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Title: Decoding Natural Behavior from Neuroethological Embedding

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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? **NO**

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

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length
Number of Steps: xx
Number of Shots: xx

Introduction

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

1.1. **Yaning Han:** The scope of my research is to understand how neural dynamics encode natural behavior and how the brain controls complex actions that support survival in natural environments.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Figure 5*

What research gap are you addressing with your protocol?

1.2. **Yaning Han:** Traditional head-fixed paradigms limit our understanding of natural behavior. Our protocol updates this paradigm by enabling precise neural-behavior decoding in freely moving animals toward natural brain intelligence.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.10*

What research questions will your laboratory focus on in the future?

1.3. **Yaning Han:** We will focus on collecting rich, uncontrolled data to build digital life models, using holistic approaches to understand intelligence in complex living systems.

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

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

Testimonial Questions (OPTIONAL):

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.4. **Yaning Han:** (authors will present their testimonial statements live)

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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.5. **Yaning Han:** (authors will present their testimonial statements live)

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

Ethics Title Card

This research has been approved by the Animal Care and Use Committee at the Shenzhen Institute of Advanced Technology

Protocol

2. Platform Establishment for mTPM Imaging

Demonstrator: Yaning Han

2.1. To begin, connect the Universal Serial Bus cable of the synchronization module of the three-dimensional behavior device to the workstation of the same device **[1]**. Then, connect the synchronization module of the mTPM *(M-T-P-M)* device to its controller using one SMA *(S-M-A)* cable **[2-TXT]**.

2.1.1. WIDE: Talent connecting the Universal Serial Bus cable from the synchronization module of the three-dimensional behavior device to its workstation.

2.1.2. Talent connecting the SubMiniature version A cable between the synchronization module and the controller of the miniature two-photon microscope device. **TXT: mTPM: miniature Two-Photon Microscopy; SMA: SubMiniature version A**

2.2. Connect the TTL *(T-T-L)* output port of the synchronization module of the three-dimensional behavior device to the TTL input port of the synchronization module of the mTPM device using one SMA BNC *(B-N-C)* conversion cable **[1-TXT]**.

2.2.1. Talent connecting the SMA to BNC conversion cable between the TTL ports of both synchronization modules. **TXT: TTL: Transistor-Transistor Logic; BNC: Bayonet Neill-Concelman**

2.3. To begin calibration, adjust the shooting angle of all four cameras **[1]** so that they cover the entire base of the open field and extend their field of view at least 20 centimeters above the farthest boundary to capture mouse rearing behavior **[2]**.

2.3.1. Talent rotating and adjusting the positions of all four cameras around the open field arena.

2.3.2. Overhead shot of the four cameras positioned to cover the base and upper area around the open field.

2.4. Then, place the calibration module at the center of the shooting area **[1]**, switch off all the lights **[2],** and run the camera calibration software **[3]**.

2.4.1. Talent placing the calibration module at the center of the open field.

2.4.2. Talent switching off room lights.

2.4.3. SCREEN: SCREEN_2.4.3.mp4: 00:03-00:07, 00:49-00:52

2.5. Now, fix the mouse restrainer to the micromanipulator of the mTPM **[1]**. Using the metal plate, secure the head of the mouse to the restrainer **[2]**.

2.5.1. Talent mounting the mouse restrainer onto the micromanipulator of the miniature two-photon microscope.

2.5.2. Talent fixing the mouse's head to the restrainer using a metal plate.

2.6. Switch off all the lights **[1]**. Then, fix the mTPM to its holder **[2]** and switch on the imaging system to locate the fluorescence signal **[3]**.

2.6.1. Talent turning off the lights in the imaging room.

2.6.2. Talent mounting the miniature two-photon microscope onto its holder.

2.6.3. Talent activating the imaging system and checking the fluorescence signal on the screen.

3. Neuroethological Data Recording

3.1. Add one drop of Carbomer eye gel to the top of the cranial window **[1]**. Move the mouse using the motion platform so that the cranial window is aligned directly beneath the objective of the mTPM **[2]**.

3.1.1. Talent applying a single drop of Carbomer eye gel onto the cranial window of the mouse.

3.1.2. Talent adjusting the mouse position using the motion platform to align the cranial window under the microscope objective.

3.2. Move the micromanipulator vertically to locate the imaging plane **[1]**. Then, move the micromanipulator in-plane to center the imaging plane **[2]**.

3.2.1. Talent adjusting the micromanipulator vertically.

3.2.2. Talent moving the micromanipulator laterally to center the imaging plane.

3.3. Then, fix the upper base to the mTPM **[1]**. Apply adhesive to glue the lower base to the upper base and secure it to the cranial window **[2]**.

