

Video Article

Calcium Imaging of Odor-evoked Responses in the *Drosophila* Antennal LobeAna F. Silbering¹, Rati Bell¹, C. Giovanni Galizia², Richard Benton¹¹Center for Integrative Genomics, University of Lausanne²Department of Biology, University of KonstanzCorrespondence to: Richard Benton at richard.benton@unil.chURL: <http://www.jove.com/video/2976/>

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The antennal lobe is the primary olfactory center in the insect brain and represents the anatomical and functional equivalent of the vertebrate olfactory bulb¹⁻⁵. Olfactory information in the external world is transmitted to the antennal lobe by olfactory sensory neurons (OSNs), which segregate to distinct regions of neuropil called glomeruli according to the specific olfactory receptor they express. Here, OSN axons synapse with both local interneurons (LNs), whose processes can innervate many different glomeruli, and projection neurons (PNs), which convey olfactory information to higher olfactory brain regions.

Optical imaging of the activity of OSNs, LNs and PNs in the antennal lobe - traditionally using synthetic calcium indicators (e.g. calcium green, FURA-2) or voltage-sensitive dyes (e.g. RH414) - has long been an important technique to understand how olfactory stimuli are represented as spatial and temporal patterns of glomerular activity in many species of insects⁶⁻¹⁰. Development of genetically-encoded neural activity reporters, such as the fluorescent calcium indicators G-CaMP^{11,12} and Cameleon¹³, the bioluminescent calcium indicator GFP-aequorin^{14,15}, or a reporter of synaptic transmission, synapto-pHluorin¹⁶ has made the olfactory system of the fruitfly, *Drosophila melanogaster*, particularly accessible to neurophysiological imaging, complementing its comprehensively-described molecular, electrophysiological and neuroanatomical properties^{2,4,17}. These reporters can be selectively expressed via binary transcriptional control systems (e.g. GAL4/UAS¹⁸, LexA/LexAop^{19,20}, Q system²¹) in defined populations of neurons within the olfactory circuitry to dissect with high spatial and temporal resolution how odor-evoked neural activity is represented, modulated and transformed²²⁻²⁴.

Here we describe the preparation and analysis methods to measure odor-evoked responses in the *Drosophila* antennal lobe using G-CaMP²⁵⁻²⁷. The animal preparation is minimally invasive and can be adapted to imaging using wide-field fluorescence, confocal and two-photon microscopes.

Video LinkThe video component of this article can be found at <http://www.jove.com/video/2976/>**Protocol****1. Mounting Block Assembly**

1. The plexiglass mounting block (Figure 1A) should be made to the exact specifications given in the blueprint (available from http://neuro.uni-konstanz.de/jove_mountingblock_blueprint) to allow easy mounting and dissection of the fly. Thread through the screws from the back but do not extend them beyond the front of the block; their position will be adjusted during the preparation of the animal (see 2.7).
2. Using a precision drill, make a dent in the upper border of the circular chamber for the fly, digging under the surface to make space for the fly's body, reducing the thickness of the block at this point.
3. Cut a copper grid with a scalpel perpendicular and close to the bottom of the slit to create a "collar". Make sure the two resulting flaps are level, as this will help insertion of the fly.
4. With a toothpick, place a small drop of superglue onto the mounting block above the circular chamber for the fly. Place the copper grid onto the glue and position so that the slit is centered on the block (Figure 1A). Press down gently and remove any excess glue. With a pair of tweezers, add tiny drops of glue under the flaps on each side and fold the grid over the front of the block so that the slit is pointing straight downwards. Make sure the top of the grid is level with the block. Allow to dry completely.
5. Assemble a wire holder: cut a plastic coverslip in half and remove a central piece to make a "U" shape. Use beeswax to glue a piece of wire across the top of the "U"; do not make the wire too taut.
6. Construct the antennal shield: make a hole in a plastic coverslip with a paper hole-punch. Trim the edges of the coverslip to 0.5 x 0.7 cm. Glue the pierced plastic coverslip to sticky tape and then place a drop of ethanol on the tape exposed through the hole to remove the adhesive.

