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Title: Preparing Adult *Drosophila melanogaster* for Whole Brain Imaging During Behavior and Stimuli Responses

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**, **Zeiss stemi 508**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **53**

Introduction

1. Introductory Interview Statements

NOTE: Videographer did not get headshots, please use a screen grab from the shoot.

REQUIRED:

1.1. **Alexandra Woller**: Our protocol allows imaging of the whole fly brain during behavior, which is important for understanding how neurons in different brain areas interact to shape behavior **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Paul Bandow**: Unlike other published protocols, this protocol was developed specifically to access the whole brain while the fly is active and sensing via smells, tastes, or visual stimulations **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Head Slot Creation

2.1. To create a head slot, after printing the holder with a 3D printer **[1-TXT]**, place a piece of sticky tape rectangularly on a flat surface under a stereomicroscope **[2]** and cut an approximately 5-millimeter x 1-centimeter piece **[3]**.

2.1.1. WIDE: Talent placing holder onto bench **TEXT: See text for holder printing details**

2.1.2. Talent placing tape onto surface under microscope

2.1.3. SCOPE: Tape being cut Videographer NOTE: 2 takes

2.2. Use two scalpels fixed together in parallel to cut a 400- x 400-micrometer neck slot in the middle of the longer side of the tape **[1]** and place the tape over the flatter side of the hole on the bottom side of the holder **[2]**.

2.2.1. SCOPE: Neck slot being cut

2.2.2. SCOPE: Tape being placed over flatter side of hole Videographer NOTE: 2 takes

2.3. Use forceps to push the tape approximately 500 micrometers around the hole **[1]** and use black nail polish to cover the top of the tape and holder **[2]**.

2.3.1. SCOPE: Tape being pushed around hole

2.3.2. SCOPE: Top being covered

2.4. After letting the nail polish dry for at least an hour, use a rolled tissue to add approximately 1 microliter of grease to the head slot **[1-TXT]**.

2.4.1. SCOPE: Grease being added to head slot **TEXT: Grease outside of slot will prevent glue sticking** Videographer NOTE: 2 takes, closer shot in 2nd one

2.5. To prepare the body slot, cut an approximately 2-centimeter piece of wide tape into two pieces **[1]** and cut 1.5-millimeter-wide slices **[2]**.

2.5.1. SCOPE: Tape being cut
2.5.2. SCOPE: Slices being cut Videographer NOTE: 2 takes

2.6. Then cut out a 0.3-millimeter-deep shoulder-and-body slot **[1]** and make sure that the tape fits the holder **[2]**.

2.6.1. SCOPE: Slot being cut Videographer NOTE: 3 takes
2.6.2. SCOPE: Slot being checked in holder

3. Fly Placement

3.1. To fix a fly into the holder, first place a shallow container with ice under the dissecting microscope **[1]** and place the holder upside down onto a piece of laboratory tissue placed over the ice **[2]**.

3.1.1. WIDE: Talent placing dish under microscope
3.1.2. Talent placing tissue/holder onto ice

3.2. Aspirate a one to 4 days old female fruit fly from its vial **[1]** and blow the fly onto unmelted ice **[2]**.

3.2.1. Fly being aspirated
3.2.2. Fly being blown onto ice

3.3. When the fly stops moving, use dull forceps to grasp the fly at the base of wings **[1]** and slide the fly into the holder with the neck inside the slot **[2]**. Videographer NOTE: closer on 2nd run

3.3.1. SCOPE: Fly being grasped *Videographer: Important step*
3.3.2. SCOPE: Fly being slid into holder *Videographer: Important step*

3.4. The eyes should be equally positioned on either side of the slot **[1]**. If needed, add 1 microliter of grease to the top of the head to prevent the glue from reaching the back of the head **[2]**. Videographer NOTE: closer on 2nd run

3.4.1. SCOPE: Shot of eyes in position
3.4.2. SCOPE: Grease being applied

3.5. Then cover the body with a tissue and some ice to make sure the fly does not move **[1-TXT]**.

3.5.1. Body being covered **TEXT: Optional: Place tape just below head to prevent legs from reaching head**

4. Securing the Head

4.1. To secure the head, place it at an approximately 20-degree angle from a fully posterior view **[1]** and use a rolled-up tissue to apply UV-glue around the head while avoiding soiling the sensory area of interest **[2]**.

