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Preparing adult Drosophila melanogaster for whole brain imaging during behavior and stimuli responses

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TITLE:

Preparing adult *Drosophila melanogaster* for whole brain imaging during behavior and stimuli responses

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SUMMARY:

We present a method specifically tailored to image the whole brain of adult *Drosophila* during behavior and in response to stimuli. The head is positioned to allow optical access to the whole brain, while the fly can move its legs and the antennae, the tip of the proboscis, and the eyes can receive sensory stimuli.

ABSTRACT:

We present a method developed specifically to image the whole *Drosophila* brain during ongoing behavior such as walking. Head fixation and dissection are optimized to minimize their impact on behavior. This is first achieved by using a holder that minimizes movement hindrances. The back of the fly's head is glued to this holder at an angle that allows optical access to the whole brain while retaining the fly's ability to walk, groom, smell, taste and see. The back of the head is dissected to remove tissues in the optical path and muscles responsible for head movement artefacts. The fly brain can subsequently be imaged to record brain activity, for instance using calcium or voltage indicators, during specific behaviors such as walking or grooming, and in response to different stimuli. Once the challenging dissection, which requires considerable practice, has been mastered, this technique allows to record rich data sets relating whole brain activity to behavior and stimulus responses.

INTRODUCTION:

Imaging brain activity using various techniques have deepened the understanding of brain

function. In humans, brain imaging techniques have important limitations: while functional magnetic resonance imaging (fMRI) offers spatio-temporal resolution far below single neuron resolution, fast techniques such as electroencephalography (EEG) only allows indirect and partial access to the brain[1]. In sufficiently big animal models such as rodents, recording of fluorescent activity sensors (e.g., GCaMP) using head-mounted microscopes permits to observe brain activity while the animal is moving in its environment[2]. Nevertheless, these techniques currently give access only to a small portion of the brain. Head-fixed animals can be imaged more comprehensively, but coverage is still partial (e.g., the cortex surface[3]). It is only in small animals, such as the zebrafish larvae, *C. elegans* and *Drosophila* that the whole brain can be imaged with temporal and spatial resolution at the level of or close to single neurons[4].

D. melanogaster is particularly promising because it has long been used as a genetic model organism[5] and powerful genetic tools have been developed[6]. Complemented by the new large scale anatomical network derived from electron microscopy[7], the fly could provide unique opportunities to study complex brain dynamics generated on a large scale network[8]. Although the cuticle is not transparent, and must thus be removed to image the brain, in vivo functional imaging has become more and more common place since the first study in 2002[9] and several protocols have already been published. However, these methods involve either separating the fly head from the body[10], severely restricting the fly's movements and/or responses to stimuli[11–15], or only permitting a small part of the brain to be imaged[9,16,25–27,17–24]. To complement these nevertheless powerful approaches, we recently developed a preparation to image the whole brain during behavior and responses to various stimuli[28].

Here, we build upon this study to present a method specifically developed to image the whole brain while the fly performs semi-naturalistic behavior (i.e. walking and grooming) and responds to sensory stimuli. This is achieved by using an observation holder designed to give access to the whole brain from the dorsal-posterior side, while leaving the antennae and proboscis intact, and permitting the fly to move its legs to walk (e.g., on an air-cushioned ball). Steps for dissecting the back of the head have been refined for speed, reproducibility, and to minimize their effect on the viability and mobility of the fly.

PROTOCOL:

All steps are performed under a stereomicroscope.

1. Preparing the holder

1.1. Print the holder 'FlyholderVJove.stl' (see **Supplementary Material**) with a 3D printer or have it printed using online services (**Figure 1A**). Both SLS (Nylon PA12) and multijet fusion (PA12) are suitable.

1.2. Create the head slot.

1.2.1. Place a piece of sticky tape rectangularly on a flat surface. Cut a slice of approximately 5 mm x 1 cm. Cut the neck slot (~400 x 400 µm) in the middle of the longer side of the tape using fixed parallel blades (two scalpel blades stuck together) to ensure the same width in every holder (**Figure 1B**).

1.2.2. Place the tape over the flatter side of the hole in the holder on the bottom side (see **Figure 1C** and **Figure 1D**). The tape can be deformed by pushing it down with forceps ~500 µm around the hole; this will further minimize hindering later fly movements.

1.2.3. Cover the tape and the holder from the top with black nail polish to prevent the buffer from leaking out. The black nail polish will also protect the fly's eyes from the excitation light of the microscope. Let the nail polish dry at least an hour before using the holder.

1.2.4. Once the nail polish is dry, add ~1 µL of grease in the head slot using a rolled tissue to ensure the glue will not wet the back of the fly's head (**Figure 2A**). Make sure not to put grease outside of the slot that would prevent the glue from sticking.

1.3. Create the body slot (**Figure 2B**).

1.3.1. Optionally, prepare the tape that will be used to position the fly's body (see below) in advance. Use the design shown in **Figure 2B** top to cut a piece of ~2 cm wide tape in two and cut 1.5 mm wide slices. Cut out 0.3 mm deep shoulders and body slot. Make sure that it fits the holder.

NOTE: The variability between fly sizes (in particular with sex, age, genotype or species) can make it necessary to adjust the tape design. If the head tends to be ill-centered, it can be helpful to add a V-shaped piece of tape temporarily over the neck slot (**Figure 3**). Also apply a few microliters of grease inside the neck slot and on the V-shaped tape.

2. Placing the fly

NOTE: One to four days old female flies are ideal because the female head is bigger and thus easier to dissect than the male head, and younger flies have softer cuticle. For walking experiments the activity of the fly can be increased by matching the experiments with times of higher circadian activity (ZT0 or ZT11), by using saline containing glucose (such as 103 mM NaCl, 3 mM KCl, 5 mM TES, 8 mM trehalose 2 H_2O, 10 mM glucose, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2 \cdot 2\ H_2O$, 4 mM $MgCl_2 \cdot 6\ H_2O$), by starving the fly up to 24h with a water only environment, and by heating the environment to ~28 °C during the experiment. Clipping the wings at least one days in advance also helps to decrease attempts to fly and thus increase the frequency of walking bouts[7,29,30].

2.1. Fill a Petri dish or pipette tip box lid with ice, place a laboratory tissue on top of the ice and set the holder upside down on it.

2.2. Transfer a fly on ice to paralyze it by sucking it from its vial into a tube and blowing it onto the ice (make sure the ice is not melted which would drown the fly).

2.3. When the fly stops moving, use dull forceps to slide it into the holder with the neck inside the slot (the fly can be held at the basis of the wing) as in **Figure 4**, left. The eyes should be at equal positions in respect to the sides of the slot (**Figure 4**, middle). If needed, add 1 µL grease on the top of the head to prevent the glue (see next step) from reaching the back of the head and remove later.

