FINAL SCRIPT: APPROVED FOR FILMING

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Title: In Vivo Imaging of Neural Activity in Unanesthetized *Drosophila* Adult Flies

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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 something similar? **YES, all done**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES, all done**

Current Protocol Length
Number of Steps: 26
Number of Shots: 57

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Isaac Cervantes-Sandoval:** We are trying to understand the molecular, cellular, and circuit basis of natural memory forgetting, aiming to uncover how the brain actively erases or suppresses memories to maintain cognitive flexibility.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.3*

What are the most recent developments in your field of research?

1.2. **Isaac Cervantes-Sandoval:** Recent research has shown that forgetting is not merely a passive decay of memories, but rather a highly regulated and active biological process that requires specific patterns of neuronal activity.

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

What are the current experimental challenges?

1.3. **Prachi Shah:** A major challenge is linking specific circuit manipulations to dynamic memory processes, while integrating connectomic, genetic, and behavioral data in a rigorous and interpretable way.

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

What significant findings have you established in your field?

1.4. **Prachi Shah:** We helped establish that forgetting is an active, biologically regulated process. Our work identified specific dopaminergic neurons and molecular pathways required for normal forgetting in the *Drosophila* brain.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.5*

What research gap are you addressing with your protocol?

1.5. **Prachi Shah:** Our protocol enables functional imaging in flies without anesthesia, preventing unwanted non-specific effects by the anesthetics. We use this approach to investigate the neural correlates underlying memory formation and active memory forgetting.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.6*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Assembly of Behavioral Experimentation Tools for *Drosophila* Neurobiology Studies

Demonstrator: Prachi Shah

2.1. To begin, use Dremel tools and a diamond saw blade to cut a 22-gauge hypodermic metal tubing to a length of approximately 10 centimeters **[1]**. With a Dremel 420 cut-off wheel, buff both ends of the tubing to create a smooth and clean opening that can accommodate the proboscis of the fly **[2]**.

2.1.1. WIDE: Talent using a Dremel tool and diamond saw blade to cut the 22-gauge tubing to 10 centimeters.

2.1.2. Talent using a Dremel 420 cut-off wheel to polish the tubing ends.

2.2. Wrap the cut tubing around a 15-milliliter centrifuge tube to form the desired curved shape **[1]**. Then cut a 7-centimeter-long piece of 12-gauge hypodermic metal tubing **[2]**.

2.2.1. Talent manually bending the metal tubing around the 15-milliliter centrifuge tube.

2.2.2. Talent cutting the 12-gauge tubing with Dremel tools.

2.3. Now use a razor blade to trim the end of a 2-microliter pipette tip to fit the 22-gauge metal tubing **[1]**. Fit the 12-gauge tubing into the other end of the pipette tip **[2]**.

2.3.1. Talent trimming the pipette tip with a razor blade.

2.3.2. Talent inserting the 12-gauge tubing into one end of the pipette tip.

2.4. Next, mix a small amount of epoxy resin and hardener together **[1]**. Apply the epoxy to the junctions where the small metal tubing meets the pipette tip and where the larger tubing connects to the other end **[2]**. Allow the epoxy to fully cure overnight before connecting the assembly to a micromanipulator holder and adjusting the angle as necessary **[3]**.

2.4.1. Talent mixing epoxy resin and hardener.

2.4.2. Talent applying epoxy to the joints at both ends of the pipette tip.

2.4.3. Talent adjusting angle on micromanipulator.

2.5. To build a shock and odor delivery pipette, cut 1 milliliter off a 1 by 100 glass pipette at the 3-milliliter mark, using a Dremel diamond tool **[1]**. Then cut a small rectangular acrylic sheet measuring 24.5 millimeters by 8 millimeters with a thickness of one-eighth inch **[2]**.

2.5.1. Talent cutting the pipette with the Dremel diamond tool.

2.5.2. Talent cutting the acrylic sheet to specified dimensions.

2.6. Cut a copper shock grid to fit onto the rectangular acrylic piece **[1]**. Solder two electrical wires to opposite ends of the copper grid **[2]**.
  2.6.1. Talent trimming the copper grid to fit the acrylic.
  2.6.2. Talent soldering wires onto the copper grid.

2.7. Now, place the copper grid on the acrylic piece **[1]** and bend it slightly to accommodate the fly's abdomen and legs **[2]**.
  2.7.1. Talent inserting the copper grid onto the acrylic grid.
  2.7.2. Talent positioning and curving the copper grid into the acrylic.

2.8. Use electrical tape to attach the copper grid to the acrylic piece **[1]**. Then use a hot glue gun to attach the glass pipette to the shock grid, ensuring it is straight and centered **[2]**.
  2.8.1. Talent taping the grid to the acrylic.
  2.8.2. Talent hot gluing the glass pipette in place.