3.3.1. Talent attaching the upper base to the body of the miniature two-photon microscope.

3.3.2. Talent applying glue and pressing the lower base into place against both the upper base and the cranial window.

3.4. To ensure structural stability, fill the gap between the two bases and the metal plate bracket attached to the mouse's head using a high-performance acrylic structural adhesive **[1-TXT]**. Then assess the bond's stability by gently probing the base with tweezers **[2]**.

3.4.1. Talent applying high-performance acrylic structural adhesive to fill the gaps between the two bases and the metal plate bracket. **TXT: Allow the adhesive to be cured for 30 min**

3.4.2. Talent using tweezers to gently probe the fixed base after curing.

3.5. After that, add one drop of Carbomer eye gel into the base chamber **[1]**. Observe the neuronal fluorescence through the mTPM **[2]**. If the fluorescence is not clearly visible, remove the adhesive using a cranial drill to detach the base, then repeat the procedure until clear fluorescence is achieved **[3]**.

3.5.1. Talent applies a single drop of Carbomer eye gel into the base chamber.

3.5.2. SCOPE: SCOPE_3.5.2.mp4: 00:04-00:12

3.5.3. Talent using a cranial drill to remove adhesive and detach the base, preparing for repeat application.

3.6. Then, secure aluminum foil with tape between the fiber of the mTPM and the cranial window **[1]**.

3.6.1. Talent positioning and taping a strip of aluminum foil between the optical fiber and the cranial window.

3.7. Switch on the room light **[1]** and test the clarity of the frames captured by the mTPM **[2]**.

3.7.1. Talent turning on the overhead room lights.

3.7.2. SCREEN: SCREEN_3.7.2.mp4: 00:02-00:08

3.8. To put the mouse in an open field, inflate at least 10 helium balloons and tie each one separately with cotton twine **[1]**. Then, detach the metal plate from the mouse restrainer **[2]**.

3.8.1. Talent inflating helium balloons and tying each with a piece of cotton twine.

3.8.2. Talent detaching the metal plate from the restrainer to release the mouse.

3.9. Hold the mouse gently by its tail using one hand **[1]**. With the other hand, support the

optical fiber of the mTPM **[2]**.

3.9.1. Talent holding the mouse by its tail.

3.9.2. Talent using the other hand to support the microscope's optical fiber.

3.10. Carefully place the mouse into the open field **[1]**. Suspend the helium balloons by attaching the cotton twine to the fiber **[2]**. Adjust the number of balloons so that the mouse can move and explore the open field without restriction **[3]**.

3.10.1. Talent gently lowering the mouse into the open field arena.

3.10.2. Talent tying the cotton twine from helium balloons to the microscope fiber.

3.10.3. Shot of the mouse freely exploring the open field with balloon-suspended fiber.

3.11. Close the door of the mTPM enclosure to reduce external disturbances **[1]**.

3.11.1. Talent closing the enclosure door around the open field setup.

3.12. Start the mTPM recording software and the synchronization software **[1]**. Set the file paths and recording parameters according to the platform establishment procedure **[2]**.

3.12.1. SCREEN: SCREEN_3.12.1.mp4: 00:01-00:02, 00:08-00:11

3.12.2. SCREEN: SCREEN_3.12.2.mp4: 00:08-00:26

3.13. Start the recording of the mTPM through the recording software **[1]**. Check the synchronization software to verify that time markers are accurately recorded for each two-photon frame **[2]**.

3.13.1. SCREEN: SCREEN_3.13.1.mp4: 00:00-00:08

3.13.2. SCREEN: SCREEN_3.13.2.mp4: 00:00-00:07

3.14. Evaluate whether the contrast of the two-photon images remains stable during recording **[1]**. Also, confirm that the mouse's movements do not disrupt the stability of the imaging frames **[2]**.

3.14.1. SCREEN: SCREEN_3.14.1.mp4: 00:09-00:18

3.14.2. SCREEN: SCREEN_3.14.2.mp4: 00:07-00:17

3.15. Now, start the customized camera synchronization script to initiate behavior recording **[1]**. Set the file path and parameters according to the platform establishment procedure

[2].

3.15.1. SCREEN: SCREEN_3.15.1.mp4

3.15.2. SCREEN: SCREEN_3.15.2.mp4: 00:04-00:24

3.16. Then, start the behavior recording using the customized synchronization script **[1]**. Confirm the presence of a time marker in the synchronization software for every 30 frames of behavior video **[2]**.