4.1.1. WIDE: Talent at microscope, adjusting head angle
4.1.2. SCOPE: Shot of head angle, then glue being applied Videographer NOTE: close shot, cut before glue touches the leg.

4.2. For taste experiments, use forceps to pull out the proboscis **[1]** and add glue at the proboscis base to prevent movement. If no taste experiment is planned, push and glue the proboscis into the head **[2]**.

4.2.1. SCOPE: Proboscis being pulled
4.2.2. SCOPE: Proboscis being glued Videographer NOTE: close shot, cut before glue touches proboscis.
~~4.2.3. SCOPE: Proboscis being pushed/glued~~

4.3. Cure the glue with UV-light for 5 seconds **[1-TXT]** and use a rolled-up tissue to carefully clean the area surrounding the head to remove any remaining liquid glue that could stick to the legs and/or soil sensory areas **[2]**. Videographer NOTE: 4.3.1 – 4.4.1 closer shots.

4.3.1. SCOPE: Glue being cured **TEXT: Optional: Roughen tape with sandpaper to increase glue adhesion**
4.3.2. SCOPE: Area being cleaned

4.4. Then use a thin strip of tape or a tissue to move the legs to the front as necessary **[1-TXT]**.

4.4.1. SCOPE: Legs being moved Videographer NOTE: 3 takes

5. Body Positioning and Hole Sealing

5.1. To position the body, remove the ice container **[1]** and turn the holder around **[2]**.

5.1.1. WIDE: Talent removing container NOTE: This and next shot together
5.1.2. Talent turning around holder

5.2. Remove the water around the fly **[1]** and, acting quickly before the fly recovers from the anesthesia, place the body slot tape over the hole **[2]** and gently push the fly's body down, taking care not to overstretch the neck **[3]**.

5.2.1. Water being removed *Videographer: Important step*
5.2.2. SCOPE: Body slot tape being placed over hole *Videographer: Important step* NOTE: This and next shot together
5.2.3. SCOPE: Body being pushed down *Videographer: Important step*

5.3. Cover any remaining large holes with tape and add approximately 1 microliter of grease to the back of the head to prevent glue from sticking in that location **[2]**.

~~5.3.1. SCOPE: Hole being covered~~
5.3.2. SCOPE: Grease being added Videographer NOTE: 5.3.2 – 5.5.2 shot with other camera, no slate

5.4. Use a rolled-up tissue to paint UV-glue around and on top of the tape and thorax **[1]** and cure the glue with UV-light for approximately 5 seconds **[2]**.

5.4.1. SCOPE: Glue being painted onto tape and thorax
5.4.2. SCOPE: Glue being cured

5.5. Carefully remove any grease and uncured glue with a laboratory tissue **[1]** and add approximately 1 milliliter of saline to the top of the head **[2]**.

5.5.1. SCOPE: Fly being wiped
5.5.2. SCOPE: Saline being added

5.6. Use forceps to push aside any air bubbles **[1]** and check for leaks by placing a coverslip over the saline and turning the holder around to check for saline on the front side **[2]**.

5.6.1. SCOPE: Air bubble being pushed
5.6.2. Coverslip being placed/holder being turned

5.7. If saline is observed, remove the saline **[1]** and fix the hole with more glue or more grease **[2]**.

5.7.1. SCOPE: Shot of saline, then saline being removed
5.7.2. SCOPE: Hole being closed

6. Head Dissection

6.1. To dissect the head, select the highest magnification **[1]** and use sharpened very fine forceps to make two cuts at the base of the central dark cuticular triangle on each side of the neck **[2]**.