2.4. Cover the body with a tissue and some ice to make sure the fly does not move during this step and the next (**Figure 4**, right). Another option to prevent the legs from reaching the head is to use a piece of tape just below the head (see **Figure 6**).

3. Securing the head

3.1. Place the head at a ~20° angle from a fully posterior view (around the lateral axis, see **Figure 5A**). This is a compromise between decreasing the depth to image and on the other side maintaining the front leg free to move and minimizing the stretch of the neck.

3.2. With a rolled-up tissue (**Figure 5B**), put UV-glue around the head while avoiding soiling the sensory area of interest (antennae, proboscis and/or eyes). For taste experiments, pull the proboscis out and add glue at its base to prevent movement. If no taste experiment is planned, the proboscis is best pushed into the head and fixed with glue to minimize movement (**Figure 6**).

3.3. Cure the glue with UV-light for 5 s. Carefully clean the surrounding of the head with a rolled-up tissue to remove remaining liquid glue that could stick to the legs and/or soil sensory areas.

NOTE: The tape can be roughed with sandpaper to increase glue adhesion if necessary.

3.4. Use a thin strip of tape or a rolled-up tissue to move the legs to the front (if they are not already) so they will not be damaged by the next step.

4. Positioning the body

NOTE: This step needs to be performed fast; before the fly recovers from anesthesia.

4.1. Remove the ice container and turn the holder around. Remove the water around the fly with a tissue.

4.2. Place the body slot tape (created in step 1) over the hole and gently push the fly's body down (**Figure 7**). Be careful to not stretch the neck too much.

5. Sealing the hole

5.1. Cover any remaining large holes with tape.

5.2. Add ~1 µL of grease to the back of the head and in the neck area to make sure no glue will wet there.

5.3. With a rolled-up tissue, paint UV-glue around and on top of the tape and on the thorax (upper dorsal part of the mesonotum) to fix it. Cure the glue with UV-light for ~5 s.

NOTE: It is important to minimize the use of UV light as it can strongly affect the fly's health.

5.4. Carefully clean grease and uncured glue with a laboratory tissue.

5.5. Put ~1 mL of saline on top of the head. Push air bubbles aside with forceps. Look for leaks by placing a coverslip over the saline and turning the holder around to check for saline on the front side. If there are any leaks, remove the saline and fix the hole (either by adding more glue or more grease).

NOTE: This can be a good time to pause if necessary. The fly can be offered a small piece of tissue or Styrofoam ball to walk on to prevent flailing and calm the fly down.

6. Dissecting the head

NOTE: Use sharpened forceps for the following steps. Very fine forceps are critical as dull forceps will make it more difficult to open the head cuticle and may lead to additional injuries on the fly's head or brain. Strong magnification can help at this stage. To that aim, one can replace the oculars of the binocular microscope with 30x oculars.

6.1. Make two cuts at the base of the central dark cuticular triangle on each side of the neck (see crosses in **Figure 8A**).

6.2. Cut around the dark triangle and remove this part of the cuticle.

6.3. The hole in the brain through which muscle 16 and the esophagus go should now be visible and move rhythmically (**Video 1** presents this rhythmic movement in a fly with fluorescent muscles). Carefully pinch the top of this area to cut muscle 16 without puncturing the esophagus. If the rhythmic movement of the brain stopped, muscle 16 was likely removed, however, the movement sometimes pause and restart later. It is thus important to pay attention to rhythmic movements and perform this step again if necessary.

6.4. Cut the remaining cuticle in small pieces and remove them carefully. Try not to pull on the cuticle too much. Instead use the forceps like a pair of scissors, to cut pieces of tissue. One can start on the medial edges, where the dark triangle was removed before and work one's way to the sides.

NOTE: Pieces of cuticle can be used to gently scrape of fat bodies if present.

6.5. Remove the air sacks one piece after the other by grabbing them with the forceps and pulling slowly and steadily.

REPRESENTATIVE RESULTS:

The preparation described above allows observation of the whole brain under a microscope for large scale 3D imaging such as classical 2 photons or confocal microscopy, but also faster techniques such as light sheet[31] and other structured illumination microscopy techniques (reviewed in[32]), or light field microscopy[28].

The access to the whole brain while observing the behavior and maintaining functional sensory organs allows to answer several questions.

First, what is the overall brain activity when the fly is at rest, during behavior, and when it responds to stimuli? As an example, we include data obtained with a light field microscope showing brain activation during responses to stimuli and behavior. For example, in **Video 2**, a calcium probe was expressed in all neurons (nsyb-GAL4 and UAS-syt-GCaMP6s (left) or UAS-GCaMP6M (right)) and a puff of odor was presented. Notice how the preparation allows to get an overview of brain activity during the response to the stimulus. The powerful genetic tools in Drosophila can be used to restrict the expression of these sensors to specific neuronal sub-types. In **Video 3**, we restricted the expression of a calcium sensor to dopaminergic and serotoninergic neurons (TH-GAL4, DDC-GAL4 and UAS-GCaMP6M). Notice the strong synchronous activity over the brain tightly correlated with the fly walking, allowed by observing the whole brain during behavior. In addition to calcium activity, other physical or chemical signals can be imaged (using for example sensors for voltage[28,33], metabolism products[34,35] or specific neuromodulators[36]).

To understand brain activity's role more specifically, we can ask what regions are involved in what behavior, response to stimuli or spontaneous patterns of activity. The data can indeed be used as an unbiased screen to extract functional regions using techniques such as principal component analysis and independent component analysis. **Figure 9A** shows different functional regions in different colors. The shape and localization of the functional regions allow to map them to anatomical templates to identify brain regions and in some cases neuron type. Furthermore, once aligned to the anatomical template, fluorescence values can be average in anatomical brain regions for quantitative analysis (see **Figure 9B**). For example, a hierarchical gaussian model applied to data in **Figure 9B** shows that regions are more active during walk (with a $\Delta F/F$ median of 0.029, 95% credible interval=[0.017 0.041]) but not during groom ($\Delta F/F$ median = -0.0049 with 95% credible interval=[-0.016 0.0059]).

Adding to the understanding depth, the simultaneous recordings of the different functional regions allowed by the preparation can be used to study the dynamical properties of the functional network. This is important because brain regions in all brains studied so far are highly recurrently interconnected and more and more studies show that even sensory areas respond to

the animal's behavioral state. Several aspects can be looked at such as functional graph properties (e.g., modules) and spatio-temporal patterns that can be fit with dynamical systems (see [8] for examples).

