

Video Article

Simultaneous Recording of Calcium Signals from Identified Neurons and Feeding Behavior of *Drosophila melanogaster*

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Abstract

To study neuronal networks in terms of their function in behavior, we must analyze how neurons operate when each behavioral pattern is generated. Thus, simultaneous recordings of neuronal activity and behavior are essential to correlate brain activity to behavior. For such behavioral analyses, the fruit fly, *Drosophila melanogaster*, allows us to incorporate genetically encoded calcium indicators such as GCaMP¹, to monitor neuronal activity, and to use sophisticated genetic manipulations for optogenetic or thermogenetic techniques to specifically activate identified neurons²⁻⁵. Use of a thermogenetic technique has led us to find critical neurons for feeding behavior (Flood *et al.*, under revision). As a main part of feeding behavior, a *Drosophila* adult extends its proboscis for feeding⁶ (proboscis extension response; PER), responding to a sweet stimulus from sensory cells on its proboscis or tarsi. Combining the protocol for PER⁷ with a calcium imaging technique⁸ using GCaMP3.0^{1,9}, I have established an experimental system, where we can monitor activity of neurons in the feeding center – the suboesophageal ganglion (SOG), simultaneously with behavioral observation of the proboscis. I have designed an apparatus ("Fly brain Live Imaging and Electrophysiology Stage": "FLIES") to accommodate a *Drosophila* adult, allowing its proboscis to freely move while its brain is exposed to the bath for Ca²⁺ imaging through a water immersion lens. The FLIES is also appropriate for many types of live experiments on fly brains such as electrophysiological recording or time lapse imaging of synaptic morphology. Because the results from live imaging can be directly correlated with the simultaneous PER behavior, this methodology can provide an excellent experimental system to study information processing of neuronal networks, and how this cellular activity is coupled to plastic processes and memory.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3625/>

Protocol

1. Constructing the FLIES

1. Shape a platform from the lid of 35 mm Falcon dish by melting the sidewall and carving it to form an appropriate angle and thickness (**Figure 1a**; **Figure 2**). Drill a hole in the platform to accept the fly head (**Figure 1a,b**), such that the mouthparts are freely exposed to the outside of the chamber (**Figure 1a**). Cut the end of a Pipetman tip, with the size matching the fly to be observed. Glue the tip to the platform as shown in **Figure 1a** to complete the FLIES (**Figure 2**).

2. Preparing the Fly for Observation

1. Starve an adult fly for 24 hours at 25 °C prior to the experiment by placing it in a vial with only a wet paper towel, if starvation is necessary.
2. Anesthetize the fly by placing it in a 15ml plastic tube standing on ice.
3. Using forceps, insert a fly into the chamber of the FLIES, and gently push the fly in until it is unable to move. Then, insert a plug to retain the fly. (**Figure 1a,b**).
4. Seal the surrounding parts of the proximal proboscis (rostrum) to the inner edge of the hole; Apply a small amount of light-curing glue (Tetric EvoFlow) to the sides and above the rostrum from the outside (arrows in **Figure 3**). Also apply the glue from the inside of the FLIES to the space between the dorsal part of the head and the inner edge of the hole (**Figure 1b**). To allow the rostrum to move freely, care should be taken to prevent any glue from touching the rostrum by using an eyelash (or something comparable) to spread the glue. Finally, cure the glue with the weakest illumination of blue light sufficient for curing to avoid damaging the fly from excessive heat. Remove the plug.
5. A thread to hold the rostrum partially lifted (arrow in **Figure 3**) may be employed to stabilize the fly's head by avoiding bump of the pharyngeal part to SOG and to expose the ventral part of the SOG, especially for imaging presynaptic terminals of Gr5a neurons⁸, which are covered by part of the pharynx when the proboscis is fully retracted.
6. Fill the surface of the platform with a sugar-free saline containing (in mM): NaCl, 140; KCl, 2; MgCl₂, 4.5; CaCl₂, 1.5; and HEPES-NaOH, 5 with a pH of 7.1¹⁰. This sucrose-free saline has similar tonicity to those of commonly-used sucrose-containing salines such as HL3.1¹¹.