6.1.1. WIDE: Talent selecting magnification
6.1.2. SCOPE: Cut(s) being made *Videographer: Important/difficult step*

6.2. Cut around the dark triangle **[1]** and remove this part of the cuticle **[2]**.

6.2.1. SCOPE: Cut being made around triangle
6.2.2. SCOPE: Cuticle piece being removed

6.3. Muscle 16 and the esophagus should be visible through this hole and moving rhythmically **[1-TXT]**.

6.3.1. SCOPE: Shot of hole

6.4. Use the very fine forceps to carefully pinch the top of this area to sever muscle 16 without puncturing the esophagus **[1]**. If the rhythmic movement stops, the muscle 16 was likely removed **[2]**.

6.4.1. SCOPE: Hole being pinched/muscle being cut *Videographer: Important/difficult step* Videographer NOTE: 2 takes
6.4.2. SCOPE: Shot of lack of movement *Videographer: Important step*

6.5. When the muscle has been excised, starting from the medial edges of the dark triangle region, use the forceps like a pair of scissors to carefully cut and remove the remaining cuticle in small pieces **[1-TXT]**.

6.5.1. SCOPE: Pieces being cut/removed *Videographer: Important step* **TEXT: Use cuticles to scrape away fat bodies as necessary**

6.6. Then use the forceps to grasp and slowly and steadily pull away one air sack at a time **[1]**.

6.6.1. SCOPE: Air sack being pulled

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
3.3., 4.1., 5.2., 6.1., 6.4., 6.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
6.1. and 6.4. require very fine forceps and some training.

Results

7. Results: Representative *Drosophila melanogaster* Whole Brain Imaging and Analysis

7.1. In this video, a calcium probe was expressed in all of the neurons of these two fruit fly brains and a puff of odor was presented **[1]**.

7.1.1. LAB MEDIA: B9_B10CombintedStacks *Video Editor: please emphasize white signals*

7.2. In this analysis, calcium sensor expression was restricted to dopaminergic and serotoninergic neurons **[1]**. Notice the tight correlation between the strong synchronous activity over the brain **[2]** and the fly's walking behavior, illustrating how the whole brain can be observed during a specific behavior **[3]**.

7.2.1. LAB MEDIA: Video3
7.2.2. LAB MEDIA: Video3 *Video Editor: please emphasize white signals in left video*
7.2.3. LAB MEDIA: Video3 *Video Editor: please emphasize sphere movement in right video*

7.3. Using principal component analysis and independent component analysis, different functional regions highlighted in different colors can be extracted **[1]**.

7.3.1. LAB MEDIA: Figure 9A

7.4. The shape and localization of the functional regions allow the regions to be mapped to anatomical templates to identify brain regions and, in some cases, neuron types **[1]**.

7.4.1. LAB MEDIA: Figure 9A *Video Editor: please outline/emphasize a few distinct regions*

7.5. Once aligned to the appropriate anatomical template **[1]**, fluorescence values can be averaged within specific anatomical brain regions for quantitative analysis **[2]**.

7.5.1. LAB MEDIA: Figure 9A
7.5.2. LAB MEDIA: Figure 9 top graph

7.6. For example, in this analysis **[1]**, regions that are more active during walking **[2]** but not during grooming can be observed **[3]**.

7.6.1. LAB MEDIA: Figure 9 bottom graph

7.6.2. LAB MEDIA: Figure 9 bottom graph *Video Editor: please emphasize green data points*

7.6.3. LAB MEDIA: Figure 9 bottom graph *Video Editor: please emphasize red data points*

Conclusion

8. Conclusion Interview Statements

8.1. **Ilona Grunwald Kadow**: Be patient! The head dissection requires practice. In my lab, people require, on average, 3-4 months to get their first useful preps **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (6.1.-6.6.)

8.2. **Alexandra Woller**: Following this protocol, we can record large scale brain activity using a fluorescence microscope. Fast volumetric methods, such as light field microscopy, are particularly indicated **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*