FIGURE AND TABLE LEGENDS:

Figure 1: Preparing the holder (step 1). A) Holder design (view from the top). **B**) Neck slot preparation. top: tape design, bottom: parallel blades used to cut the neck slot. **C**) View of the holder from below. D) Bottom view indicating where to add the neck slot tape.

Figure 2: Further preparations (step 1). A) Add grease into the neck slot to prevent glue to cover the back of the head and to prevent saline leaks. Scale bars, 1 mm. **B**) Top: shape of the piece of tape that will be used to push the body down. Bottom: make sure the tape fits the holder.

Figure 3: Placement of a v-shaped piece of tape to aids centering. (**A**) Bottom view. (**B**) Top view. Scale bar, 2 mm.

Figure 4: Place the fly (step 2). Left, use two dull forceps to place the body so that the neck is in the neck slot. Middle, align the head. The eyes lie on both sides on the edges of the slot. The fly's head is straight. Right, tuck the fly with a tissue covered with ice to keep it from moving. Scale bar, 1 mm.

Figure 5: Fix the head (step 3). **A**) Ideal angle of the head. **B**) Add UV glue around the head, avoiding sensory areas of interest. Scale bar, 1 mm.

Figure 6: The proboscis' tip and the antennae can be kept free from glue (step 3). The white circle indicates the area where the UV-glue has been applied. The arrow shows which parts are left free from glue for olfactory and gustatory experiments. Note the optional tape that prevents the legs from touching the glue area. Scale bar, 200 µm.

Figure 7: Place the back tape (step 4.2). The tape created in step 1 (see **Figure 2**, left) is used to place the body and cover the large hole in the holder. Scale bar, 1 mm.

Figure 8: Dissection steps (step 6). A-B) Cut on each side at the base of the dark triangle (crosses) and remove this part of the cuticle **C**). **D**) and **E**) remove muscle 16. Then gently remove the rest of the cuticle **F**), the air sacks **G**), and muscles. **H**) Dissected head. Scale bar, 200 µm.

Figure 9: Representative results. A) Functional regions (extracted using PCA and ICA as in [28]) for a fly expressing GCaMP6 pan-neuronally imaged with a light field microscope (25x, NA=0.95 with a matching f/12 micro lens array). Scale bar, 100 µm. **B**) Average calcium activity in large brain regions during responses to stimuli and behavior (reproduced from[28]).

Video 1: Muscle 16 movements (step 6.3). GFP was expressed in muscles. Note the pumping movement that comes from the hole just above the trachea.

Video 2: Pan-neuronal calcium activity during response to odor for two different preparations. Balsamic vinegar was puffed onto the fly. Pan-neuronal calcium activity (nsyb-GAL4 driver, UAS-syt-GCaMP6s left and UAS-GCaMP6M right) was imaged with a light field microscope (25x, NA=0.95 with a matching f/12 microlens array). Light field images were then reconstructed as described in ref.[28,37].

Video 3: Calcium activity in a neuron type subset during behavior. GCaMP6 was expressed in dopaminergic and serotoninergic neurons (with TH-GAL4 and DDC-GAL4). In combination with the fly genetic tools, the preparation allows to observe a strong increase in activity in many regions during walk.

DISCUSSION:

Drosophila is one of the rare adult animals that whole brain can be imaged during complex behaviors. Here, we present a method to prepare the fly and expose its whole brain to image ongoing whole brain activity. Several important points should be noted.

Dissecting a small animal such as *D. melanogaster* is challenging. The method thus requires a lot of practice and patience to master it. However, after training, the procedure takes less than 30 minutes and produces reproducible results.

The method we presented has additional limitations. First, tilting the fly head from its natural position leads to stretching the neck which could be damaging to connective tissue, nerves or muscle. Second, although the ventral sub-esophageal zone (SEZ) is optically accessible, it is below the semi-transparent esophagus, which decreases the intensity and resolution in this area. Finally, although the holder is out of reach in most directions, the fly still sometimes realizes its presence and pushes on it to try to escape.

Despite these limitations, the comprehensive data obtained from whole brain imaging during behavior and responses to stimuli will make it possible to decipher brain function at the level of the whole network when the animal interacts with and navigates complex, naturalistic environments.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