2. Animal Preparation

1. Flies of the appropriate genotype - i.e. bearing the desired combination of "driver" and activity-reporter transgenes - should be 1-3 weeks old; the cuticle of younger flies is too soft and difficult to cut. When experiments require direct comparison of different genotypes, and in order to

match transgene expression levels, flies should be age-matched to avoid age-related physiological differences. If gender is not important, we prefer to use female flies since they have larger heads and are easier to manipulate.

2. Anesthetize flies by cooling on ice.
3. Introduce a single fly into the mounting block. Hold the fly by the wing joint and slide it, dorsal-surface first, along the slit of the copper grid so that it is suspended by its neck (Figure 1B-C). If necessary, rotate it until it is looking down.
4. Place a fine cactus spine in front of the fly's head to prevent it from escaping (Figure 1B-C). Place the cactus spine in front of the proboscis to prevent it from moving. Fix the cactus spine to the block using beeswax. Make sure that the top of the head is level with the top of the block.
5. Fix the head of the fly to the block with a small drop of rosin (dissolved in ethanol). Place the block in a humidified box and allow the rosin to harden for about one hour.
6. Using forceps, pull the antennal plate forward and drop the wire on the holder (wax-side out; see 1.5) in the cuticular fold between the antenna and head. Fix the holder to the front of the block with beeswax.
7. Using the screws built into the block, gently push the wire holder forward to create some space between the antennal plate and the head.
8. Place the antennal shield (see 1.6) over the top of the fly's head with the hole positioned centrally. Fix with beeswax to the top of the mounting block.
9. Using a blade-splitter, cut a small hole in the tape on the shield exposing the cuticle of the fly's head behind the antenna. The hole should not extend beyond the eyes to prevent the preparation from leaking.
10. Seal the hole between the tape and the cuticle with two-component silicon. Remove any excess silicon from the top of the fly's head.
11. Place a drop of *Drosophila* Ringer's saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 36 mM saccharose, 5 mM HEPES, pH 7.3) on the head. Make sure that there are no leaks: the antennae should remain completely dry. Using a sapphire blade, cut a hole in the cuticle. First, lightly score along the cuticular fold directly behind the antenna, then cut along the eyes and across the ocelli on the back. Finally, fold the piece of cuticle over the scored edge and cut from the underside to avoid severing the antennal nerves.
12. Carefully remove glands and trachea with fine tweezers. Rinse repeatedly with *Drosophila* Ringer's, leaving a large drop on the head.

3. Odor Stimulus Delivery

1. Odor syringes: insert a cellulose pad into a 2 ml plastic syringe with clean forceps and pipette onto it (using filter tips) 20 μ l of odor, diluted as desired in an appropriate solvent, using the pipette tip to push the pad deep into the barrel. Plug and cap the syringe until required. Fresh odor dilutions should be prepared at least every 3 months (or more frequently for highly volatile and/or often-used odors), and new pads should be prepared for odor syringes just before each recording session. Do not use odor pads for more than three stimulations, since the odor concentration might decay.
2. Custom-made olfactometer (Figure 2): a primary air stream (1 l/min) is directed through Teflon tubing (0.4 mm internal diameter); 27 cm upstream of the exit of this tube, a secondary air stream (1 l/min) is added. Both air streams are generated by membrane pumps, and the flow rate is regulated by two independent flowmeters. The air streams can be humidified by passing through water in gas washing bottles, although care should be taken to avoid over-humidification (i.e. more than ~60%). The secondary air stream is directed either through an odor syringe or an empty syringe: syringes are connected to the airflow through a syringe needle (1.2 mm outer diameter) piercing the plunger. A three-way magnetic valve controlled via a valve controller unit (e.g. Harvard Apparatus VC6) is used to switch between the clean air and odor stimulus.