2.9. To build the recording chamber, take a glass microscope slide as the chamber base **[1]**. Mix resin and epoxy glue together **[2]**. Using the epoxy glue, attach neodymium magnets onto all four corners of a black acrylic chamber **[3-TXT]**.
  2.9.1. Talent placing the microscope slide on the work surface.
  2.9.2. Talent mixing resin and epoxy.
  2.9.3. Talent applying glue to attach magnets to the acrylic chamber. **TXT: Magnet dimensions: 3 mm x 2 mm**

2.10. Place an additional magnet on top of each glued magnet then glue the newly placed magnets to a glass slide using epoxy **[1]**. Hold the assembly in place with paper clips while curing **[2]**.
  2.10.1. Talent placing magnets on top of existing corner magnets and glueing them to the glass slide.
  2.10.2. Talent securing the assembly with paper clips for curing.

3. Preparation and Dissection of *Drosophila* for Neural Imaging

3.1. Remove the 200-microliter pipette tip from the aspirator **[1]**. Insert the aspirator into a vial containing the *Drosophila* and aspirate a single fly into the 1,000-microliter pipette tip **[2]**.
  3.1.1. Talent removing the 200 µL pipette tip from the aspirator.
  3.1.2. Talent aspirating a fly from a vial into a 1,000 µL pipette tip.

3.2. Replace the 200-microliter pipette tip back onto the aspirator **[1]**. Then gently blow and flick the aspirator so the fly is immobilized headfirst at the top of the 200-microliter pipette tip **[2]**.
3.2.1. Talent replacing the 200-microliter pipette tip onto the aspirator.
3.2.2. Talent blowing and flicking the aspirator to position the fly headfirst.

3.3. Next, place the dissection chamber onto the manipulator holder **[1]**. Connect the vacuum to the fly-holding tubing and adjust the flow rate to approximately 500 milliliters per minute **[2-TXT]**.
3.3.1. Talent placing the dissection chamber onto the manipulator holder.
3.3.2. Talent connecting vacuum tubing to the fly-holding tubing and adjusting flow rate. **TXT: Use a fast connector to facilitate quick connection and disconnection of the vacuum**

3.4. Now move the vacuum metal tubing to the center of the microscope's field of view **[1]**. Gently aspirate the fly's proboscis into the vacuum holder **[2]**.
3.4.1. SCOPE: Jove_3.4.1_3.4.2.mp4 00:00-00:04
3.4.2. SCOPE: Jove_3.4.1_3.4.2.mp4 00:05-00:12

3.5. Adjust the manipulator to align the fly's head with the chamber opening **[1]**.
3.5.1. SCOPE: Jove_3.5.1.mp4 00:04-00:15

3.6. Turn on the direct current power supply **[1]**. Using platinum resistance wire, apply melted myristic acid to glue the eyes and thorax to the chamber **[2]**.
3.6.1. Talent switching on the direct current power supply.
3.6.2. SCOPE: Jove_3.6.2.mp4 00:00-00:10, 00:17-00:20

3.7. Once secured, disconnect the vacuum tubing **[1]**. Remove the recording chamber from the vacuum connection using the manipulator and turn the chamber upside down **[2]**. Then glue the proboscis from below using platinum resistance **[3]**.
3.7.1. Talent disconnecting vacuum tubing.
3.7.2. Talent detaching and inverting chamber.
3.7.3. SCOPE: Jove_3.7.3.mp4 00:13-00:20

3.8. When everything has been glued, turn off the direct current power supply **[1]**. Then turn the chamber upright **[2]**.
3.8.1. Talent turning off power.
3.8.2. Talent orienting the chamber upright.

3.9. Attach the chamber to the glass slide base **[1]**. Cut a small piece of tape with scissors **[2]** and place it in front and behind the fly's head **[3]**.
3.9.1. Talent placing the chamber onto the glass slide base.
3.9.2. Talent cutting tape with scissors.

3.9.3. SCOPE: Jove_3.9.3.mp4 00:01-00:10

3.10. Rotate the chamber so the fly's head faces the experimenter at a 90-degree angle **[1]**. With a dissecting needle, make vertical incisions along the sides of the eyes **[2]**.
3.10.1. Talent turning the chamber to face the fly's head toward the experimenter.
3.10.2. SCOPE: Jove_3.10.2.mp4 00:00-00:08

3.11. Rotate the chamber horizontally **[1]**. Then make a horizontal cut across the cuticle **[2]**.
3.11.1. Shot of the chamber being rotated horizontally.
3.11.2. SCOPE: Jove_3.11.2.mp4 00:04-00:16

3.12. Now add 100 microliters of saline to the top of the fly's head **[1]**. Using sharp forceps, remove the cuticle window **[2]**. Then, remove any remaining fat or trachea with the forceps **[3]**.