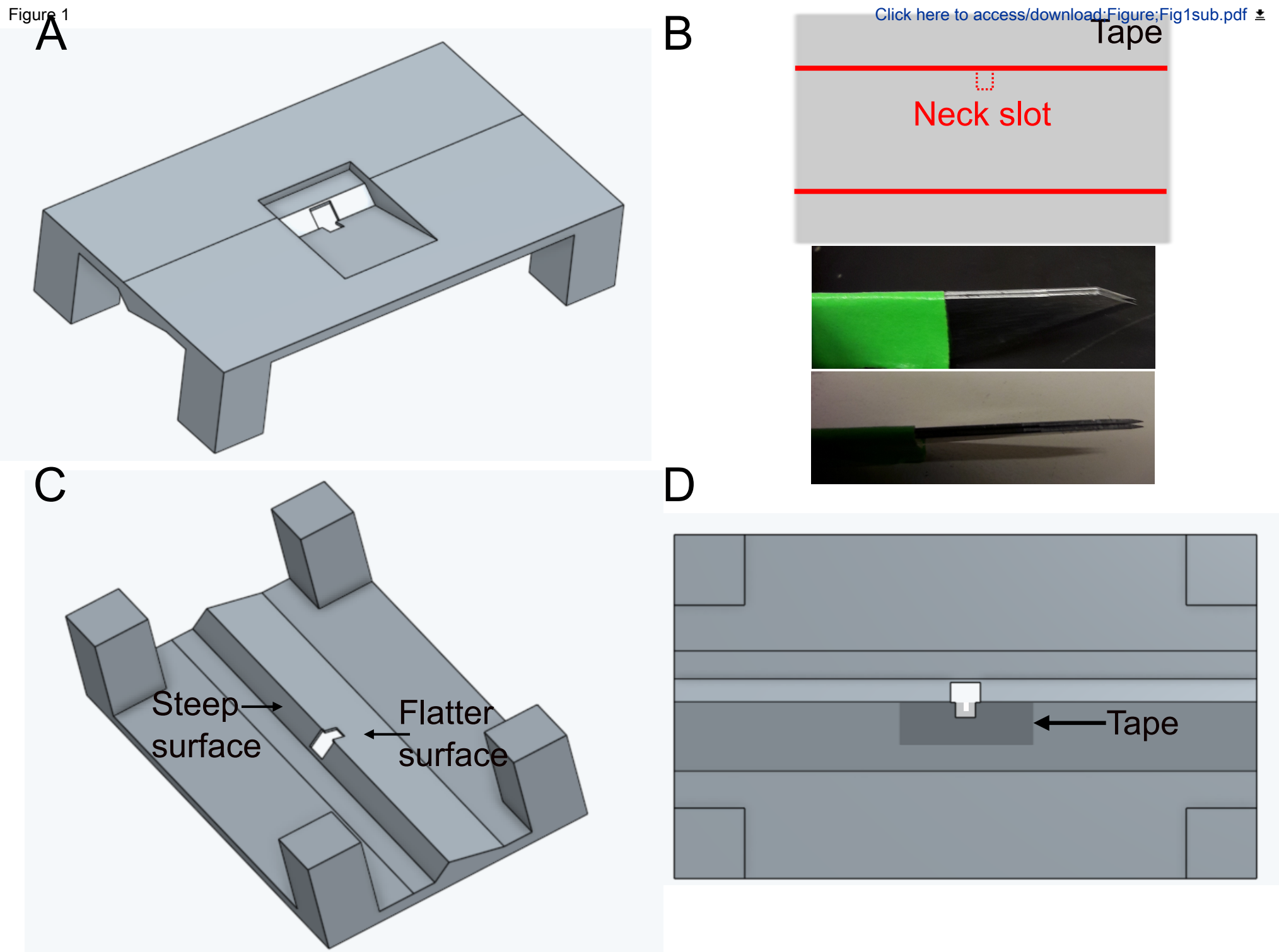

Figure 2

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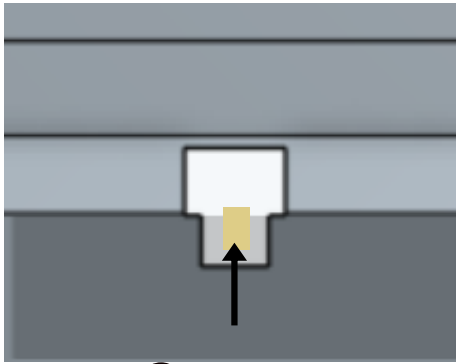

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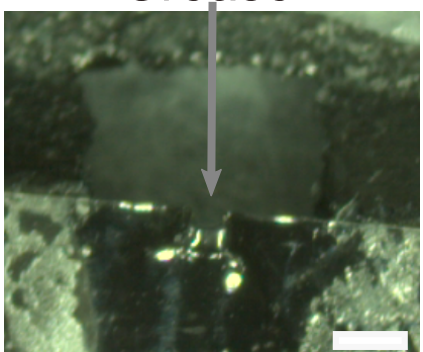

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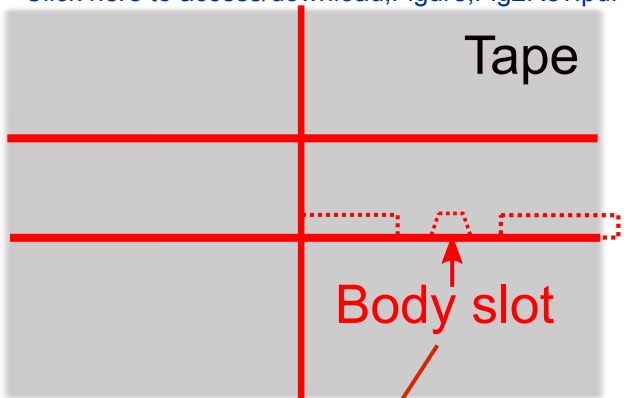


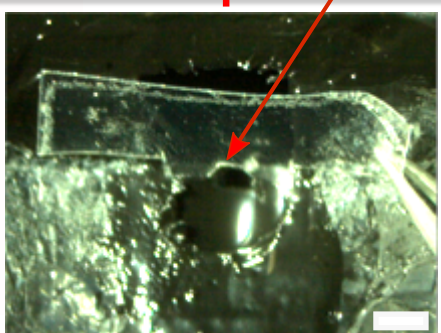
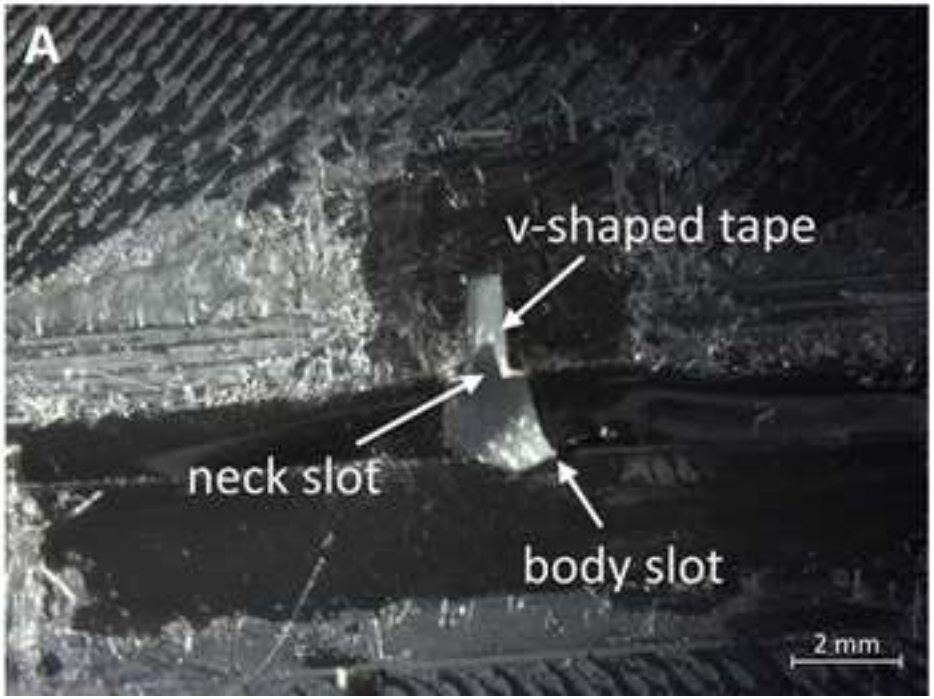