4. In vivo Imaging

1. We describe here imaging using an upright fluorescent microscope (e.g. upright fixed Stage Zeiss Axio Examiner D1) equipped with a 20x water objective (e.g. Zeiss W "Plan-Apochromat" 20x/1.0 M27 DIC) (Figure 2). For imaging with G-CaMP (excitation/emission: 488/509 nm), use a filter block with the following properties: 450-490 nm excitation filter, dichroic mirror (T495LP) and 500-550 nm emission filter (Chroma ET). Place the mounting block containing the fly under the microscope. Position the exit of the olfactometer approximately 0.5 cm in front of the fly's antennae.
2. Lower the objective until it touches the Ringer's solution on the fly's head. Looking through the viewer under bright-field illumination, position the preparation so that the hole in the head capsule is centered and its borders are in focus. Switch to fluorescent light and adjust the focus until you can see the labeled neurons (apparent from basal green fluorescence of G-CaMP).
3. Fine focus on the glomeruli of interest by acquiring images with a Charge-coupled device (CCD) camera (e.g. CoolSNAP-HQ2 Digital Camera System) mounted on the microscope using appropriate acquisition software (e.g. Metafluor) (Figure 3A). Close the diaphragm in the illumination light path to limit excitation light to the imaged area.
4. For recording odor-evoked activity, set the acquisition rate to 4 Hz and adjust the exposure time to obtain fluorescence values within the dynamic range of your CCD camera, but not lower than 1000 (arbitrary units), with at least 12-bit dynamic resolution. The exposure time should not exceed 100 ms, if possible, to reduce G-CaMP photobleaching. If the spatial resolution of your camera allows, you can increase the binning (number of wells on the chip that will be binned into one digital pixel) to reduce the exposure time. We obtain images of 350x225 pixels (with a binning of 2x2), corresponding to 210x135 μ m at the preparation. These settings are optimal to capture glomerular odor-induced responses of G-CaMP with good spatial and temporal resolution while limiting reporter bleaching.
5. Set the measurement parameters. We typically record for 10 seconds (i.e. 40 frames), with odor stimulation typically starting 4 seconds after the beginning of the acquisition period (frame 15) and lasting one second (until frame 19). A fly preparation can normally be tested with up to 25-30 different odor stimuli, often limited by an irreversible decline in the fluorescent signal of the reporter due to photobleaching. The inter-stimulus interval will depend on the strength of response observed and the rate of photobleaching. The appropriate length of inter-stimulus interval to avoid sensory adaptation can be determined approximately by repeated presentation of a positive control odor (if known) in trial experiments. Inclusion of this odor every ~10 stimuli in a given series can allow verification of the continued viability and consistent responsiveness of the preparation.

5. Data Processing and Analysis

Data is processed using ImageJ (with Mac biophotonics plug-in, www.macbiophotonics.ca) and custom written programs in Matlab and R as follows:

1. Correct for sample movement artifacts: align all the images corresponding to one animal preparation (e.g. 30 measurements of 40 frames each) using the "rigid body" mode of StackReg/TurboReg (bigwww.epfl.ch/thevenaz/stackreg) in ImageJ. This step is required to remove shifts in the position of the labeled neurons with respect to the acquisition frame within and between measurements. However, it is only possible to remove shifts in the xy plane. Data should be discarded if strong changes in focus occur.

2. Segment the stack into individual 10-second (40-frame) measurements.
3. Correct for bleaching artifacts: exposure to fluorescent light will cause fluorescence to decay during a single measurement¹⁰. In some cases, it is important to correct for this decay before analyzing the data. For each measurement calculate a background frame. This should be the mean of several frames before stimulus onset (e.g. frames 6-14). Divide each frame by this background frame to obtain the relative fluorescence changes. Calculate the mean relative fluorescence value for each frame by averaging all pixels within the area labeled by G-CaMP. Fit a double exponential to the mean fluorescence curve, giving stronger weight to the frames before the stimulus and zero weight to the time of the odor response (20 frames after stimulus onset in our case). Subtract the fitted curve from the relative fluorescence curve of each pixel. Multiply the corrected frames by the background frame to re-obtain raw data. This kind of correction may lead to a modification in the response dynamics if the signal does not return to the baseline by the end of the measurement (e.g. if inhibitory or very strong excitatory responses are evoked) (Figure 4). While correction of bleaching artifacts is useful in many cases, interpretation of temporal parameters should be made with caution if a bleach correction is applied.
4. Calculate the relative change in fluorescence ($\Delta F/F$) for each frame of each measurement as $(F_i - F_0)/F_0 * 100$, where F_0 is the background frame (see 5.3), and F_i the fluorescence value for the i^{th} frame of the measurement.
5. Calculate the mean $\Delta F/F$ within a circular region of interest in a glomerulus (Figure 3B) with a diameter of 10 pixels to obtain activity traces (Figure 5A). If desired, transform these traces into heatmaps using ImageJ (Figure 5A).
6. Calculate the peak amplitude of the response by subtracting the mean $\Delta F/F$ of four frames before stimulation from the mean $\Delta F/F$ of four frames during the peak of the response. The same procedure is used both to illustrate spatial response patterns (see Figure 3B) and to quantify the response amplitude in specific regions of interest across flies (see Figure 5B). The peak response can also be quantified by integrating the area below the curve during the time of the stimulus, or averaging the frames around the maximum of the response.