3.12.1. Talent pipetting 100 microliters of saline onto the fly's head.

3.12.2. SCOPE: Jove_3.12.2_3.12.3.mp4. 00:00-00:09

3.12.3. SCOPE: Jove_3.12.2_3.12.3.mp4. 00:21-00:34

4. Visualization Of Learning-Induced Plasticity Through Aversive Olfactory Conditioning

4.1. Place a prepared fly onto the microscope stage of a confocal microscope equipped with a laser and a water immersion objective **[1]**. With a micromanipulator, adjust the position of the shock grid and odor pipette so the fly is correctly positioned on the shock grid **[2]**.
4.1.1. Talent placing the prepared fly chamber on the stage of the confocal microscope.
4.1.2. SCOPE: Jove_4.1.2.mp4 00:00-00:18

4.2. Use the coarse Z adjustment knob to scan through the Z-axis of the brain and locate the brain region of interest **[1]**. Set the frame size to 512 by 512 pixels **[2]**.
4.2.1. ~~SCOPE/SCREEN: Live Z-stack scan through the brain is being done using the coarse Z adjustment.~~
NOTE: 4.2.1 not provided. The shot has been combined with 4.2.2
4.2.2. SCREEN: Jove_4.2.2.mp4 00:00-00:06
4.2.3. SCREEN: Jove_4.2.3.mp4 00:00-00:10

4.3. Begin recording from the neuron of interest using a custom-made or commercially available odor delivery system. Set the recording duration to 2 minutes **[1-TXT]**.

4.3.1. SCREEN: Jove_4.3.1.mp4 00:00-00:11 **TXT: Simultaneously initiate pre-training odor delivery**

4.4. Initiate the training protocol using the odor delivery system, 5 minutes after collecting pre-training responses **[1]**. Then record post-training responses about 5 to 15 minutes after training **[2]**.

4.4.1. Talent initiating training odor delivery from the software or hardware control.
AND
SCREEN: Jove_4.4.1-(1).mp4 00:00-00:20

4.4.2. SCREEN: Jove_4.4.2.mp4. 00:00-00:20

Results

5. Representative Results

5.1. The calcium indicator GCaMP6f *(G-Camp-six-F)* and the red fluorescent protein TdTomato *(T-D-tomato)* were selectively expressed in the Mushroom Body Output Neuron, with dendrites projecting into the γ *(Gamma)* and α′ *(alpha-dash)* lobes of the mushroom body **[1-TXT]**, and the neuron was visualized using the MB077C*(M-B-Zero-Seven-Seven-C)* split-Gal4 *(Gal-four)* driver line **[2]**.

5.1.1. LAB MEDIA: Figure 2A. **TXT: Mushroom Body Output Neuron: MBON γ2α′1** *Video editor: Please highlight the green fluorescent signal*

5.1.2. LAB MEDIA: Figure 2B. *Video editor: Zoom in on the black-and-white image showing the dendritic arbor and cell body labeled ROI*

5.2. Calcium responses in the Mushroom Body Output Neuron to 3-octanol were significantly reduced 5 minutes after aversive conditioning without anesthesia **[1]** and remained suppressed at 15 minutes **[2]**.

5.2.1. LAB MEDIA: Figure 2D and F *Video Editor: Show the bottom panel images of D and F*

5.2.2. LAB MEDIA: Figure 2D and F. *Video Editor: Please highlight OCT (CS+) of 15 min in both images.*

5.3. In contrast, calcium responses to 4-methyl cyclohexanol, were significantly enhanced 5 minutes post-training **[1]**, and remained elevated at 15 minutes **[2]**.

5.3.1. LAB MEDIA: Figure 2D and F. *Video Editor: Show the top panel images of D and F*

5.3.2. LAB MEDIA: Figure 2D and F. *Video Editor: Please highlight OCT (CS+) of 15 min of both images.*

5.4. Pseudo-color images demonstrated distinct fluorescence changes pre- and post-training **[1]**.

5.4.1. LAB MEDIA: Figure 2E. *Video editor: Please sequentially highlight the images of top and bottom panel from Pre, 5 min to 15 min*

5.5. In anesthetized flies, post-training calcium responses to CS+ *(C-S-plus)* were only partially reduced and responses to CS− *(C-S-minus)* were not significantly different from baseline **[1]**.

5.5.1. LAB MEDIA: Figure 2G. *Video editor: Please emphasize 5 min post training*

5.6. Quantitative analysis confirmed that CS+ response was significantly depressed post-training in anesthetized flies **[1]** but CS− responses remained statistically unchanged **[1]**.