Figure 3



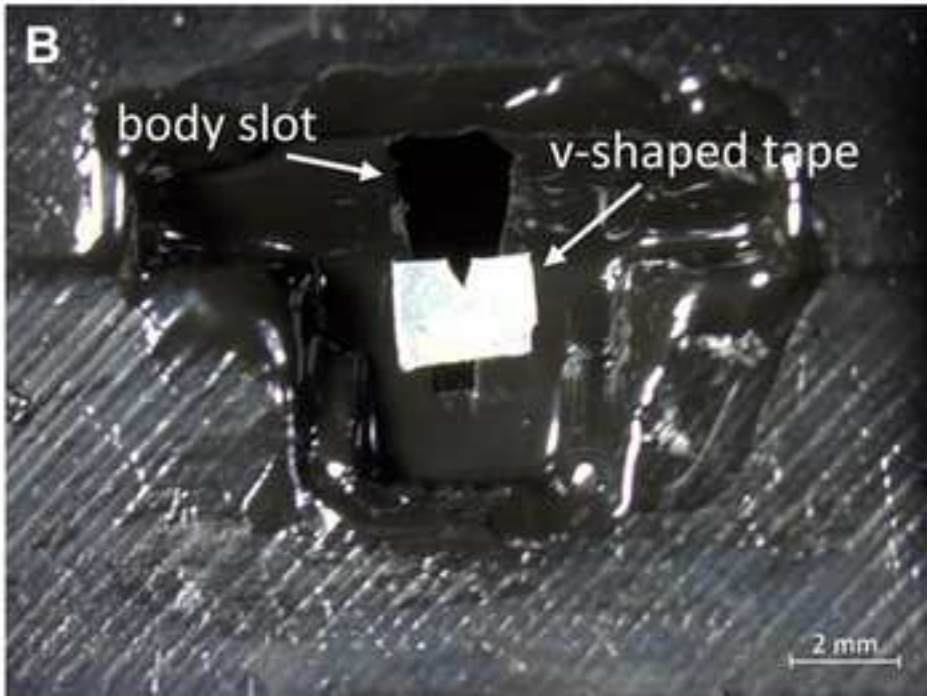
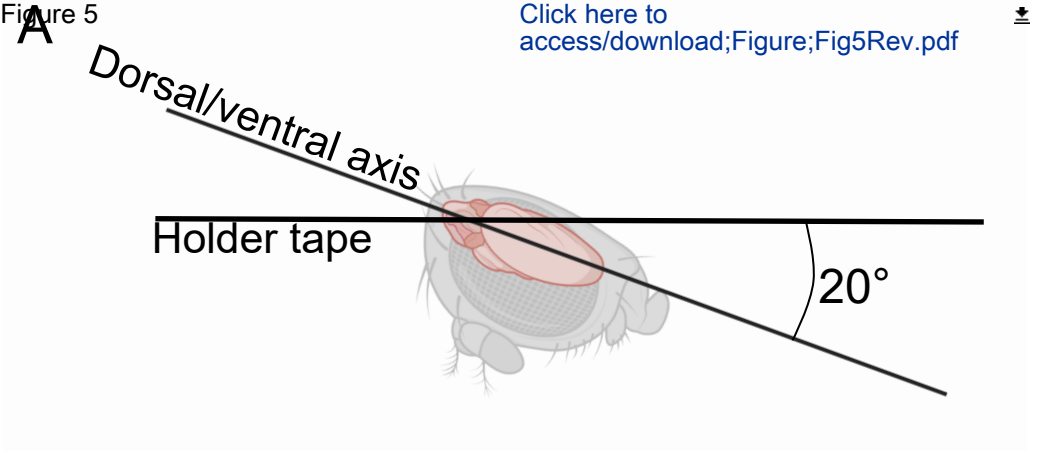


