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TITLE:

In Vivo Optical Calcium Imaging of Learning-Induced Synaptic Plasticity in *Drosophila melanogaster*

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KEYWORDS:

Drosophila melanogaster, mushroom body, synaptic plasticity, learning and memory, olfactory associative learning, classical conditioning, optical calcium imaging, two-photon microscopy, memory engram

SUMMARY:

Here we present a protocol with which pre- and/or postsynaptic calcium can be visualized in the context of *Drosophila* learning and memory. In vivo calcium imaging using synaptically localized calcium sensors is combined with a classical olfactory conditioning paradigm such that the synaptic plasticity underlying this type of associative learning may be determined.

LONG ABSTRACT:

Decades of research in many model organisms have led to the current concept of synaptic plasticity underlying learning and memory formation. Learning-induced changes in synaptic transmission are often distributed across many neurons and levels of processing in the brain. Therefore, methods to visualize learning-dependent synaptic plasticity across neurons are needed. The fruit fly *Drosophila melanogaster* represents a particularly favorable model organism to study neuronal circuits underlying learning. The protocol presented here demonstrates a way in which the processes underlying the formation of associative olfactory memories, i.e., synaptic activity and their changes, can be monitored in vivo. Using the broad array of genetic tools available in *Drosophila*, it is possible to specifically express genetically encoded calcium indicators in determined cell populations and even single cells. By fixing a fly in place, and opening the head capsule, it is possible to visualize calcium dynamics in these cells

whilst delivering olfactory stimuli. Additionally, we demonstrate a set-up in which the fly can be subjected, simultaneously, to electric shocks to the body. This provides a system in which flies can undergo classical olfactory conditioning – whereby a previously naïve odor is learned to be associated with electric shock punishment – at the same time as the representation of this odor (and other untrained odors) is observed in the brain via two-photon microscopy. Our lab has previously reported the generation of synaptically localized calcium sensors, which enables one to confine the fluorescent calcium signals to pre- or postsynaptic compartments. Two-photon microscopy provides a way to spatially resolve fine structures. We exemplify this by focusing on neurons integrating information from the mushroom body, a higher-order center of the insect brain. Overall, this protocol provides a method to examine the synaptic connections between neurons whose activity is modulated as a result of olfactory learning.

INTRODUCTION:

Deciphering where and how the information is acquired in the brain through learning and subsequently stored as memory constitutes one of the most challenging tasks in neuroscience[1]. Neuroscientific research has led to the concept of a change in synaptic transmission as the neuronal substrate that underlies learning and memory formation[2,3]. It is hypothesized that, during learning, synaptic connections between neuronal ensembles that are active during the perception of a stimulus become modified such that their combined activity pattern can be retrieved during memory recall, thereby instructing future behavioral action[4]. These "engram cells" and their synapses are often distributed across brain regions and levels of processing, which makes it difficult to assign observed changes in synaptic transmission to the learning of a task or a stimulus. To localize and visualize those synaptic changes that are causally linked to a specific learning task one needs an appropriate model system that allows for precisely confining those synapses.

For such an endeavor, *Drosophila melanogaster* is particularly suitable because it combines relative brain simplicity, behavioral richness, and experimental accessibility. Among the well-established model organisms, *Drosophila* is situated between the nematode *C. elegans* and genetically tractable mammals like mice in terms of neuronal complexity. The stereotypic number of neurons (~300) and limited behavioral repertoire is observed in *C. elegans*. Mammals, on the other hand, have millions of neurons and staggering behavioral complexity. The brain of the fruit fly is, with its ~100,000, neurons significantly smaller than the brains of most vertebrates, and many of the neurons are individually identifiable[5]. Yet, *Drosophila* demonstrate a broad spectrum of complex behaviors, including an ability to exhibit robust associative olfactory learning and memory formation, first described over 40 years ago[6]. In the course of this classical conditioning procedure, groups of flies are subjected to an odor as the conditioned stimulus (CS^+) while they receive a punishing electric shock as the unconditioned stimulus (US). A second odor (CS^-) is then presented without any punishment. Thereby, the animals learn to avoid the odor associated with the punishment, which can be tested in a subsequent choice situation between the two odors, CS^+ and CS^-. Work on dissecting the neuronal substrate underlying this behavior in *Drosophila* has identified the mushroom bodies (MB) as the primary site of the "engram"[7-10] and, therefore, the circuitry of this brain region was and is the subject of intense research in order

to uncover the logic by which a memory engram is acquired and stored (recently reviewed in[11, 12]).

