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Title: Whole-brain 3D activation and functional connectivity mapping in mice using transcranial functional ultrasound imaging

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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, all done**

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 videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

Current Protocol Length

Number of Steps: 27
Number of Shots: 30, 18 SC

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Adrien Bertolo:** Functional ultrasound is a new neuroimaging modality that allows the mapping of cerebral blood volume in the living rodent brain. Using ultrafast plane wave imaging, we can measure whole brain hemodynamic responses with unmatched spatiotemporal resolution and sensitivity. This protocol explains how to perform transcranial functional Ultrasound imaging in mice both for anesthetized and awake animal experiments.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. Videographer's note: Use last one.

1.2. **Mohamed Nouhoum:** Compared to other whole brain functional imaging techniques such as fMRI, Functional ultrasound provides high portability, ease of use, and allows experiments in awake and freely moving subjects, avoiding anesthesia bias and enabling behavioral studies.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.9.1.*

OPTIONAL:

1.3. **Silvia Cazzanelli:** Until recently, fUS imaging was only used in collaboration with ultrasound experts. Now, this technology is available to the wide neuroscience community, thanks to commercially available scanners and dedicated software for preclinical brain imaging, making fUS pretty easy to use without any background in ultrasound.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.1.3.* Videographer's note: Use last one.

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the European Community Council Directive of 22 September 2010 and the local ethics committee (Comité d'éthique en matière d'expérimentation animale number 59, 'Paris Centre et Sud').

Protocol

2. Animal Preparation

2.1. For an anesthetized imaging session, apply eye ointment to the mouse eyes to avoid any cataract formation **[1]** and shave the mouse head using a trimmer **[2]**. Apply some depilatory cream **[3]** and rinse after a couple of minutes. Repeat this until the hair is completely removed **[4]**. *Videographer: This step is important!*

2.1.1. Talent applying eye ointment to mouse.

2.1.2. Talent shaving the mouse head.

2.1.3. Talent applying depilatory cream.

2.1.4. Talent rinsing the cream off.

2.2. Insert subcutaneous pins in the limbs for electrocardiogram recording **[1]** and place centrifuged ultrasound gel on the head **[2]**. *Videographer: This step is important!*

2.2.1. Talent positioning the mouse on the frame Videographer's note: For 2.2.1, use 2nd part.

2.2.2. Talent inserting pins into limbs.

2.2.3. Talent placing gel on the mouse's head.

2.3. For awake head-fixed mice experiments, place the anesthetized animal in a stereotaxic frame on a 37-degree Celsius heating pad **[1]**. Apply protective gel for the eyes **[2]** and subcutaneously administer lidocaine under the scalp using a 26-gauge needle, then wait a few minutes **[3-TXT]**. *Videographer: This step is important!*

2.3.1. Talent positioning the mouse on the frame.

2.3.2. Talent applying gel to the eyes.

2.3.3. Talent injecting lidocaine. **TEXT: 0.2 mL, 2 %**

2.4. Make an incision following the sagittal suture from behind the occipital bone to the beginning of the nasal bone **[1]**. Then use surgical scissors to excise the skin over both hemispheres **[2]**.

2.4.1. Talent making the incision.

2.4.2. Talent excising skin. Videographer's note: 2.4.2 was filmed with 2.4.1

2.5. Clean the skull with 1% iodine solution and remove any remaining periosteum **[1]**. Using the headplate as a template, mark two holes in the skull to position the anchoring screws **[2-TXT]**.

2.5.1. Talent cleaning the skull.

2.5.2. Talent drilling a hole in the skull. **TEXT: 1 mm diameter**

2.6. Position the headplate with the screws **[1]** and use dental cement to fix the screws and the headplate in the front and back of the frame to maintain good grip of the implant **[2]**. *Videographer: This step is difficult and important!*

2.6.1. Talent positioning the headplate. Videographer's NOTE: from 12/43/30/00 including 2.6.2.

2.6.2. Talent fixing screws with dental cement.

2.7. Remove the animal from the stereotaxic frame after the cement is dry **[1]** and reverse the anesthesia by a subcutaneous injection of 1 milligram per kilogram of atipamezole **[2]**. Administer prophylactic meloxicam for post-operative pain **[3-TXT]**.

2.7.1. Talent removing the mouse from the frame.

2.7.2. Talent administering meloxicam. **TEXT: 5 mg/kg/day, s.c.**

2.7.3. Talent injecting atipamezole into the mouse.

2.8. Place a magnetic 3D-printed cap over the headplate for protection and allow the mouse to recover for 4 to 6 days before the beginning of habituation to the mobile home cage **[1]**. Place the animal in a recovery cage on a heating pad for a few hours, then return the mouse to its home cage with littermates **[2]**.

Videographer's NOTE: For step 2.8, steps 1 and 2 were inverted.