Figure 4

Click here to access/download;Figure;Fig4VRev.tif

Figure 5



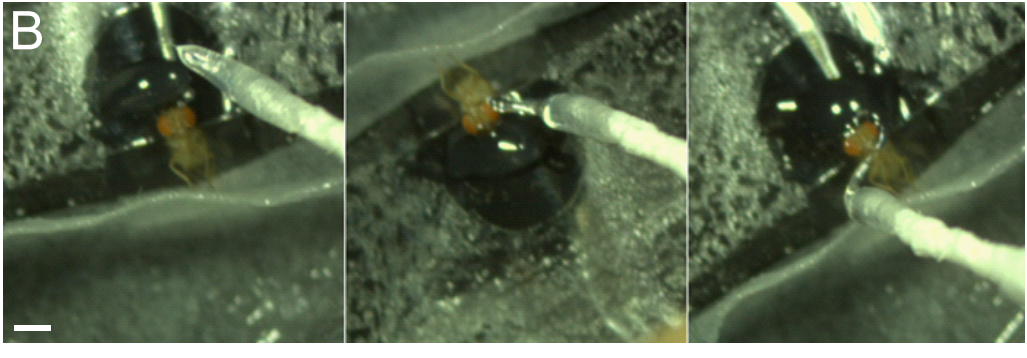


Figure 6

Click here to access/download;Figure;Fig6.tif

Figure 7

Click here to access/download;Figure;Fig7VRev.tif

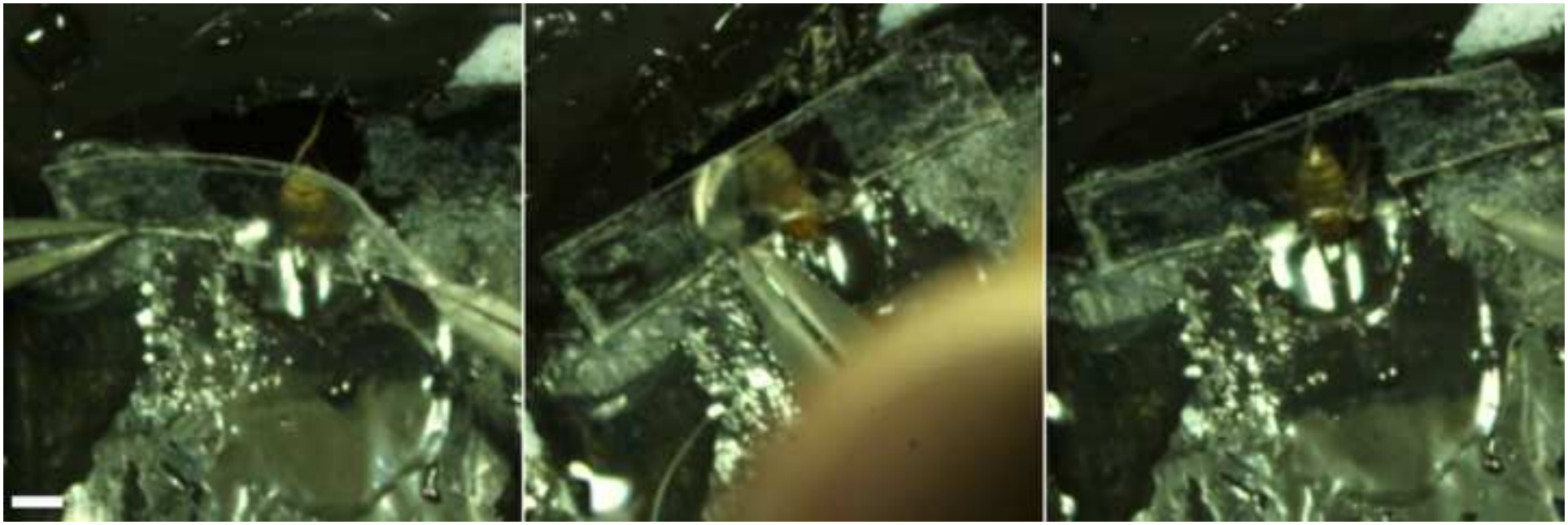


Figure 8

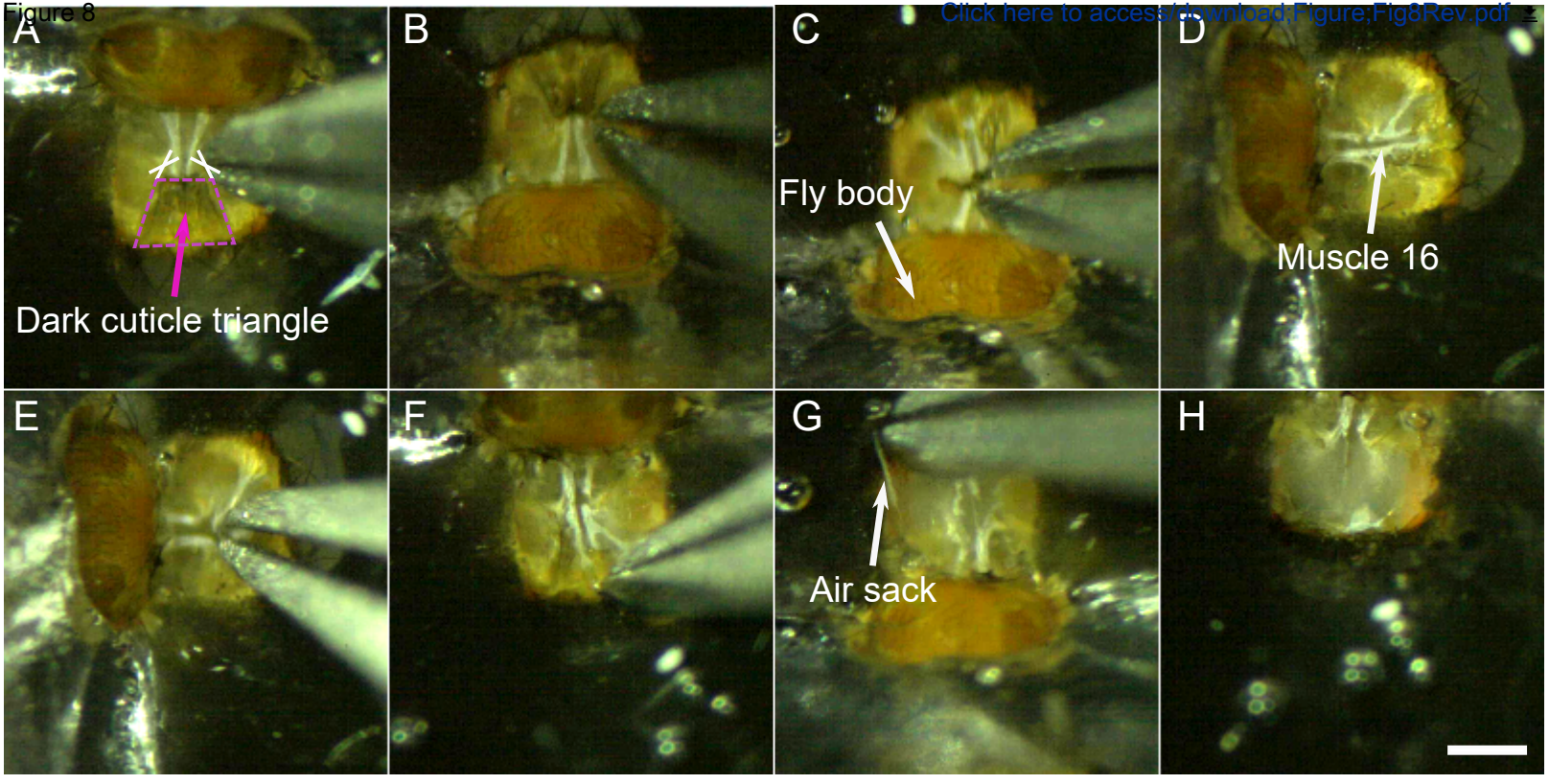
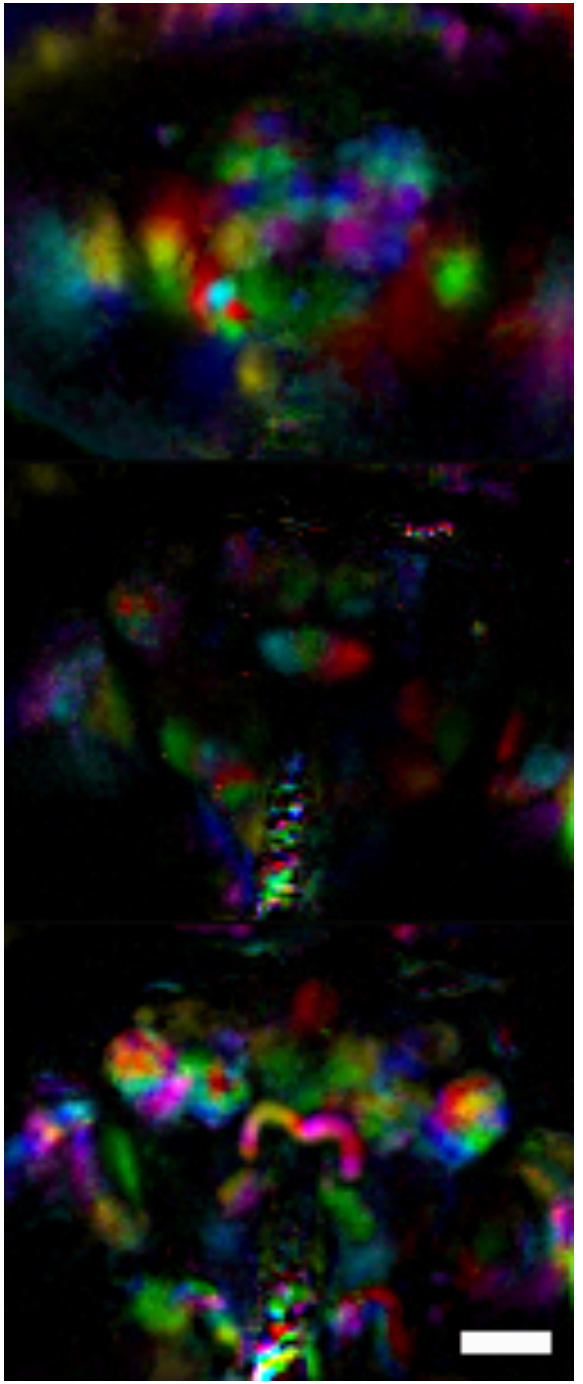