The *Drosophila* MB consists of ~2,000 intrinsic neurons (Kenyon cells) per hemisphere, organized in parallel axonal projections[13]. Axons of olfactory projection neurons are extended to the lateral protocerebra and to the MB calyces, the main dendritic input site of the MB and receive olfactory input from antennal lobes. The long, parallel axons bundle of Kenyon cells constitute the peduncle and the lobes. Most Kenyon cells bifurcate forming horizontal β/β'-lobes by extending one collateral towards the midline of the brain, and the vertical α/α'-lobes by extending second collateral projecting in the dorsal-anterior direction. The other group of Kenyon cells forms the horizontal γ-lobes[13] of the MB where the learning process and subsequent short-term memory formation could be localized[10]. The MB lobes receive afferent input and provide efferent output, both of which are typically restricted to distinct compartmental sub-regions along the Kenyon cell axons[14-16]. In particular, afferent dopaminergic MB input neurons have been shown to mediate value-based, e.g., punitive, reinforcing effects in associative olfactory learning[15,17]. Stereotypic and individually identifiable efferent MB output neurons from the mushroom body lobes integrate information across large numbers of Kenyon cells, target diverse brain areas and bear behavior-instructive appetitive or aversive information[15]. This neuronal architecture has led to a concept of the organization of the associative engram. Odors are relatively precisely encoded by sparsely activated ensembles of Kenyon cells. The coincident activity of these Kenyon cell ensembles and release of dopamine - evoked by punishing stimuli - modulates transmission from Kenyon cell presynapses onto MB output neurons such that the animals will subsequently avoid this particular smell[10,12]. We use this rather precisely defined and localized engram as a paradigmatic case to illustrate how these learning-dependent changes in synaptic activity can be determined and monitored.

The value of *Drosophila* as a model system relies strongly on the unmatched genetic toolbox that allows one to express transgenes for identifying, monitoring, and controlling single neurons within complex circuits[18]. The advent of techniques for neuronal activity monitoring - such as calcium imaging, discussed here - have allowed for the determination of neuronal activity patterns in response to a specific stimulus. By combining specific Gal4-driven expression of genetically encoded calcium indicators (GECIs) with olfactory stimulation, one can visualize the odor-evoked calcium dynamics of neurons of interest[19]. In this protocol, it is shown that by further coupling this technique with a classical conditioning paradigm, it is possible to examine these olfactory responses in the context of learning. Learning-induced plasticity can be further dissected using GECIs that are not only localized to a single specific neuron, but also to specific subcompartments of a neuron. Pech et al.[20] established a selection of tools that allow exactly this. By targeting GCaMP3[21] to either the pre- or postsynapse – via linkage to the vertebrate Synaptophysin or *d*Homer, respectively[20] – the differential modulation of these sites can be distinguished. This localization confers, in this context, an advantage over most GECIs that are ubiquitously present throughout the cytosol - e.g., GCaMP[22], GCaMP3[21], or GCaMP6[23] - because it means that pre- and postsynaptic transients can be distinguished from the overall integrated calcium influx that occurs as a result of neuron activation. This can provide clues about the location and types of plasticity that occur as a result of or that cause learning and memory

formation. As an example, the protocol provided here shows the value of this tool in deciphering the modulation of MB output neurons during olfactory associative learning by targeting the expression of the calcium sensor to only the postsynapse. By monitoring, within an individual fly, odor-evoked activity before and after olfactory conditioning a direct comparison can be drawn between a naïve odor response and a learned odor response. Whilst fixed in the same imaging chamber, flies are exposed to a selection of odors. Then, they receive an aversive associative conditioning protocol in which one of these odors is paired with electric shock (becoming the CS^+) and another odor is presented without reinforcement (becoming the CS^-). Finally, the flies are again exposed to the same odors as in the first step. Calcium dynamics are observed using two-photon microscopy.

PROTOCOL:

1. Transgenic fruit flies, *Drosophila melanogaster*

1.1. Cross female virgin and male flies (raised at 25 °C in 60% relative humidity on a 12 h light/dark cycle) carrying the desired Gal4 and UAS constructs[25], respectively, to produce flies in which specific neurons of interest express a genetically encoded calcium indicator.

1.2. Age the female progeny of the above cross until they are in the range of 3-6 days post-eclosion. Female flies are preferable because of their slightly larger size.

2. Preparation of the fruit fly for in vivo calcium imaging

2.1. Select a single female fly and anesthetize on ice for no longer than 5 min.

2.2. Using fine forceps, place the fly in the imaging chamber (demonstrated in **Figure 1c**). Ensure the thorax and legs are in contact with the electrical wires in the bottom of the chamber and that the head lays flat. Fix the position of the fly using clear adhesive tape.

NOTE: This protocol requires a custom-built chamber in which the flies are fixed, with the head capsule accessible for opening, and the flies able to receive both odor stimulations to the antennae and electric shocks to the thorax and legs (**Figure 1**).

2.3. Using a surgical scalpel blade fixed to the scalpel handle (see **Table of Materials**), cut a window in the tape around the head of the fly, leaving the antennae covered and only the anterior-most portion of the thorax exposed.

2.4. Surround the sides and back of the head with blue-light curing glue, carefully manipulated using an insect pin held by concave-convex jaws. Set the glue using a blue light-emitting LED lamp.

2.5. Check that the glue is completely set and clear any residual unhardened glue from the dorsal surface of the fly head.

2.6. Apply a drop of Ringer's solution[24] (see **Table of Materials**) to cover the exposed cuticle of the head.

2.7. Using a very fine-bladed stab knife, cut through the cuticle. For the most efficient removal of the cuticle, first cut across the posterior of the head using the ocelli as a starting point. Then, cut up each side, medial to the eyes, to form a flap of the cuticle that can be easily torn off using forceps.