6. Representative Results

We used the protocols above to record and analyze propionic acid-evoked responses of flies expressing G-CaMP1.6²⁸ under the control of the promoter for the olfactory co-receptor *IR8a*, which is active in several different populations of OSNs^{29,30} (Figures 3-5). Stimulation with 1% propionic acid elicits increases in G-CaMP fluorescence - indicating increases in intracellular calcium - in OSNs innervating two glomeruli, DC4 and DP11 (Figure 3B). We illustrate different ways of representing data from multiple flies in Figure 5: line activity traces, heatmaps and a bar plot of peak responses. Propionic acid-evoked responses have distinct peak amplitudes and temporal dynamics in DC4 and DP11. Moreover, although responses are generally robust, variability between individual animals can also be observed in both glomeruli (see heatmaps in Figure 5A). Quantification of the peak responses (Figure 5B) allows a simple statistical comparison between different glomeruli and different odorants across animals. However, odor-evoked calcium responses can have non-uniform temporal dynamics, and quantifying their magnitude with a single value will disregard this complexity.

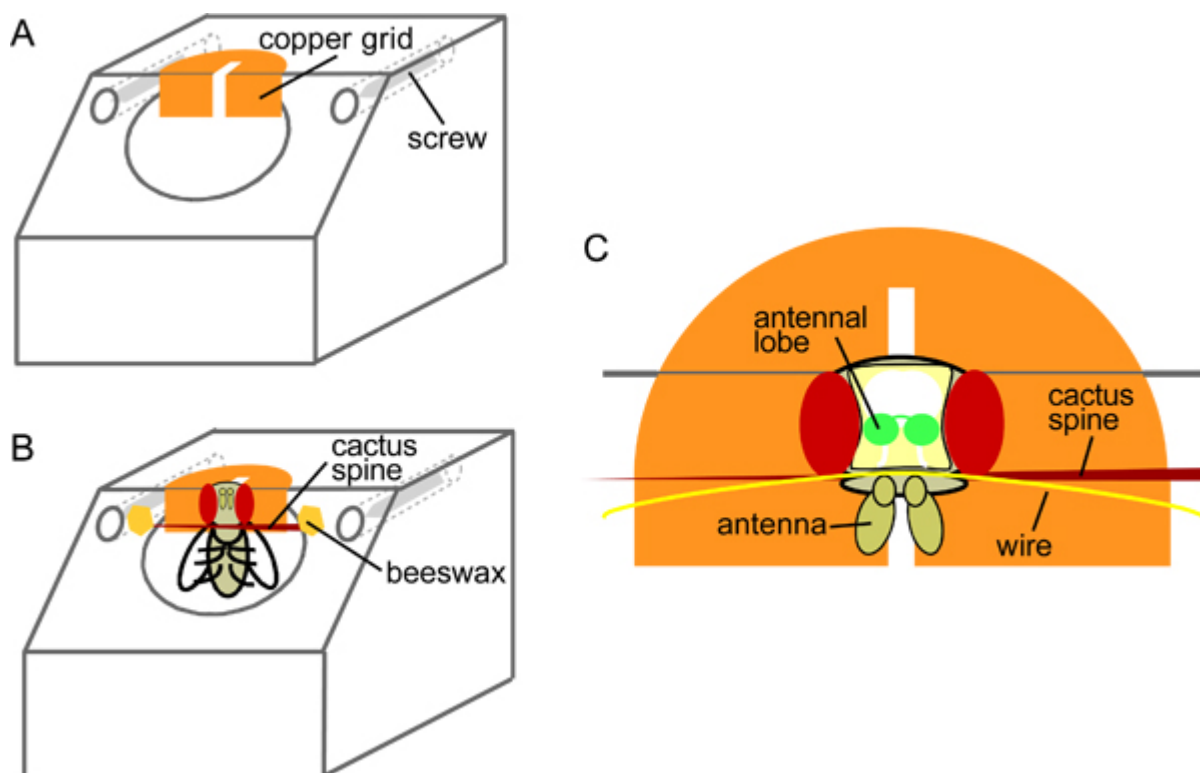


Figure 1. Schematic of the mounting block (A) before and (B) after introduction of the fly, and (C) a top view in close-up (adapted from³¹).

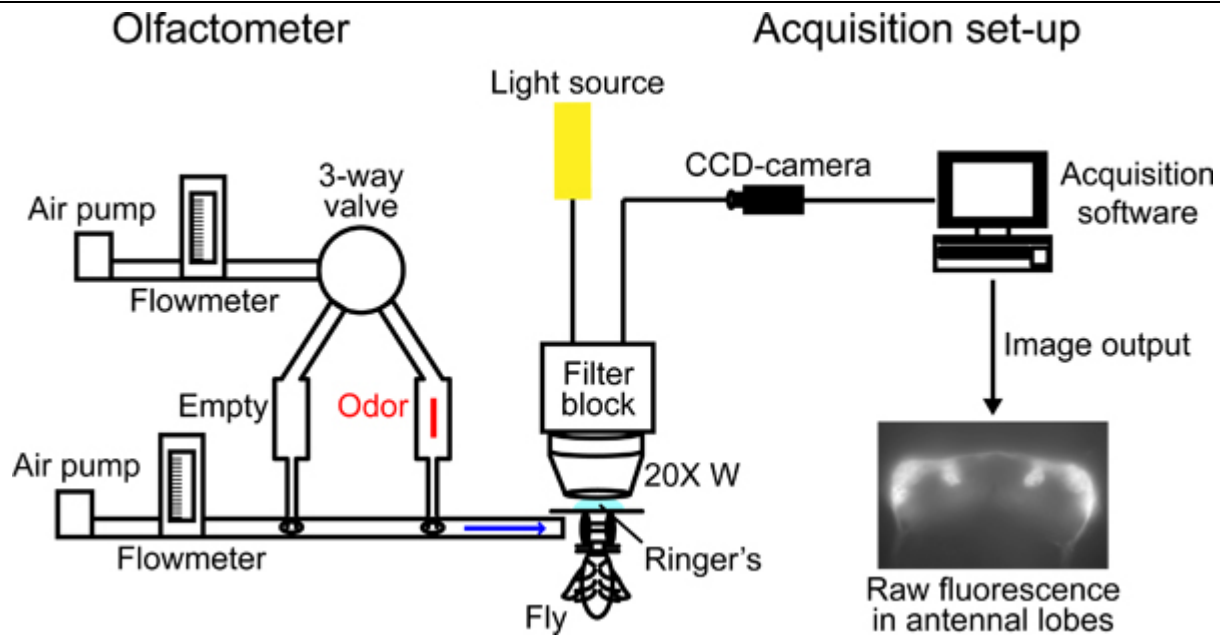


Figure 2. Schematic of the experimental set-up.