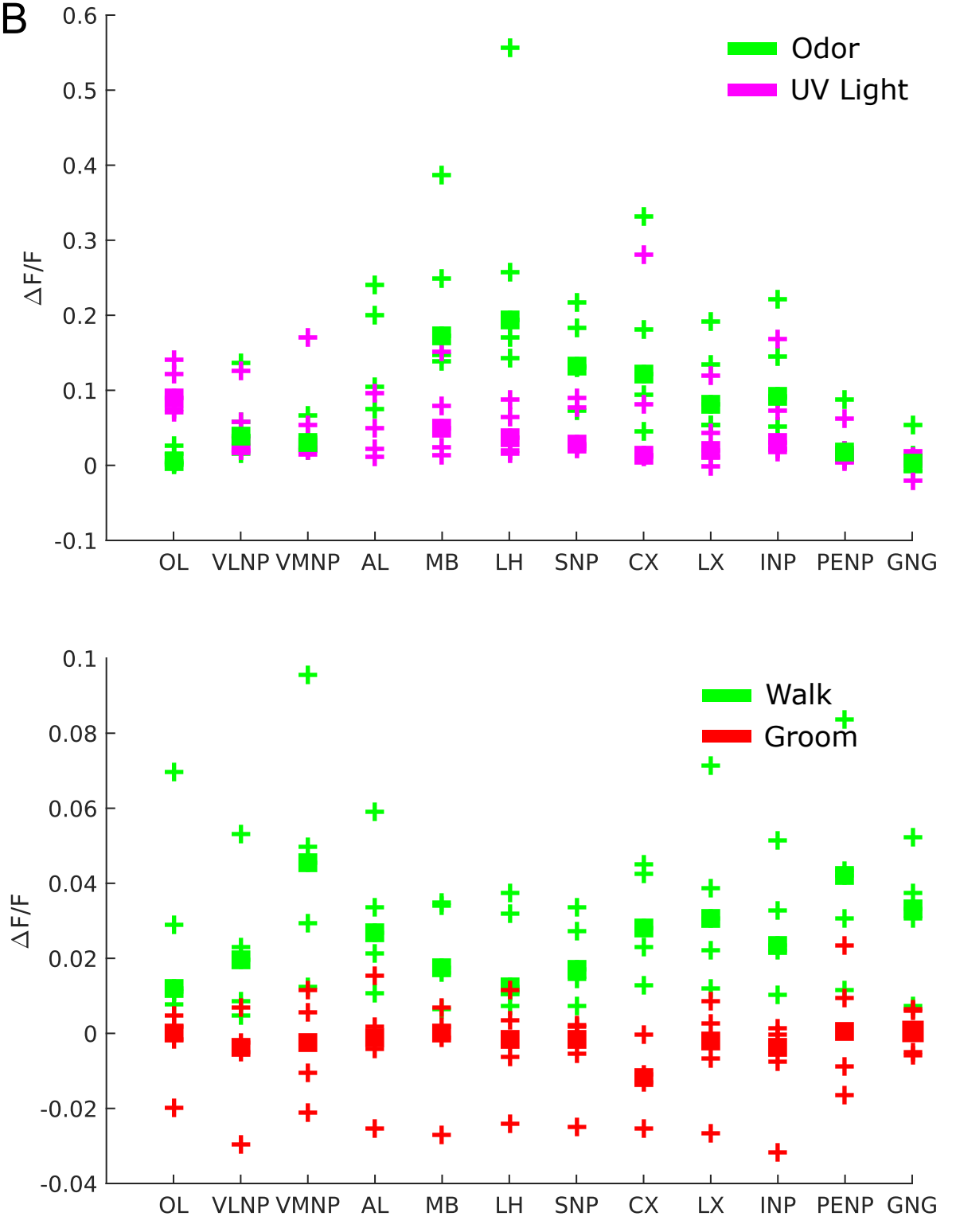
Figure 9

Click here to access/download;Figure;Figure9.pdf

A



B



Video 1

Click here to access/download

Video or Animated Figure

Muscle16_Movie.avi

Video 2

Click here to access/download

Video or Animated Figure

B9_B10CombinedStacks.avi

Video 3

Click here to access/download

Video or Animated Figure

Video3.avi

Name of Material/ Equipment	Company	Catalog Number
#5 forceps	FST by DUMONT	11252-30
#55 forceps	FST by DUMONT	11255-20
30x oculars	yegren	WF30-9-30-H
AHOME/UV flashlight	Shenzhen Yijiawan Technology Co., L	B07V2W9543 (ASIN)
Fotoplast Gel/UV Glue	Dreve Otoplastik GmbH	44791
Gloss Finish Transparent Tape	3M Scotch	
KIMTECH Science/Precision wipes	Kimberly-Clark Professional	7552
KL 1500 LCD/Microscope light	Schott	
Leica MS5 Microscope	Leica	
Nail Lacqueur	Opi Products Inc., N. Hollywood	6306585338
Saline:	Sigma Aldrich	
Scalpel	Werner Dorsch GmbH	78 621; B07SXCXWFS (ASIN)
Vacuum grease	Dow corning	0020080 /100 gr

Comments/Description

straight tip 0.05 x 0.02 mm, Dumoxel, 11 cm long
straight tip 0.05 x 0.02 mm, Inox, 11 cm long
WF30X/9 High Eye-point Eyepiece Wide Field View Ocular Optical Lens for Stereo Microscope or Biological Microscope 30X, 30mm without Reticle

365 nm
GHS07, GHS08

11 x 21 cm

WF30X/9
black

soft handle
Moly Kote 111 Compound Grease Grease Valve Stamp 100 g

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully proofread the manuscript.

2. Please specify which video is Movie 1: Muscle16_movie.avi or pbio.2006721.s001.avi?

We have changed the name to Video 1 to match the Jove nomenclature.

3. Please provide a legend for Movie 1 and Movie 2.

We have added legends for the videos.

4. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

We have added scale bars.

5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

This has been corrected.

6. Please revise the title for conciseness.

We have modified the title to "Preparing adult Drosophila melanogaster for whole brain imaging during behavior and stimuli responses". We believe that all the remaining elements are important to identify the study.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added details throughout the protocol.

8. 1.1: Does the printing material matter?

We have added details for the printing material: "Both SLS (Nylon PA12) and multijet fusion (PA12) are suitable."