2.8. Remove any excess cuticle that may block the brain region of interest.

2.9. Carefully clear the dorsal surface of any trachea using fine forceps, avoiding disruption of the brain tissue itself. Remove and refresh Ringer's solution[24] (see **Table of Materials**) as required to clear the area of tissue debris.

2.10. Place the hypodermic odor delivery needle in position, approximately 1 cm from the head of the fly. Ensure there is nothing that could obstruct odor delivery to the antennae.

2.11. At the microscope, connect the imaging chamber to the odor-delivery system via the hypodermic odor delivery needle.

2.12. To allow the fly to fully to recover from anesthesia and surgery, and to adapt to the air flow, implement a 10 min resting period at this stage.

3. In vivo calcium imaging

3.1. Use a multiphoton microscope equipped with an infrared laser and a water immersion objective (see **Table of Materials**), installed on a vibration-isolated table. For the visualization of GFP-based calcium indicators, tune the laser to an excitation wavelength of 920 nm and install a GFP band-pass filter.

3.2. Using the coarse Z adjustment knob, scan through the Z axis of the brain and locate the brain region of interest. Use the crop function to focus scanning on only this area to minimize scan time, and to rotate the scan view such that the anterior of the head is facing downwards.

3.3. Adjust the frame size to 512 x 512 px, scan speed to > 4 Hz, and scan region (in the Y dimension) so that the neurons of interest are covered.

4. Visualization of odor-evoked calcium transients through olfactory conditioning

4.1. Use an odor delivery system[19,26] that can deliver several odor stimuli in a temporally precise manner.

4.1.1. Use an additional computer to control the device, and to communicate with the imaging microscope software to coordinate odor stimulations with image capture during experiments.

4.1.2. Initiate a pre-programmed macro package capable of linking the image acquisition software and odor delivery program (e.g., a VBA macro package installed in the microscope control software, see **Table of Materials**) that is responsive to an external input trigger provided by the initiation of an odor delivery protocol in a separate program).

4.2. Start the measurement by monitoring "pre-training"/naïve odor-evoked calcium transients at a resolution of 512 x 512 px and a frame rate of 4 Hz. Deliver a 2.5 s odor stimulus flanked by additional image acquisition for 6.25 s preceding odor onset (to establish an F_0 baseline value) and 12.5 s after odor offset. Repeat this with a second odorant and then with a third odorant.

4.3. Continue 3 min after this measurement with classical conditioning ("training") the fly.

4.3.1. Select one of the odors presented in the "pre-training" phase to become the CS^+ odor and another to become the CS^- odor. Present the CS^+ odor using the computer-controlled odor-deliver system for 60 s alongside twelve 90 V electric shocks.

4.3.2. After a 60 s break, present the CS^- odor alone for 60 s. Use as odorants 4-methylcyclohexanol and 3-octanol. Do not present the third odorant (e.g., 1-octen-3-ol) during this training as it is used as control only before (step 4.2.) and after (step 4.4) the training phase.

4.4. Measure the "post-training" odor-evoked calcium transients again by repeating the "pre-training" odor stimulation protocol (step 4.2.) 3 min after finishing the training phase (step 4.3).

NOTE: The timing of this step should reflect the time of interest after memory formation (e.g., carry out this step 3-4 min after the conditioning step to investigate short-term memory). Typically, flies can survive for several hours in this preparation.

4.5. Save imaging files in an appropriate format (e.g., Tiff) for later image analysis.

5. Image analysis

5.1. To analyze images, open Tiff (or similar) files in the image analysis software such as Fiji[27].

5.2. Align the stacks using a movement correction algorithm to ensure minimal movement in the X and Y directions. Discard any recordings that show strong movement in the Z axis.

5.3. Select the tool of the software used to mark a region of interest (ROI) around the area that is to be examined. This should be as precise as possible to limit the influence of background fluorescence.

5.4. Use the respective tool of the software to extract temporal fluorescence intensity data as data files from the selected ROI, such that a value is generated for each frame of the recording.

5.5. Open the data using a data analysis program to calculate the $\Delta F/F_0$ values for each odor trace. F_0 = average fluorescence in the 2 s before odor onset. ΔF = the difference between the raw fluorescence in a given frame and F_0. From these values, calculate a $\Delta F/F_0$ value for each frame which can be plotted against time to reflect relative fluorescence throughout the odor stimulus period.

REPRESENTATIVE RESULTS:

An example of images acquired with the above protocol can be seen in **Figure 2**. *d*Homer-GCaMP3 is expressed in an MB output neuron whose dendrites innervate the compartment 1 of the MB γ-lobe (the neuron is termed MVP2[28, 29]) and is genetically targeted using the split-Gal4 line MB112C[16]. Also, demonstrated is the difference in the subcellular localization of a cytosolic and the post-synaptically localized calcium indicator. When comparing **Figures 2a** and **Figure 2f**, one can clearly observe the specific, compartmentalized expression of the dHomer-GCaMP3 sensor[14] – with no expression in the axonal compartments of the neuron, and a punctuated signal visible in the dendritic compartment. Clear – though the lower amplitude – odor responses can be seen in flies expressing *d*Homer-GCaMP3 (**Figure 2**, lower panels), compared to flies expressing cytosolic GCaMP6f[23] (**Figure 2**, upper panels). This demonstrates that the tool is effective for the visualization of olfactory responses at the level of the postsynapse, and, therefore, provides an additional specificity to the examination of the neural circuits underlying, in this case, odor encoding and olfactory learning.