- Open the head capsule using a tungsten blade and forceps with sharpened tips like scissors, which is a custom-made device and originally designed by Dr. Kageyuki Yamaoka, Japan, to clip the cuticle and trachea to expose the SOG (**Figure 4**). The ventral edge of the head cuticle was cut as ventral as possible whereas the dorsal edge cut was not as critical. First, make an incision through the posterior edge using a tungsten blade. Then, cut through the side and the anterior edges using the sharpened forceps. The cut cuticle piece should be lifted with the forceps and removed. Antennae and antennal nerves should be cut out by the sharpened forceps.
- In order to avoid movement artifacts during recordings, the brain must be stabilized through the removal of certain parts of the fly. First, selectively remove air sacks between the SOG and cuticle. Cut the oesophagus at the end toward the pharynx and at the end going into the SOG to remove the connection between the pharynx and the brain. Then pull out Muscle 16¹², and finally, detach any trachea connecting the brain and the cuticle.
- We set our FLIES on the stage of an Olympus BX51WI microscope connected to a spinning disk confocal microscope system (Improvision in PerkinElmer) (**Figure 5**). A water immersion lens, e.g. 40X/0.8 N.A, was immersed into the bath (**Figure 6**).

3. Ca²⁺ Imaging of the Brain

- Ca²⁺ imaging using GCaMP3.0^{1,9} was performed through the method established by Marella *et al.*⁸ (**Figure 6**). Using a spinning disk confocal microscope (Improvision), take a single optical section at 4Hz with an exposure time of 122ms using 491nm excitation laser, focusing at the region of interest (a cell body of a motor neuron, or an interneuron, or presynaptic terminals of gustatory sensory neurons) with Velocity software, ver. 4.3 (Improvision).

4. Stimulating the Proboscis

- Aspirate 100mM aqueous sucrose solution into a hypodermic needle with a small wick (made from Japanese Washi paper, see **Table of specific reagents**) protruding from the tip (**Figure 7**).
- Discharge a small drop of sucrose solution onto the wick, then, aspirate it, immediately before applying the soaked Washi paper wick to the tip of the proboscis. The Washi paper wick should only be applied for an instant to avoid satiation (**Figure 8**). Monitor the Proboscis Extension Response (PER) behavior using a CCD camera attached to a dissection microscope (**Figure 5**) simultaneously with Ca²⁺ imaging. Data must be taken within one hour after starting the dissection because the PER response is not maintained more than one hour under these conditions.

5. Representative Results

Motor neurons of the rostrum protractor, a pair of major muscles responsible for lifting the rostrum for proboscis extension, have been reported to show robust responses to sweet stimuli¹³. **Figure 9** shows Ca²⁺ signals through GCaMP3.0^{1,9} expressed by a Gal 4 driver, E49¹³, from a cell body of the motor neuron. As shown in the video, a robust PER was observed in synchrony with an increase in Ca²⁺ signal.

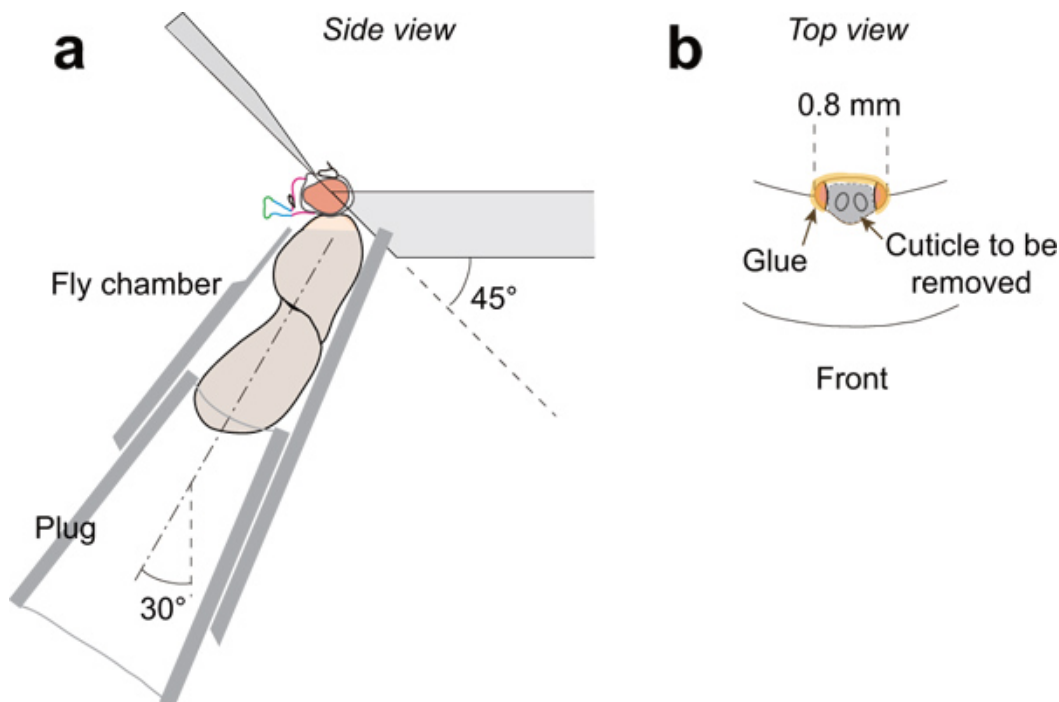
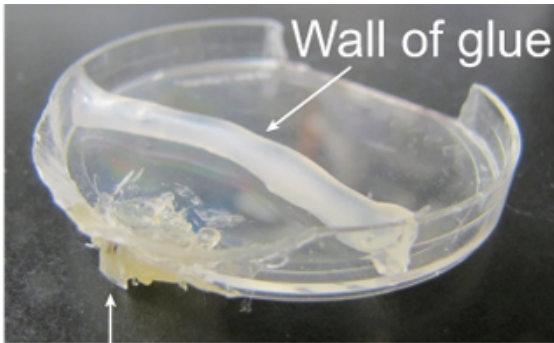


