focus to visualize two rows of sensilla on the labellum (note that it is difficult to see both rows in one image). L8 is in the top row and is flanked by the I7 and I8 sensilla (Figure 11A). The lower row includes multiple S-type sensilla including S6, which is in between L3 and L4, and is also flanked by S5 and S7 (Figure 11B).

Problem 5

Cannot identify the md-L neuron. Refer to part 2: calcium imaging of md-L neuron: step 36.

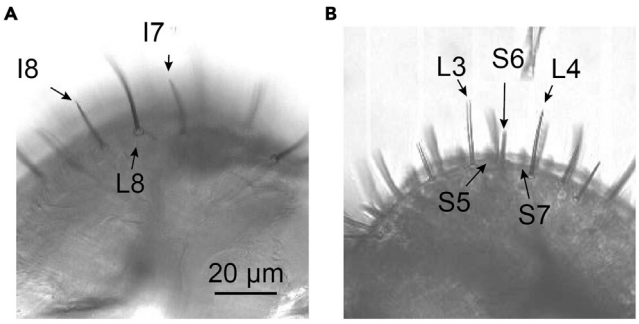

Figure 11. Images of a fly labellum, with the identities of several sensilla in the middle indicated
(A) Top row of sensilla on the labellum.
(B) Lower row of sensilla on the labellum.

Potential solution

The soma of the md-L neuron is located in the outer layer of the middle part of labellum in approximately the same layer of focus as the two rows of sensilla (Figures 6 and 11B). Use tdTomato, which is much brighter than GCaMP6f, to visualize the md-L soma and the many dendrites that extend to most L-type sensilla.

Problem 6

The md-L neurons do not exhibit a calcium response. Refer to part 2: calcium imaging of md-L neuron: steps 40 and 50.

Potential solution

The labellum may be damaged or the tip may also not be making contact in the right place. Therefore, carefully dissect the head. Normally, the md-L neuron remains responsive for at least 30 min.

Problem 7

Check the health of the preparation for calcium imaging. Refer to part 2: calcium imaging of md-L neuron: step 36.

Potential solution

An abnormally high basal level of GCaMP6f fluorescence may indicate that the neuron is damaged. If the basal level of GCaMP6f fluorescence is undetectable, this indicates that the neuron has died.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Craig Montell (cmontell@ucsb.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101806.

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AUTHOR CONTRIBUTIONS

Q.L. and C.M. prepared and wrote this protocol, and Y.L. prepared the video.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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