An example of the latter can be seen in **Figure 3**. In this experiment, *d*Homer-GCaMP3 is expressed as in **Figure 2** - in the mushroom body output neuron MVP2. This is a neuron known to be modulated through olfactory learning such that presynaptic depression as a result of aversive olfactory conditioning is reflected in a deceased postsynaptic response to the trained odor[28,29] and here a confirmation of this result is shown using this in vivo calcium imaging protocol. The calcium traces shown in **Figure 3** represent exemplary data from one individual fly. Note that the noise level and amplitude can vary between individual preparations (e.g., compare **Figure 2e** and **Figure 3c-e**). Therefore, a within-animal comparison of pre- vs. post-training provides a way to account for inter-individual variability. Of course, the protocol is suitable to be transferred to imaging of other neurons of interest.

FIGURE AND TABLE LEGENDS:

Figure 1: Construction of a mounting chamber and preparation of a fly for imaging. (**a**) Steps for the construction of the fly mounting chamber. The base is formed of a standard microscope slide (1) covered with a fine plastic mesh (2). Two electrical wires carrying opposing charges (3) are glued to the slide on either side. The wires are stripped and bent to cross the slide (4) running parallel with one another but not in contact. Layers of clear adhesive tape (5) are added until reaching the height of a fly (approximately 1 mm). A channel is cut through the tape vertically (6), approximately 1 mm wide to fit the width of a fly. An elevated platform made of clear adhesive tape (7) is built just above the electrical wires to form a pillow for the fly's head whilst the thorax is on top of the wires. (**b**) Schematic illustration of the fly positioned under the two-

photon microscope. The odor stimulation is achieved through a hypodermic needle positioned in front of the fly's head (inserted through the channel in the tape (6) and on top of the platform (7) to direct the odors to the antennae). (**c**)-(**h**) Fixing and opening of the fly head. (**c**) A fly fixed inside the imaging chamber, inside the channel (Scale bar = 1 mm) and held in place by a fresh piece of clear adhesive tape over the whole fly. (**d**) Magnified view of the fly in position. Scale bar = 0.1 mm. (**e**) A window is cut into the tape around the head of the fly. (**f**) The head is further fixed with blue light-curing glue. (**g**) The head capsule is covered with Ringer's solution and opened. (**h**) Completed preparation of the brain with excess tissue and trachea (white tissue visible in (**g**) removed.

Figure 2: Postsynaptically-localized calcium visualized using *d*Homer-GCaMP3. (**a**) Confocal microscopy images showing the expression of cytosolically localized GCaMP6f (i) and postsynaptically localized *d*Homer-GCaMP3 (ii) in the MB output neuron MVP2. Green arrow indicates the γ1 subregion of the MB γ-lobe. Scale bars = 15 µm. (b)-(d) Images captured using two-photon excitation microscopy of the genotypes above, showing the in vivo fluorescence of the respective calcium sensors. (**b**) F_0 (baseline fluorescence) images, demonstrated as an average intensity projection of the 2 s before odor onset. Scale bars = 10 µm. (**c**) F_{odor} images (fluorescence during odor stimulation), demonstrated as an average intensity projection over the odor response period (2.5 s). (**d**) ΔF images generated by subtracting F_0 image from F_{odor} image, to show actual odor response signal. (e) $\Delta F/F_0$ traces from the above flies of the respective genotypes through an odor stimulation (grey bar).

Figure 3: Learning-induced postsynaptic plasticity visualized via *d*Homer-GCaMP3. (**a**) Schematic of conditioning protocols used in these experiments. Naïve odor responses, before conditioning ("pre") are measured first. Then each fly experiences one of three possible conditioning protocols: "Paired", where one odor (CS^+) is paired with electric shocks (US) and another (CS^-) is not reinforced; "Odors only", where only the odors are presented, both without reinforcement, to control for odor exposure effects; and "Shocks only", where only electric shocks are presented without any odor stimuli, to control for shock exposure effects. After this conditioning phase, flies are then exposed to the "Pre" odors again to test for altered odor-evoked activity ("Post"). (**b**) An example of odor response modulation as a result of the paired odor/shock presentation. Strong suppression of the response to the CS^+ odor can be seen in the γ1 subregion (dotted line). (**c**)-(**e**) Odor response traces from the same fly as in (**b**), showing that this suppression effect is only observed in the case of the trained odor.