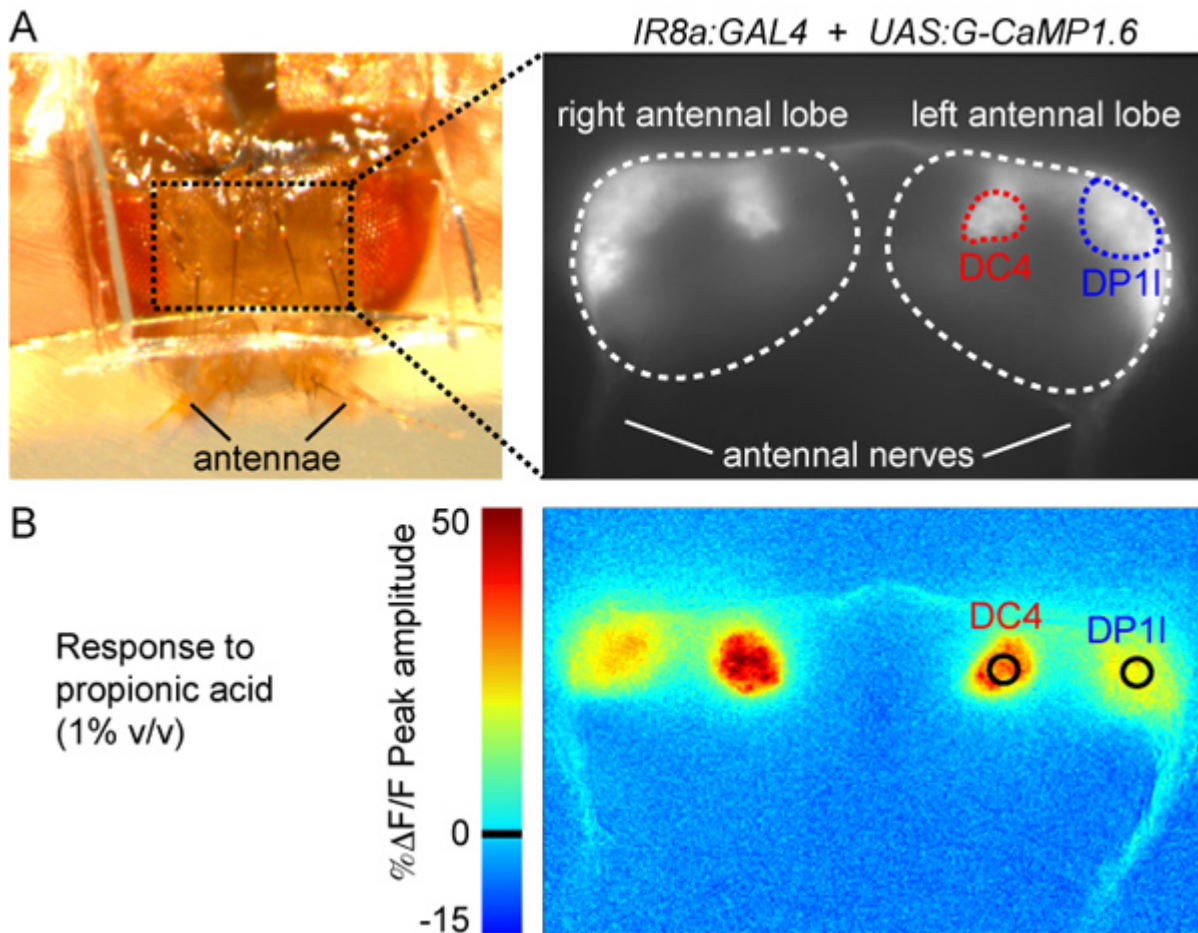


Figure 3. (A) Top view of the fly preparation after step 2.8 (left panel) and raw fluorescence image of the antennal lobes labeled with G-CaMP1.6 (Genotype: *IR8a promoter:GAL4; UAS:G-CaMP1.6*). DC4 and DP11 glomeruli can be distinguished by their morphology, size and position and are marked in red and blue, respectively.

(B) Color-coded image of the response to propionic acid (1% v/v). The image corresponds to the $\Delta F/F$ of the peak of the response calculated as explained in step 5.6 (see also Figure 5A). The colorscale on the left indicates the $\% \Delta F/F$ value. The black circles indicate the position and size of the region of interest used to quantify the responses (see Figure 5).