9. 1.2: What are the dimensions of the sticky tape?

We have added the width of the sticky tape: "Cut slices of approximately 5 mm x 1 cm."

10. 1.3: Create the body slot how? Please quantitate how much grease to use.

We have added precisions on how to cut the tape for pushing the body down. "Use the design shown in Fig. 2A top to cut a piece of ~2cm wide tape in two and cut 1.5 mm wide slices. Cut out 0.3 mm deep shoulders and body slot."

We have added precision on the grease quantity: "add a few microliters of grease in the head slot using a rolled tissue to ensure the glue won't wet the back of the fly's head. Make sure not to put grease outside of the slot that would prevent the glue from sticking.

11. 2.1: How much is used?

We have added modified to: "Fill a Petri dish or pipette tip box lid with ice."

12. Please specify all volumes and amounts used throughout.

We have added the volumes for grease and saline.

13. Please revise the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

We have drastically revised the section Representative Result, with a new structure, new original examples, and an example of analysis of the outcome. We have clarified why these representative results are specifically enabled by the technique described in the protocol.

14. Please avoid bulleted lists in the Representative Results.

We have rewritten the whole section and removed the bullet list.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor

or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

We have added a .docx file with the link to the policy for figure 9, and we have added the citation in the caption.

16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

We have corrected the formatting.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Woller et al. describes methods to making a fly holder, fixing and dissecting the fly for the whole brain imaging technique. Even thought their description of the protocol is detail, actual video demonstrating each and every step of the procedure is strongly desired. Many of the written protocol cannot replace actual demo.
Major Concerns:
Although fly preparation is critical for the whole brain imaging, I wonder if authors are considering inclusion of steps to modify the microscope that enables light-field microscopy. Fly head fixation and dissection alone has been described multiple times in other studies in detail, although the described methods and procedures are not identical to others and that may appeal to some readers.

We thank the reviewer for their interest in our Jove protocol. The protocol we present is the first (to our knowledge) to show how to prepare the fly for whole brain imaging during behavior, and each step has been specifically developed and optimized for this purpose. We have made this clearer in the abstract and in the text.

“a method specifically developed to image the whole brain while the fly performs semi-naturalistic behavior (i.e. walking and grooming) and responds to sensory stimuli.”

Although light field microscopy is particularly indicated for fast large-scale 3D brain imaging, other slower technique with higher spatial resolution might also be used in combination to the preparation technique we describe. We thus believe that our unique preparation that allows for whole brain imaging during behavior is valuable in itself, independent of the imaging technique used.

We have added a small paragraph making this point clearer:
"The preparation described above allows to observe the whole brain under a microscope for large scale 3D imaging such as classical 2 photons or confocal microscopy, but also faster techniques such as light sheet[31] and other structured illumination microscopy techniques (reviewed in [32]), or light field microscopy [28]. "

Minor Concerns:

Inclusion of the brain activity video is impressive, but there is no description of how this was done.

We have added descriptions of the representative results in the text and captions.

Reviewer #2:

Manuscript Summary:

The authors report a beautiful protocol on a method for optically imaging large parts of the Drosophila brain while the animals can be stimulated with a variety of sensory cues and while the animals are free to move their legs. The article is nicely introduced and advantages and disadvantages are mentioned. The protocol is sufficiently detailed and very clearly written. It will be of interest to the broad community of researchers working with Drosophila. I have no major points of concern and recommend that the protocol will be published.

Major Concerns:

None.

Minor Concerns:

The authors write in the introductory section that "... functional imaging is becoming more and more common place and several protocols have already been published". That is correct, and I totally agree with it. Actually, functional brain imaging in Drosophila is common place since more than 15 years. But I would find it appropriate to cite those studies that have made this advance possible. As it reads now it seems as if the studies cited (mostly from recent years) have accomplished to make optical imaging in Drosophila feasible. Of course, it is a common phenomenon that every ~ 10-15 years a new generation of scientists re-invents a particular technique with significant improvements when compared with previous approaches. This is also the case here: novel sensors, better microscopes and more elaborated brain peparations are of advantage so that a larger brain volume can be monitored and the flies can move their legs. Still, the foundations have been set with the invention of in vivo optical imaging in Drosophila: The authors might want to cite these papers to show on which shoulders the entire, nowadays commonly used technique actually rests, and what the actual very valuable advance in the protocol presented actually is:

- Fiala et al. (2002), Current Biology, Volume 12, Issue 21, Pages 1877-1884. The first in vivo optical imaging study in Drosophila at all.
- Fiala and Spall (2003), Science's STKE Vol. 2003, Issue 174, pp. pl6. The first in vivo optical imaging protocol in Drosophila.
- Strutz et al. (2012). Calcium imaging of neural activity in the olfactory system of Drosophila. In J.-R. Martin (Ed.), Neuromethods / Genetically Encoded Functional Indicators (pp. 43-70). doi:10.1007/978-1-62703-014-4_3. The tilting of the fly head and

the holder described in the present manuscript strongly resembles the preparation technique described by Antonia Strutz from Silke Sachse's lab.

Also, those techniques that are designed not to image the whole brain, but only small numbers of neurons with precision, would be good to mention, e.g. Hancock et al. (2019), J. Vis. Exp. 152, e60288, or Bilz et al. (2020), Neuron Volume 106, Issue 6, 963-976. This would highlight the difference of the presented protocol and demonstrate that is offers the advantage of visualizing large parts of the brain while the animal is free to move the legs.

My comments are not intended to overduly criticize the manuscript but to help improving it .

We thank the reviewer for these positive comments and for pointing out the lack of acknowledgment of early studies. We have modified the text: “in vivo functional imaging has become more and more common place since the first study in 2002“ and added all the references pointed by the reviewer.

Reviewer #3:

Manuscript Summary:

This manuscript describes a protocol for dissection of adult Drosophila melanogaster for live whole brain imaging during behavior. There are a few different protocols around to achieve this same goal, but a detailed, well documented protocol including all critical

steps (and technical drawing allowing one to reproduce the stage) is a welcome addition.

Major Concerns:

None

Minor Concerns:

Drosophila should be italicized

We have corrected the non-italicized instance.

A few spelling and grammar mistakes need to be fixed (ex: KimwiPe vs KimwiPPe, use A piece of tape, a few more)

We have corrected these mistakes.

The raw imaging data is an important validation of this protocol. If possible, a few original example should be shown (ideally different preparations responding to the same stimulus, to get an idea of variability?) --rather than re-produce data from previous publications by this group

We have added several original datasets, as well as a dataset showing region activity during responses to stimuli and behavior for several flies to give an idea of the variability and of how to analyse the data.

Fly holder design