DISCUSSION:

The dissection of the neural circuitry underlying learning and memory is a prominent goal in the field of neuroscience. The genetic accessibility of *Drosophila* and the breadth and ease of behavioral testing makes this an ideal tool to investigate such phenomena. Here, a method is presented with which it is possible to visualize, within individual flies, the modulation that occurs at a subcellular level as a result of olfactory conditioning. By carrying out both pre-training and post-training visualization of odor-evoked calcium dynamics, first established by our group[17], it is possible to draw a direct, within-animal comparison between naïve and learned odor responses

and, therefore, examine the plasticity of neurons of interest. This confers an advantage to this protocol over *en masse* assessments (used in classical olfactory conditioning) because pre- and post-training states can be quantitatively assessed for individuals, which allows one to determine inter-individual variability. Moreover, the training procedure performed directly under the microscope is largely equivalent to the classical olfactory conditioning paradigm typically used in behavioral experiments and does not involve artificial optogenetic stimulation of neurons. Of course, handling the small flies and carefully opening the head capsule without damaging the brain tissue while keeping the sensory organs intact requires a level of expertise that can be gained by meticulous and repeated training, not unlike the extent seen in physiological assessments such as electrophysiology.

Additionally, the potential to use localized calcium indicators to examine plasticity at the single neuron level is demonstrated – adding greater precision to the neuronal circuit dissection. This is exemplified by showing a learning-induced depression of a postsynaptic response in a well-investigated MB output neuron[28,29]. *Drosophila* strains expressing a variety of synaptically localized fluorescence sensors under UAS control are available, e.g., expressing the presynaptically localized sensor Synaptophysin-GCaMP3 or the red fluorescent sensor of synaptic transmission Synaptophysin-pHTomato[20]. Of course, the variety of fluorescence sensor proteins can be implemented using the protocol described above.

Optical imaging techniques are in general limited by the temporal and spatial resolution of the microscopic image acquisition system (e.g., a two-photon microscope) and the temporal resolution of the sensor itself (e.g., the kinetics of the calcium sensor). Electrophysiological recordings, e.g., using patch clamp electrophysiology from somata of neurons in the *Drosophila* brain, still offer an unmatched temporal resolution, but without providing any spatial precision. Due to the specificity of the gene expression in neurons of interest combined with the subcellular localization of the sensor, optical imaging techniques can potentially complement electrophysiological techniques to uncover neuronal plasticity underlying learning and memory formation. The continuous progress in the development of fluorescence sensors will perhaps provide the possibility to fuse sensor proteins with faster kinetics or higher signal-to-noise ratios (e.g., GCaMP6 variants[23]) with synaptically localized proteins like *d*Homer. Also, recent advances in establishing microscopic techniques with faster acquisition rates or higher spatial resolution will prove invaluable in further fine-tuning of our approach.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