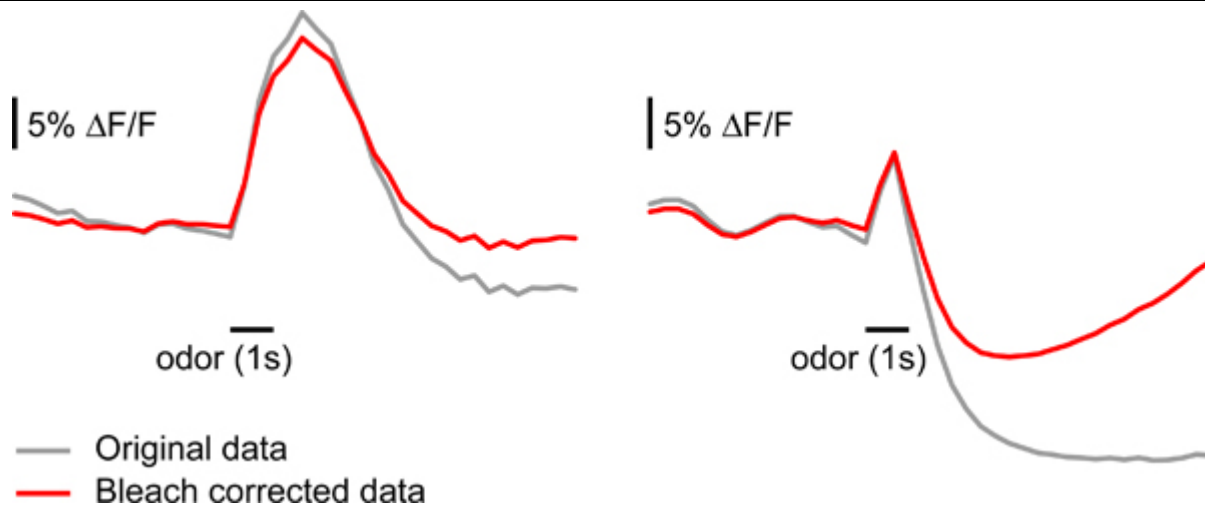


Figure 4. Correcting bleaching of the fluorescent reporter signal. Examples of the effect of bleach correction as described in step 5.3. Sample data on the left panel can be corrected without major changes in the shape of the response. Sample data on the right panel is severely affected by bleach correction, likely because the signal drops and is maintained below baseline after odor stimulus termination.