3.16.1. SCREEN: SCREEN_3.16.1.mp4: 00:02-00:12

3.16.2. SCREEN: SCREEN_3.16.2.mp4

3.17. Check that all four video streams from the cameras are synchronized correctly **[1]**. Verify that the video capture parameters of the three-dimensional behavioral tracking system are set properly **[2]**.

3.17.1. SCREEN: SCREEN_3.17.1.mp4: 00:00-00:10

3.17.2. SCREEN: SCREEN_3.17.2.mp4: 00:00-00:04

3.18. Once the behavioral recording stops automatically, manually switch off both the mTPM recording and synchronization software to conclude the trial **[1]**.

3.18.1. SCREEN: SCREEN_3.18.1.mp4

Results

4. Results

4.1. Correlation coefficient matrices showed no distinct neuron-specific patterns for subject poses, object poses, or body distances, indicating weak correspondence between neural signals and behavioral metrics **[1].**

4.1.1. LAB MEDIA: Figure 2F.

4.2. All neuron-behavior correlation coefficients fell between minus 0.3 and 0.3, confirming weak associations under naturalistic conditions **[1].**

4.2.1. LAB MEDIA: Figure 2G**.** *Video editor: Highlight the range of correlation values along the y-axis (from -0.3 to 0.3) across the three stacked dot plots.*

4.3. CEBRA *(Zi-Bra)*-derived neural embeddings form intricate patterns, incorporating components from multiple joint embeddings **[1].** CEBRA embeddings demonstrated consistent alignment of behavioral and neural variables across three mouse pairs, particularly for body distance and social motifs **[2].**

4.3.1. LAB MEDIA: Figure 5A, last column. *Video editor: Show the far-right panel in each row under "S1-embedding,"*

4.3.2. LAB MEDIA: Figure 5B and C. *Video editor: Highlight the "Distance" and "SBeA Categories" columns in 5B. Emphasize the lower RMSE bars for body distance and social motifs after alignment in 5C.*

4.4. The decoding error for body distance embeddings was significantly higher than subject and object poses but remained within expected tracking error limits **[1].** Joint embeddings of neural activity with various behavioral variables revealed high decoding accuracy across subject poses, object poses, and motifs **[2].**

4.4.1. LAB MEDIA: Figure 5D. *Video editor: Highlight the taller bar labeled "Social" and compare it visually to the shorter bars labeled "Subject" and "Object."*

4.4.2. LAB MEDIA: Figure 5E.

4.5. Cosine similarity analysis using the S1–subject pose embedding as a reference showed

lower alignment for object-related motifs, suggesting primary encoding of self and social behavior **[1].**

4.5.1. LAB MEDIA: Figure 5F. *Video editor: Highlight the downward-pointing bar for "S1–Object motifs".*

1. **synchronization**
   IPA: /ˌsɪŋ.krə.nəˈzeɪ.ʃən/
   Phonetic spelling: *sing-kruh-nuh-ZAY-shun*

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2. **module**
   IPA: /ˈmɑː.dʒul/ or /ˈmoʊ.dʒul/
   Phonetic spelling: *MOH-jool* (or *MAH-jool*)

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3. **microscope**
   Pronunciation link: https://dictionary.cambridge.org/pronunciation/english/microscope (Cambridge Dictionary)
   IPA: /ˈmaɪ.krə.skoʊp/ (Cambridge Dictionary)
   Phonetic spelling: *MY-kruh-skohp*

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4. **acrylic**
   Pronunciation link: https://dictionary.cambridge.org/pronunciation/english/acrylic (Cambridge Dictionary)
   IPA: /əˈkrɪl.ɪk/ (Cambridge Dictionary)
   Phonetic spelling: *uh-KRIL-ik*

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5. **objective** (as in microscope objective)
   IPA: /əbˈdʒɛk.tɪv/
   Phonetic spelling: *uh-JEK-tiv*

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6. **fluorescence**
   IPA: /flʊˈrɛs.əns/
   Phonetic spelling: *flu-RES-uhns*

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7. **platform**
   IPA: /ˈplæt.fɔːrm/ or /ˈplæt.fɔrm/
   Phonetic spelling: *PLAT-form*

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8. **embeddings** (neural embeddings)
   IPA: /ɛmˈbɛd.ɪŋz/
   Phonetic spelling: *em-BED-ings*

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9. **decoder / decoding**
   - **decoder** IPA: /dɪˈkoʊ.dər/ — *di-KOH-der*
   - **decoding** IPA: /dɪˈkoʊ.dɪŋ/ — *di-KOH-ding*

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10. **correlation**
    IPA: /ˌkɔːr.əˈleɪ.ʃən/
    Phonetic spelling: *kor-uh-LAY-shun*