Figure 1. Schematic of the Fly brain Live Imaging and Electrophysiology Stage (FLIES). **a**, The FLIES is built such that the angle of the wall allows for the fly head to come out straight. Three segments of the fly proboscis are color-coded; the rostrum is magenta. The fly is inserted into the chamber with forceps, and a piece cut from the end of a Pipetman tip is plugged into the back of the chamber to block the fly from escaping. **b**, The hole is bored to conform to the fly's head. The remaining gaps are sealed with light-curing glue as indicated to ensure that there are no leaks. After the glue has cured, the Pipetman tip as a plug is removed. Saline is poured into the FLIES such that the surface of the brain is

completely covered. It should be ensured that there is no saline leaking out onto the anterior end of the fly head. The shaded area surrounded by the dashed line in panel **b** shows the part to be dissected out.



Fly chamber

Figure 2. Photograph of the FLIES. The crescent shaped wall of glue (from a glue gun) is made to control the flow of saline solution during perfusion, and to reduce the amount of saline used.



Figure 3. Frontal view of a fly mounted in the FLIES. This image shows how a fly is set in the FLIES, and how the light-curing glue is applied around the head of the fly (arrows) to create a saline-proof seal. If any saline leaks through to the proboscis of the fly, then the experiment will fail. The thread (arrowheads) tethered to the stage is to hold the proboscis in position, so that it is not completely retracted. Otherwise it would obstruct the more ventral portion of the SOG and cause movement artifacts.