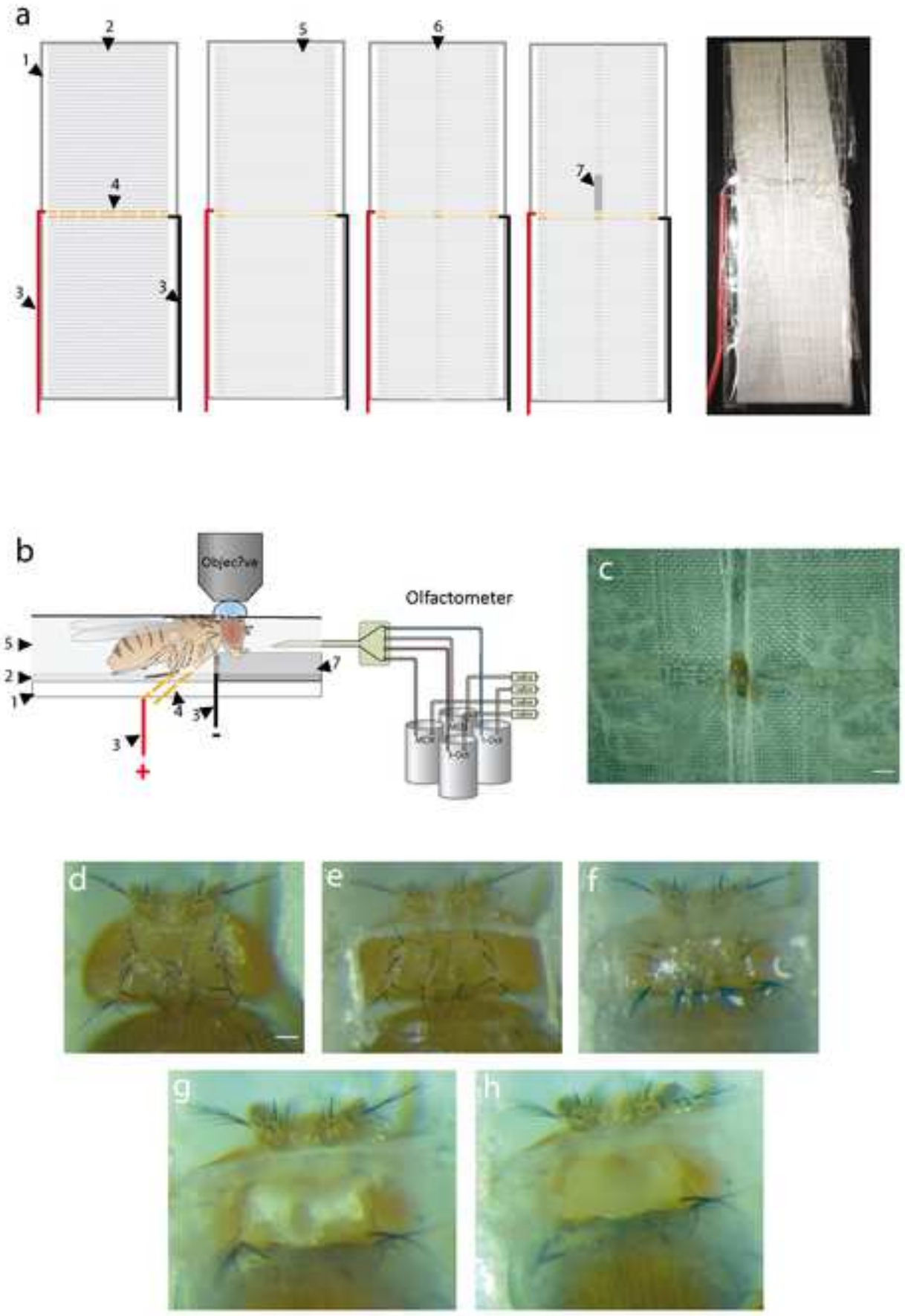


Figure 2

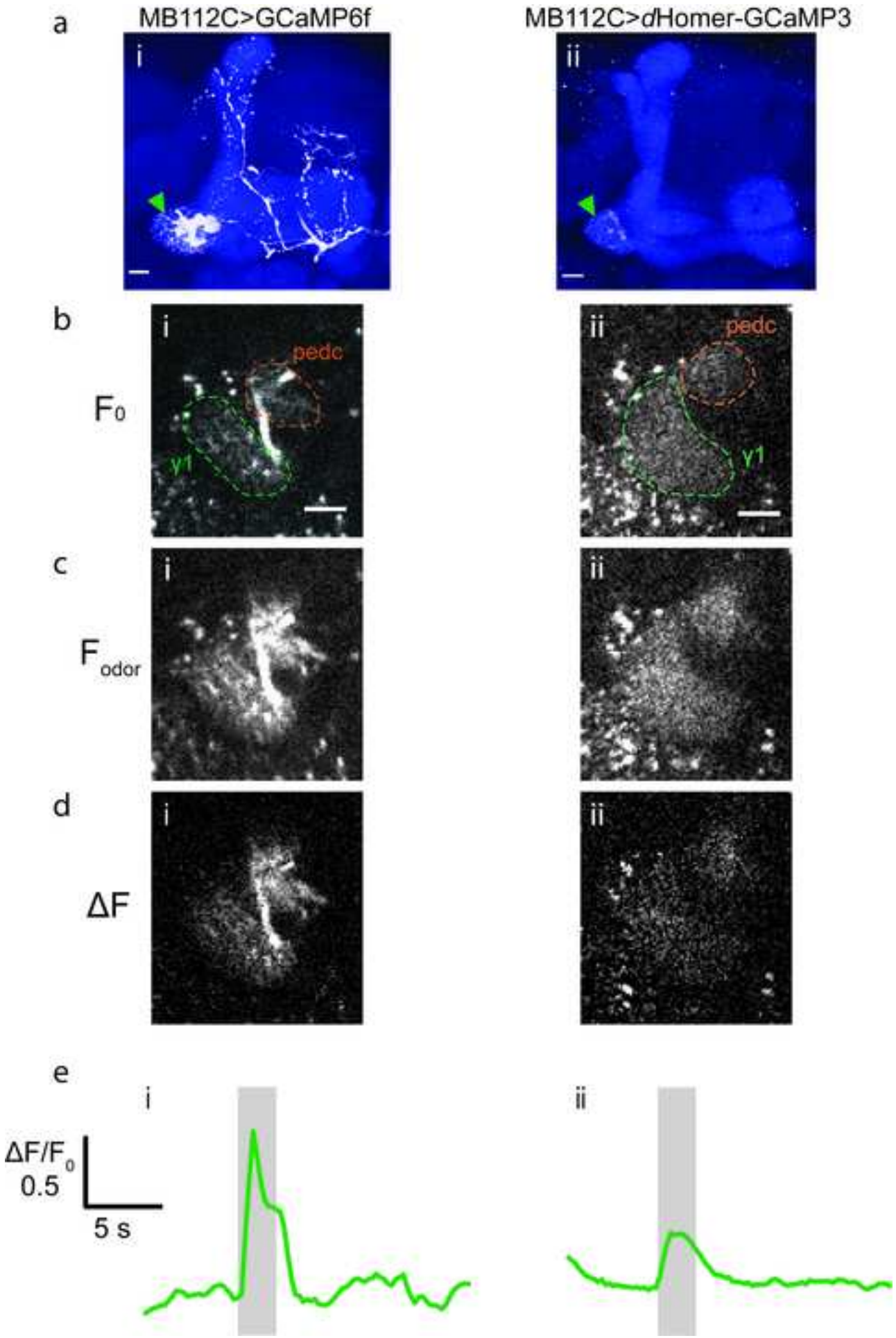
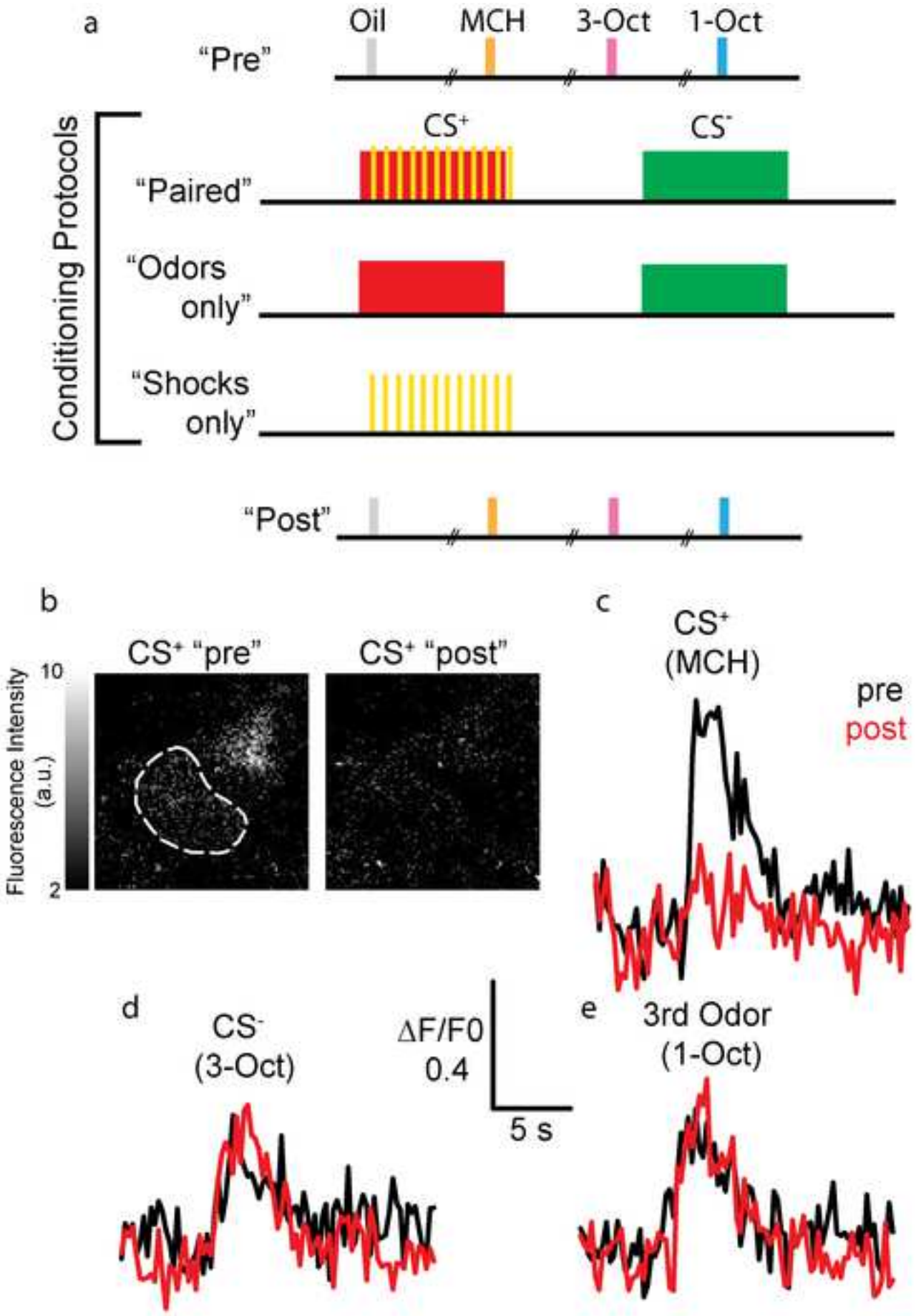