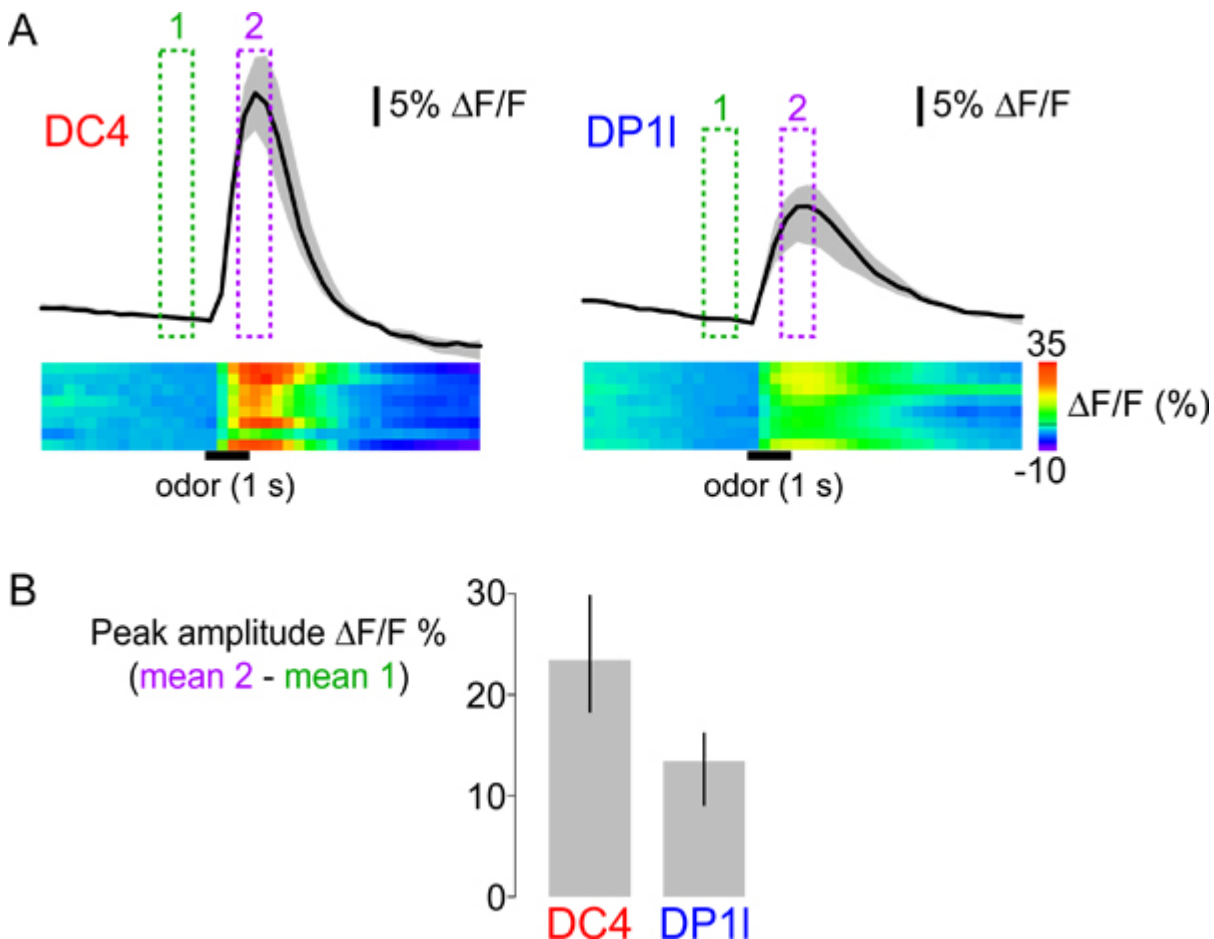


Figure 5. (A) Top: temporal dynamics of calcium responses of glomeruli DC4 (left) and DP11 (right) to propionic acid (1% v/v). The black traces show the median of the mean $\Delta F/F$ within the region of interest (see Figure 3B) across eight animals. The grey surface indicates the range between the first and third quartiles of the distribution. Presentation of median and quartile values provides more information about the variability and distribution of observed responses than mean and standard error. The green and magenta boxes indicate the frames averaged to calculate the peak responses shown in Figure 3B, and quantified in Figure 5B. Bottom: the heatmaps show the response data for all individual animals in separate rows, with $\Delta F/F$ values represented by the colorscale (far right). The horizontal black bars indicate the time and duration of the stimulus. (B) Median peak responses to propionic acid across eight animals for DC4 and DP11 glomeruli. The error bars indicate the range between first and third quartiles of the distribution.

Discussion

The preparation and analysis methods described here can be used to analyze odor-evoked responses in any population of neurons in the antennal lobe (OSNs, LN and PNs) for which a corresponding driver transgene is available. While we describe its application using a fluorescence microscope, optical imaging of this preparation can equally be performed using confocal or two-photon microscopy^{22,32}. Indeed,

these latter instruments alleviate the light-scattering problem of wide-field microscopy, which can reduce the resolution of images, especially when large numbers of neurons express the reporter. This preparation usually permits recordings of odor-evoked responses for at least half an hour, but this time can be significantly longer or shorter depending on the number and length of measurements recorded, the exposure time used for each frame and the inter-stimulus interval. Furthermore, two-photon excitation can reduce photobleaching of the fluorescent reporter, as well as cellular phototoxicity, to permit measurement over longer time periods^{22,32}.

Regardless of the choice of microscope, movement of the fly brain within the head capsule is the most recurrent problem when imaging in intact animals. While movement correction can correct minor issues (step 5.1), it is usually better only to record from highly stable preparations. Strategies to reduce movement artifacts include: (i) properly constraining the proboscis of the fly with the cactus spine; (ii) immobilizing the fly's legs by fixing them to the stage with eicosane (prior to step 2.6) (eicosane can be melted at ~37 °C; use a silver wire to extend and refine the tip of the soldering iron for this purpose); (iii) nicking the oesophagus (visible between the antennal nerves) with fine tweezers.

In addition to G-CaMP1.6 used here, a number of optimized versions of G-CaMP and other calcium indicators are available, which differ in their signal-to-noise ratio, baseline fluorescence and temporal dynamics^{12,28,33}. The precise choice of sensor should take into account all of these properties, the type of neurons to be analyzed and the biological question being addressed. Current sensors report calcium changes that correlate fairly well with action potentials^{12,34}, and their further improvement, coupled with increasing numbers of specific genetic driver lines³⁵ will continue to enhance the potential of optical imaging of neural activity in the intact fly.

Disclosures

The authors have nothing to disclose.

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