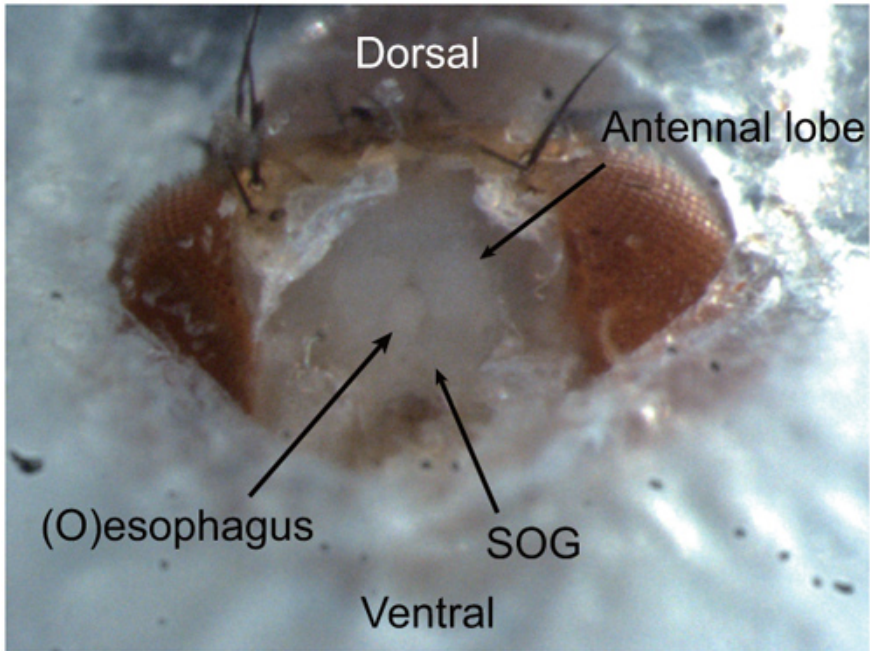


Figure 4. Top-view of the FLIES with a dissected fly. This image shows the exposed SOG of a fly following cuticle removal. The esophagus, Muscle 16 and tracheae connected to the brain, as well as selected air-sacks have also been removed to prevent them from disturbing the brain, which can lead to artifacts in the calcium signals.

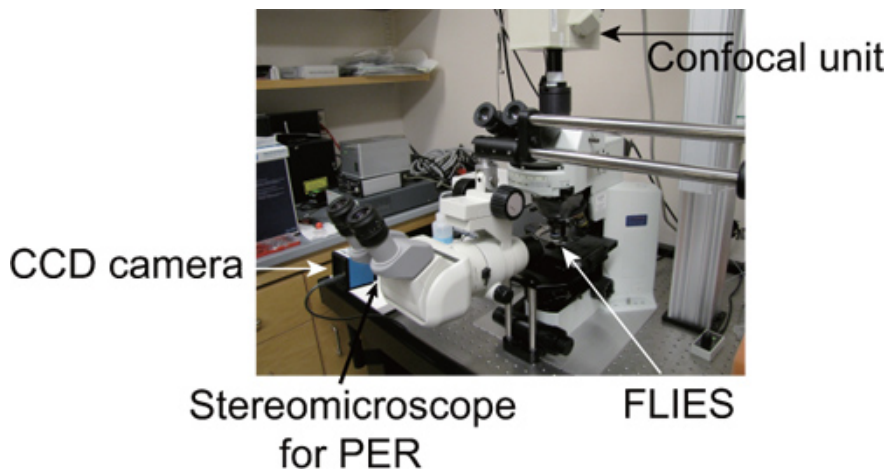


Figure 5. The monitoring apparatus. This apparatus is assembled from an Olympus BX51WI microscope attached to a spinning disk confocal microscope system (Improvision), and a side-mounted Nikon SMZ800 microscope. This setup allows for the live-imaging of brain activity during PER behavior.

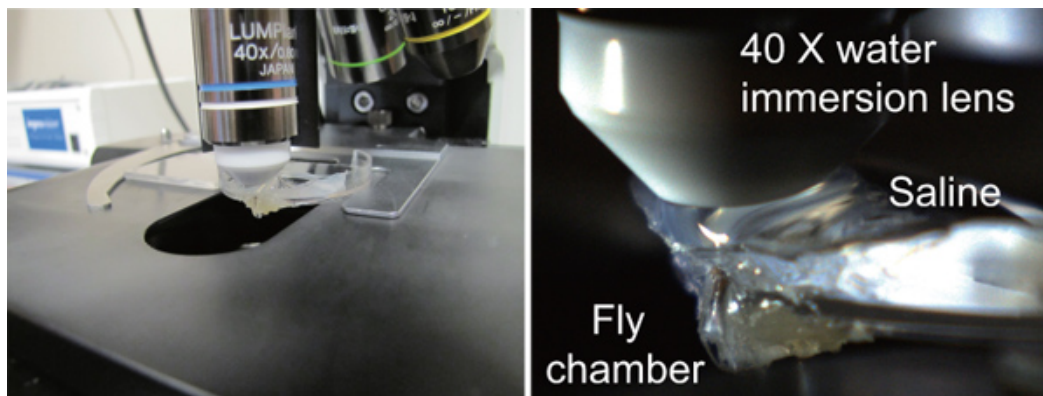


Figure 6. Photographs to show the side-view of the FLIES. The thin layer of saline solution is sandwiched between the 40X water-immersion lens and the fly.