Figure 3



Name of Material/ Equipment

1-Octen-3-ol

3-Octanol

4-Methylcyclohexanol

Bandpass filter for EGFP (525/50 nm)

Clear adhesive tape

Concave-convex jaws

Fine forceps

Hypodermic needle

Insect Minutien pins

Kentoflow

Microscope slide

Mineral oil

Mode-locked Ti-Sapphire laser Chameleon Vision 2

Multiphoton Microscope LSM 7MP equipped with BiG detectors

Plan-Apochromat 20x (NA = 1.0) water immersion objective

Ringer's solution

Stab knife

Surgical scalpel blade

Surgical scalpel handle

Visual Basics of Applicatons (VBA) software to receive a trigger from the odor-delivery device and the electric sh

Company

Sigma-Aldrich, St. Louis, MO, USA

Sigma-Aldrich, St. Louis, MO, USA

Sigma-Aldrich, St. Louis, MO, USA

Carl Zeiss Microscopy GmbH, Jena, Germany

Tesa SE, Norderstedt, Germany

Fine Science Tools, North Vancouver, Canada

Fine Science Tools, North Vancouver, Canada

Sterican - B. Braun, Melsungenk, Germany

Fine Science Tools, North Vancouver, Canada

Kent Express Dental Supplies, Gillingham, UK

Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Sigma-Aldrich, St. Louis, MO, USA

Coherent Inc., Santa Clara, CA, USA

Carl Zeiss Microscopy GmbH, Jena, Germany

Carl Zeiss Microscopy GmbH, Jena, Germany

n.a.