2.8.1. Talent placing a cap over the headplate.

2.8.2. Talent placing the animal in a recovery cage.

Videographer's NOTE: 2.8.2 was placed before 2.8.1 from 12/51/50.

2.9. On day 4 and 5 post-recovery, repeatedly clamp the mouse to the mobile home cage and gradually increase the head-fixed time, starting from 5 minutes and up to 30 minutes **[1]**. Apply some saline and ultrasound gel on the imaging window to habituate the mouse. Repeat this process on day 6 post-recovery **[2]**. *Videographer: This step is difficult and important!*

2.9.1. Talent clamping the mouse to the mobile home cage.

2.9.2. Talent applying saline and ultrasound gel on the imaging window.

3. Probe positioning

3.1. Start the software and create an experiment session. Go to the **Move Probe** menu to adjust the **[1]** position of the ultrasound probe **[2]** using the navigation keyboard **[3]**.

3.1.1. SCREEN: 62267_screenshot_1. 0:20 – 0:28.

3.1.2. SCREEN: 62267_screenshot_2. 0:10 – 0:14.

3.1.3. Probe coming closer to the mouse head *Videographer: This step is important!*

3.2. Start the **Live View** acquisition and adjust probe position if needed via real time imaging of the animal cerebral blood volume, or CBV. Align the brain at the center of the image, then optimize the imaging parameters to capture the highest signal-to-noise ratio.

3.2.1. SCREEN: 62267_screenshot_3. 0:03 – 0:16.

3.2.2. SCREEN: 62267_screenshot_3. 0:17 – 0:27.

4. Angiographic scan and atlas registration

4.1. Open the **Angio 3D** option in the acquisition software. On the preset panel, adjust the first slice, last slice, and step size scanning parameters in order to scan the whole brain and start the acquisition **[1]**.

4.1.1. SCREEN: 62267_screenshot_4. 0:03 – 0:41. *Video Editor: Speed this up after 0:19.*

4.2. Leave the acquisition software open and start the software for data analysis and visualization, then load the angio 3D scan. Navigate through the acquisition volume using the 3-views panel and select the **Coronal Scan Direction**, antero-posterior or postero-anterior **[1]**.

4.2.1. SCREEN: Data analysis software opened, angio 3D scan loaded, and Coronal Scan direction selected. *Videographer: Film the screen for this shot.*

4.3. Go to the **Brain Registration Panel** and load the mouse reference template for the registration process. Register the scan on the **Allen Mouse Common Coordinates Framework** using the fully automatic or the manual registration modes **[1]**.

4.3.1. SCREEN: 62267_screenshot_5. 0:13 – 0:35.

4.4. Check the result by looking at the superposition of the angio 3D scan and the reference template or by looking at the superposition of the scan and the Allen reference atlas using the **Atlas Manager** panel. Save the registration as a .bps file **[1]**.

4.4.1. SCREEN: 62267_screenshot_5. 1:00 – 1:31. *Video Editor: Speed this up from 1:00 – 1:25.*

5. Brain Positioning System (BPS)

5.1. In the IcoStudio software, make sure that the angiographic scan and its .bps file are loaded **[1]**.

5.1.1. SCREEN: 62267_screenshot_6. 0:04 – 0:07.

5.2. Go to the **Brain Navigation Panel**. In the **Atlas Manager** panel, navigate through the mouse **Allen brain atlas** with the parent-child tree navigator. Find the anatomical targeted regions and select them to superimpose them to the scan in the 3-views **[1]**.

5.2.1. SCREEN: 62267_screenshot_6. 0:08 – 0:24.

5.3. Visualize the targeted regions in the 3-view panel and choose an imaging plane that overlaps the targeted regions for the experiment by manually setting two markers on the coronal position that includes the regions of interest **[1]**.

5.3.1. SCREEN: 62267_screenshot_6. 0:25 – 0:51.

5.4. Click on **Brain Positioning System**, or BPS, to extract the resulting motor coordinates, which correspond to the probe position, to image the targeted plane. Check the preview of the image which is computed from the angio scan **[1]**.

5.4.1. SCREEN: 62267_screenshot_6. 0:52 – 1:04.

5.5. In the IcoScan software, enter the **Probe positioning** panel and click on **Enter BPS coordinates**, then apply the extracted coordinates, causing the probe to move and align on the targeted imaging plane **[1]**.

5.5.1. SCREEN: 62267_screenshot_7. 0:02 – 0:27.

5.6. Perform a live view acquisition and check that the current imaging plane corresponds to the prediction **[1]**.

5.6.1. SCREEN: 62267_screenshot_7. 0:28 – 0:39.

6. Task-evoked experiment: whisker stimulation

6.1. Predefine the stimulation sequence, including time of stimulation, inter-stimulation time, and number of repetitions **[1]**.