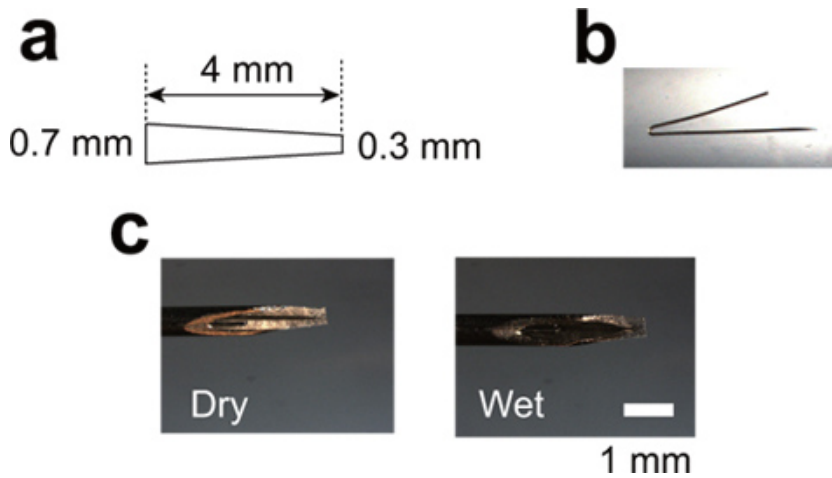


Figure 7. Schematic for making the Washi paper wick. Gampi-Washi paper is a traditional Japanese parchment that is extremely thin and smooth (Haibara, Japan). It is also very strong and can retain a large amount of liquid. The Washi paper must be cut into a trapezoid with very specific dimensions; 0.3 mm and 0.7 mm in width, and 4-5 mm in length (a). This trapezoid is then inserted from the 0.7 mm side into the tip of a 23 G hypodermic needle and can also be stabilized through the insertion of an insect pin, which is bent to a V-shape (b). c. The Washi paper becomes transparent following exposure to a liquid, making it ideal for this experiment.

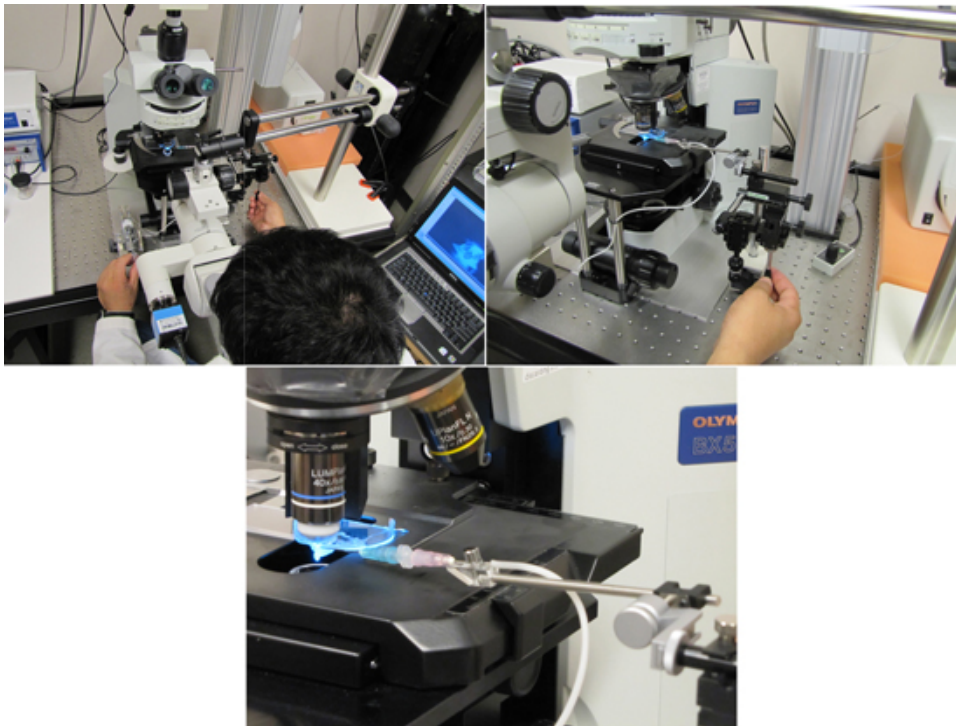


Figure 8. Stimulation of the proboscis on the FLIES with the Washi-wick. The Washi paper wick at the tip of a hypodermic needle is applied for an instant using a Joystick manipulator with one hand. The needle is connected through a flexible tube to an injector manipulated with the other hand, for aspirating and discharging sucrose solution.