Sharpoint, Surgical Specialties Corporation, Reading, PA, USA

Swann-Morton, Sheffield, UK

Swann-Morton, Sheffield, UK

Cutom-written and available upon request

Catalog Number

O5284

218405

153095

10053-09

11412-11

4665120

26002-10

953683

0656.1

M8410

421452-9900-000

n.a.

72-1551

0303

0907

n.a.

Comments/Description

Chemical used as odorant

Chemical used as odorant

Chemical used as odorant

Standard claer adhesive tape

Blade Holders with concave-convex jaws

Forceps with tip 0.1 x 0.06mm

1.20x40mm

Diameter 0.1mm, tip 0.0125mm

Blue light-curing glue

Standard objective slide 76 x 26 mm

Used as diluent for odorants

Tunable infrared femtosecond laser

Multiphoton microscope, multiple companies provide similar devices.

Objective W "Plan-Apochromat" 20x/1.0 DIC M27 70mm

5mM KCl, 130mM NaCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 5mM Hepes-NaOH, 36mM sucrose, pH = 7.4

5.0mm Straight restricted blade depth

Product No. 11

Product No. 7S/S

n.a.

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Author(s): Clare E. Hancock, Florian Bilz, André Fiala

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Institution:	University of Goettingen
Title:	In vivo optical calcium imaging of learning-induced synaptic plasticity in Drosophila melanogaster.
Signature:	André Fiala
Date:	May 17th, 2019

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Journal of Visualized Experiments
- Dr. Vineeta Bajaj-
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USA

18th of June, 2019

Response to Reviewers

Dear Dr. Bajaj,

We would like to thank you and the four reviewers for the thorough review process. The reviewers' comments have helped to improve the manuscript substantially. We were also glad to read that all four reviewers were in general very positive about our manuscript. All comments made by you and the reviewers have been addressed in the new version of the manuscript, as outlined below.

Editorial comments:

1. We have proofread the manuscript or proper English language. We have also changed British English to American English throughout.
2. We have indicated email addresses for each author on the title page of the manuscript.
3. We have indicated all commercially available products in the Table of Materials and Reagents.
4. It was checked that the text does not match previously published text.
5. The protocol section was changed to imperative tense throughout.
6. Personal pronouns were removed in the protocol section.
7. Single line spaces were included in the protocol section.
8. Discussions about the protocol were removed to the Discussion section.
9. Large paragraphs of text in the protocol section were removed.
10. It is made clearer how particular steps are performed.
11. The steps for using software are made as explicit as it is reasonably possible.
12. Text in the protocol section has been shortened.
13. The odorants used are indicated as well as how they are presented.
14. About 2.75 pages are highlighted to indicate the filmable content.
15. There are no copyrights on any pictures. All pictures are our own and have not been published before.
16. The discussion section was revised according to your suggestions.
17. The Journal titles in the reference list were expanded.
18. All microscopic images in the figures contain scale bars now.
19. The figures are now changed (odor instead of odour and s instead of sec).
20. The materials in the table are sorted now alphabetically.

Reviewer #1:

1. The highlighted section (approximately 2.75 pages) is intended to indicate the filmable part of the protocol.
2. The exact composition of the Ringer's solution together with the reference of its first description is now indicated.
3. In the discussion section we have now explained in greater detail why we have used GCaMP3 fused with a postsynaptic protein. In fact, there is no fusion construct that would target GCaMP6 to the postsynaptic compartment. We have discussed that this will be a potentially valuable improvement in the future.

Reviewer #2:

1. The terms "razor blade" and "blade holder" were replaced by "surgical scalpel blade" and "surgical scalpel handle".
2. The term "needle" was replaced by "hypodermic needle"′.
3. The reason for using female flies is mentioned now. In fact, female flies are just slightly larger than males.
4. The word "retrained" was replaced by "restrained".

Reviewer #3:

1. New pictures of the fly preparation are included now together with a more details explanation of the assembly of the preparation chamber and the opening of the head capsule (figure 1).
2. Spelling errors were corrected.
3. The reviewer's helpful suggestions for changing some sentences we have included throughout.

Reviewer #4:

1. The design of the imaging chamber we have described now in greater detail and explained the assembly in figure 1. Over the years our group has designed a variety of imaging chambers. The latest version is actually extremely simple but is the most efficient construction because it enables one to perform the preparation very quickly in a few minutes.
2. It was asked how exactly the electric shock system works. We have explained that in more detail in the new version if the manuscript. However, the explanations are not extensive because the entire shock system consists literally of two wires and a power supply only. The arrangement we have described better in now in figure 2 and hope that it will become entirely clear once the video is being provided.
3. The difference in noise level and also in the amplitude of the signal is due to inter-individual variability (e.g., induced by small motions of a preparation). This is indicated now in the section "representative results".
4. The emission filter and the detector used are now indicated in the table of materials.
5. The frame rate used for optical imaging is now indicated.
6. Critical steps in the experimental process have been indicated in greater detail now.

Sincerely yours,

André Fiala