5.6.1. LAB MEDIA: Figure 2H. *Video editor: Please highlight the light blue column corresponding to "Post" in the bottom graph*

5.6.2. LAB MEDIA: Figure 2H. *Video editor: Please highlight the light green column corresponding to "Post" in the top graph*

5.7. Plasticity was significantly higher in non-anesthetized flies compared to anesthetized ones **[1]**.

5.7.1. LAB MEDIA: Figure 2I.

Pronunciation Guide:

1. Drosophila

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/drosophila
- **IPA:** /drəˈsɑːfələ/
- **Phonetic Spelling:** druh-SAH-fuh-luhmerriam-webster.commerriam-webster.com+5merriam-webster.com+5merriam-webster.com+5

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2. Dremel

- **Pronunciation link:** https://www.howtopronounce.com/dremel
- **IPA:** /ˈdrɛməl/
- **Phonetic Spelling:** DREM-uhlmerriam-webster.com+2oed.com+2merriam-webster.com+2

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3. Myristic Acid

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/myristic%20acid
- **IPA:** /mɪˈrɪstɪk ˈæsɪd/
- **Phonetic Spelling:** mih-RIS-tik ASS-idmerriam-webster.com+6merriam-webster.com+6merriam-webster.com+6

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4. Proboscis

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/proboscis
- **IPA:** /prəˈbɑːsɪs/
- **Phonetic Spelling:** pruh-BAH-sishowtopronounce.com

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5. Epoxy

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/epoxy
- **IPA:** /ɪˈpɑːksi/
- **Phonetic Spelling:** ih-POK-seemerriam-webster.comoed.com+10it.howtopronounce.com+10howtopronounce.com+10

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6. Micromanipulator

- **Pronunciation link:** https://www.howtopronounce.com/micromanipulator
- **IPA:** /ˌmaɪkroʊməˈnɪpjəˌleɪtər/
- **Phonetic Spelling:** MY-kroh-muh-NIP-yuh-lay-ter

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7. Confocal Microscope

- **Pronunciation link:** https://www.howtopronounce.com/confocal-microscope
- **IPA:** /kɒnˈfoʊkəl ˈmaɪkrəˌskoʊp/
- **Phonetic Spelling:** kon-FOH-kul MY-kroh-skohp

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8. Aversive Olfactory Conditioning

- **Pronunciation link:** https://www.howtopronounce.com/aversive-olfactory-conditioning

- **IPA:** /əˈvɜːrsɪv ɒlˈfæktəri kənˈdɪʃənɪŋ/
- **Phonetic Spelling:** uh-VUR-siv ol-FAK-tuh-ree kun-DISH-uh-ning

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9. GCaMP6f

- **Pronunciation link:** https://www.howtopronounce.com/gcamp6f
- **IPA:** /ˈdʒiː kæmp sɪks ɛf/
- **Phonetic Spelling:** GEE-kamp six-effmerriam-webster.com+22howtopronounce.com+22howtopronounce.com+22merriam-webster.com

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10. TdTomato

- **Pronunciation link:** https://www.howtopronounce.com/tdtomato
- **IPA:** /ˌtiː diː təˈmeɪtoʊ/
- **Phonetic Spelling:** TEE-dee tuh-MAY-toh

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11. Mushroom Body Output Neuron

- **Pronunciation link:** https://www.howtopronounce.com/mushroom-body-output-neuron
- **IPA:** /ˈmʌʃruːm ˈbɒdi ˈaʊtpʊt ˈnjʊərɒn/
- **Phonetic Spelling:** MUSH-room BOD-ee OUT-put NYOO-ron

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12. MB077C

- **Pronunciation link:** https://www.howtopronounce.com/mb077c
- **IPA:** /ɛm biː ˈzɪərəʊ ˈsɛvən ˈsɛvən siː/
- **Phonetic Spelling:** EM-bee zero-seven-seven-seemerriam-webster.com+3howtopronounce.com+3merriam-webster.com+3

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13. Split-Gal4

- **Pronunciation link:** https://www.howtopronounce.com/split-gal4
- **IPA:** /splɪt gæl fɔːr/
- **Phonetic Spelling:** split GAL-four

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14. 3-Octanol

- **Pronunciation link:** https://www.howtopronounce.com/3-octanol
- **IPA:** /θriː ˈɒktənɒl/
- **Phonetic Spelling:** three OK-tuh-nol

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15. 4-Methylcyclohexanol

- **Pronunciation link:** https://www.howtopronounce.com/4-methylcyclohexanol
- **IPA:** /fɔːr ˈmɛθəlˌsaɪkloʊˈhɛksənɒl/
- **Phonetic Spelling:** four METH-uhl-sy-kloh-HEK-suh-nolmerriam-webster.com

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16. Pseudo-color

- **Pronunciation link:** https://www.howtopronounce.com/pseudo-color
- **IPA:** /ˈsuːdoʊ ˈkʌlər/
- **Phonetic Spelling:** SOO-doh KUL-er