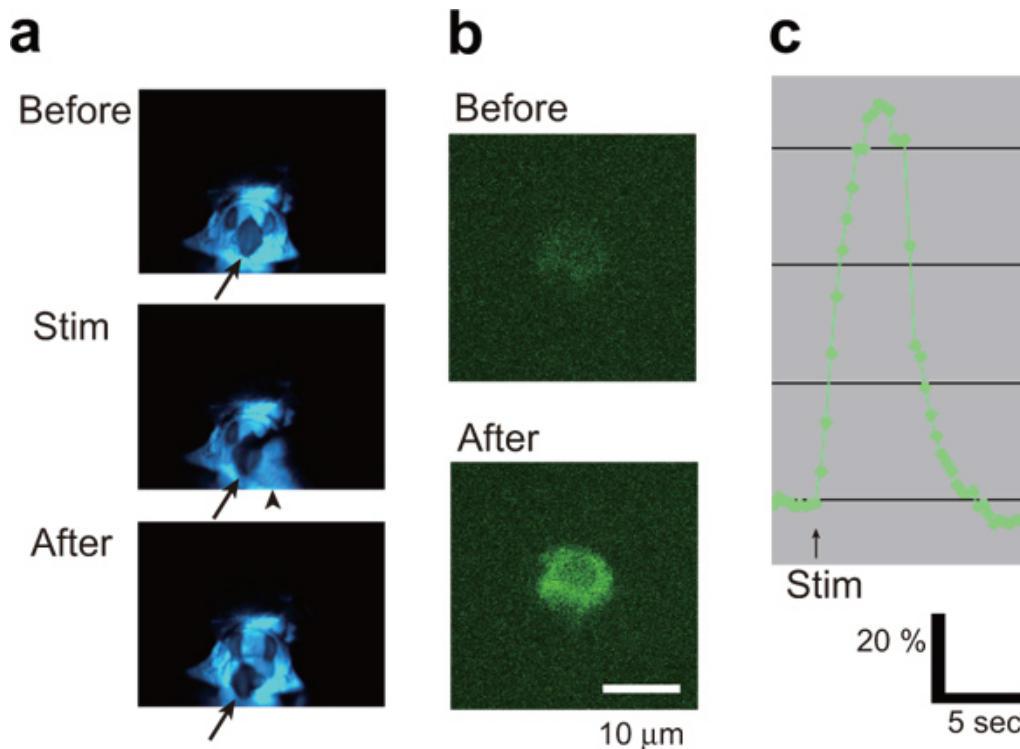


Figure 9. Representative results. **a**, PER behavior recorded through the CCD camera installed on the stereomicroscope. Frontal views of a starved fly with an extended proboscis (arrows) before the stimulation, at the stimulation, and after the stimulation with a wick containing 100 mM sucrose aqueous solution (arrowhead). Illumination is accomplished by a 491 nm laser used for GCaMP fluorescence. **b**, Sucrose-induced GCaMP 3.0 response in the motor neuron for the protractor of rostrum muscle in the starved state. The top panel, before the stimulation; the bottom panel, immediately after the stimulation. Scale bar, 10 μ m. **c**, Quantification of the sucrose-induced GCaMP 3.0 response. The motor neuron responds to a sucrose stimulus by a sharp increase in fluorescence followed by a restoration phase of several seconds to the base line.

Discussion

The FLIES allows for simultaneous recording of Ca^{2+} signals and PER behavior. Even with the brain exposed to saline normal PER behavior was observed. Using the Gampi-Washi wick instead of a Kimwipe wick used in the original capillary method⁷ facilitates a highly reproducible and stable PER behavior and avoids the need to become skilled in making and choosing a good Kimwipe wick. The experimental tips stated above allowed us to successfully avoid disturbances by movement of the proboscis, leading to a very stable recording of Ca^{2+} signals with low noise. Occasionally, tissue removal was not sufficient to expose cells or suppress movement adequately, leading to poor results. However, once we were skilled, more than 80 % of preparations produced good results. This methodology is not only for Ca^{2+} imaging, but can also be adapted to any live imaging while observing PER behavior. For example, we can access any cell through the use of an electrode to directly record activity. Combined with two-photon excitation microscopy, we are able to perform time lapse imaging of synaptic structure, which can be related to behavioral changes. Therefore, this method of brain imaging with behavioral observation is not only valuable for the functional dissection of the neuronal network, but could be used as a powerful tool to correlate synaptic plasticity¹⁴ to the mechanisms behind memory.

Disclosures

I have nothing to disclose.

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