6.1.1. SCREEN: 62267_screenshot_8. 0:06 – 0:31. *Video Editor: Speed this up.*

6.2. Run a 3D fUS *(spell our 'F-U-S')* sequence by defining the total time of acquisition, the number of positions, and the dead-time between positions **[1]**. For automatic stimulation synchronized with the acquisition system through TTL input, select the **Trig-IN** option before starting the acquisition **[2]**.

6.2.1. SCREEN: 62267_screenshot_8. 0:32 – 0:42.

6.2.2. Close-up of the mouse during whisker stimulation.

6.3. Open the acquisition in IcoStudio software and enter the **Activation map** menu, then fill the activation pattern field with start and end times and compute the activation map. Adjust the display parameters for visualization and export the activation map as a .h5 file for off-line analysis **[1]**.

6.3.1. SCREEN: 62267_screenshot_9. 0:02 – 0:55. *Video Editor: Speed this up from 0:38 – 0:53.*

7. 4D functional connectivity

7.1. Run a 3D fUS sequence by defining the total time of acquisition, the number of imaging plane positions, and the dead-time between positions **[1]**.

7.1.1. SCREEN: 62267_screenshot_10. 0:15 – 0:30.

7.2. Save the acquisition and load it in the IcoStudio software. If necessary, load the .bps file and the Allen mouse brain coordinate framework. In the **Atlas manager**, select regions of the atlas as regions of interest **[1]**.

7.2.1. SCREEN: 62267_screenshot_10. 0:02 – 0:25.

7.3. Enter the **Functional Connectivity** menu and select the desired regions in the ROI manager. Visualize the results as connectivity matrix or seed-based correlation map, then select and adjust the bandwidth filters as desired and export correlation results for statistical analysis **[1]**.

7.3.1. SCREEN: 62267_screenshot_12. 0:10 – 0:50. *Video Editor: Speed this up from 0:13 – 0:25.*

Results

8. Results: Transcranial Activation Maps and rCBV Time Course Following Whiskers Stimulation in an Anesthetized and Awake Behaving Mouse

8.1. This protocol was used for 3D quantification of cerebral hemodynamic variations transcranially in the mouse brain. Whisker stimulation was selected as an example of a sensory stimulation-evoked response **[1]**.

8.1.1. LAB MEDIA: Figure 4 A.

8.2. Significant activation was determined with the resolution of a general linear model, or GLM, using a default mouse hemodynamic response function **[1]**.

8.2.1. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the orange regions in the brain images.*

8.3. The total trial time was 760 seconds **[1]**, with a 60 second baseline **[2]**, an 80 second stimulation **[3]**, and a 60 second recovery time, repeated 5 times **[4]**.

8.3.1. LAB MEDIA: Figure 4 C.

8.3.2. LAB MEDIA: Figure 4 C. *Video Editor: Emphasize the part of the plot before the first peak.*

8.3.3. LAB MEDIA: Figure 4 C. *Video Editor: Emphasize the peaks.*

8.3.4. LAB MEDIA: Figure 4 C. *Video Editor: Emphasize the parts of the plot between the peaks.*

8.4. Using a voxel-wise time course of the contralateral primary somatosensory cortex, the barrel field region, or S1BF, revealed a 15 to 20% increase of the CBV compared to baseline **[1]**.

8.4.1. LAB MEDIA: Figure 4 B.

8.5. The same paradigm was applied in a head-fixed behaving mouse in the mobile homecage using the awake preset of IcoScan **[1]**. The activation map after a multiple whisker stimulation experiment is shown here **[2]**.

8.5.1. LAB MEDIA: Figure 5 C and D.

8.5.2. LAB MEDIA: Figure 5 A.

8.6. Significant activation was determined with the resolution of a GLM using a default mouse hemodynamic response function **[1]**.

8.6.1. LAB MEDIA: Figure 5 A and B.

8.7. The temporal correlations of normalized low-frequency spontaneous CBV fluctuations between 3D brain regions in a ketamine-xylazine anesthetized mouse are shown here **[1]**.

8.7.1. LAB MEDIA: Figure 6 A.

8.8. Seed-based analysis in the dorsal hippocampus revealed a significant interhemispheric connectivity between the right and left hippocampus as well as deep retro-hippocampal regions and piriform cortices **[1]**.

8.8.1. LAB MEDIA: Figure 6 B.

8.9. A seed region selected in the S1BF also resulted in a symmetrical correlation pattern **[1]**.

8.9.1. LAB MEDIA: Figure 6 C.

Conclusion

9. Conclusion Interview Statements

9.1. **Sophie Pezet:** The critical point for successful experiments is the animal's preparation, in particular, the level of anesthesia for experiments involving anesthetized animals and the protection of the skull for experiments in awake animals.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.2.1, 2.8.2*

9.2. **Zsolt Lenkei:** fUS enabled us to study important brain functions in awake animals dealing with fundamental questions on sleep, learning or behavior, but also the pharmacological modulation of functional connectivity for drug discovery.

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