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TITLE:

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

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KEYWORDS:

Functional ultrasound; functional connectivity; whole brain imaging; imaging platform.

SUMMARY:

This protocol describes the quantification of volumetric cerebral hemodynamic variations in the mouse brain using functional ultrasound (fUS). Procedures for 3D functional activation map following sensory stimulation as well as resting-state functional connectivity are provided as illustrative examples, in anesthetized and awake mice.

ABSTRACT:

Functional ultrasound (fUS) imaging is a novel brain imaging modality that relies on the high-sensitivity

measure of the cerebral blood volume achieved by ultrafast doppler angiography. As brain perfusion is strongly linked to local neuronal activity, this technique allows the whole-brain 3D mapping of task-induced regional activation as well as resting-state functional connectivity, non-invasively, with unmatched spatio-temporal resolution and operational simplicity. In comparison with fMRI (functional magnetic resonance imaging), a main advantage of fUS imaging consists in enabling a complete compatibility with awake and behaving animal experiments. Moreover, fMRI brain mapping in mice, the most used preclinical model in Neuroscience, remains technically challenging due to the small size of the brain and the difficulty to maintain stable physiological conditions. Here we present a simple, reliable and robust protocol for whole-brain fUS imaging in anesthetized and awake mice using an off-the-shelf commercial fUS system with a motorized linear transducer, yielding significant cortical activation following sensory stimulation as well as reproducible 3D functional connectivity pattern for network identification.

INTRODUCTION:

Over the last two decades, neuroimaging has become an important tool for studying brain function and organization, enabling researchers to make important discoveries in the field of neuroscience. Today, functional magnetic resonance imaging (fMRI) has become the gold standard clinical neuroimaging technique to assess task or drug-evoked brain activation and to map functional connectivity at rest. While human fMRI has high reliability and sensibility, mouse fMRI remains technically challenging for numerous reasons[1]. First, fMRI has a poor spatial and temporal resolution. The small size of the mouse brain necessitates the use of strong magnetic fields using expensive scanners to achieve reasonable spatial resolution. Second, maintaining stable physiological parameters within the narrow range allowing efficient neuro-vascular coupling is very difficult in anesthetized mice. Finally, the blood oxygen level dependent (BOLD) signal on which fMRI studies rely has relatively poor sensitivity, leading to low signal-to-noise ratio when applied to mice and often requires repeated stimulus presentation over long acquisition to detect small variations. The mouse being the most widely used animal model in biomedical preclinical research, these limitations are partly responsible for the translational gap in neuropsychiatry, hindering new promising therapeutic targets on the bench to be transposed into effective treatments on bedside.

Functional ultrasound (fUS) is a recently developed neuroimaging technique based on ultrafast doppler[2]. By directly sampling cerebral blood volume, this technique allows probing brain activity in real-time through the neurovascular coupling. Compared to other neuroimaging techniques, fUS yields a spatial resolution of 100 µm and a temporal resolution in the tens of milliseconds. This technique allows whole-brain imaging of complete coronal sections of the mouse brain, completely non-invasively. Furthermore, it is fully compatible with conscious and behaving animals[3–5]. One of the main current limitations of fUS is its 2D feature, allowing to record a single coronal plane at the same time. While volumetric 3D fUS using 2D matrix array transducers has already been successfully demonstrated in rats[6] and confirmed in mice[7], its current lack of sensitivity requires a full craniotomy as well as averaging an important number of trials to detect a slight change of activity. Alternatively, linear transducers can be stepped across multiple positions and perform functional imaging plane by plane to cover the whole brain. However, this technique requires numerous experimental paradigm repetitions and as such long acquisition times (3-4 hours for the mouse brain)[8,9].

In the present work, we describe a robust experimental platform including a commercially available

functional ultrasound scanner and a fast plane-switching linear transducer with procedures to acquire 3D fUS data in anesthetized and awake mice, allowing volumetric and transcranial functional mapping of the mouse brain, non-invasively, without contrast-agent and within short acquisition times. We illustrate this feature by mapping somatosensory cortex activation following whisker stimulation as well as resting-state functional connectivity. Aside from animal preparation and data collection, we also describe the procedure for visualization, atlas registration and analysis of real-time fUS signals.

PROTOCOL:

All the procedures presented here have been performed in agreement with the European Community Council Directive of 22 September 2010 (010/63/UE) and our local ethics committee (Comité d'éthique en matière d'expérimentation animale number 59, 'Paris Centre et Sud', project #2017-23). Adult mice (male C57BL/6 Rj, age 2-3 months, 20-30 g, from Janvier Labs, France) were housed 4 per cage with a 12h light/dark cycle, constant temperature at 22 °C and food and water ad libitum. Before the beginning of the experiments, animals are given a one-week minimum acclimatization period to housing conditions.

1. Animal preparation for anesthetized fUS imaging

1.1. Anesthesia

1.1.1. Weigh the mouse.

1.1.2. Prepare a mixture of ketamine and xylazine at 10 mg/mL and 2 mg/mL, respectively, in sterile saline. Administer 0.2 mL of the ketamine/xylazine solution intraperitoneally using a 26 gauge needle and 1 mL disposable syringe. After a few minutes, position the animal onto the stereotaxic frame, making sure that the head is flat.

1.1.3. Administer a second volume of anesthetics to reach a total dose of 100 mg/kg ketamine and 20 mg/kg xylazine (taking the initial dose into account).

NOTE: Anesthesia should last for 1 h. To maintain a steady sedation for a longer time, inject 0.05 mL of the ketamine/xylazine mixture every 30 min intraperitoneally.

1.2. Animal preparation for anesthetized imaging session

1.2.1. Apply some eye ointment (e.g., Ocry-Gel) to the mouse eyes to avoid any cataract formation during the imaging session. Shave the mouse head using a trimmer. Apply some depilatory cream and rinse after a couple of minutes. Repeat until the hair is completely removed.

1.2.2. Insert subcutaneous pins in the limbs for electrocardiogram (ECG) recording. Place centrifuged ultrasound gel (1500 rpm, 5 min) on the head.

1.2.3. Monitor the depth of anesthesia during the complete duration of the experiments (anesthesia

induction included). Maintain the temperature of the animals at 37 °C by using a heating blanket coupled to a rectal probe.

1.2.4. Monitor the following physiological parameters which are indirect indicators of the depth of anesthesia: Heart rate (220-250 beats per minute - monitored through the electrocardiogram thin electrodes implanted subcutaneously), and Respiratory rate (130-140 breaths per minute - monitored using a spirometer connected to the ECG acquisition system).

NOTE: A description of the experimental setup is depicted in **Figure 1**.

[Place Figure 1 here]

2. Animal preparation for awake head-fixed mice experiments

2.1. Headplate surgery

2.1.1. Place the anesthetized animal (steps 1.1-1.2) in the stereotaxic frame on a heating pad (37 °C). Apply protective gel for the eyes and administer lidocaine s.c. (0.2 mL, 2 %) under the scalp skin using a 26-gauge needle and wait a few minutes.

NOTE: Monitor the anesthesia level every 10-30 min by response (absence of) to a firm toe pinch.

2.1.2. Perform an incision following the sagittal suture from behind the occipital bone to the beginning of the nasal bone. Using surgical scissors, excise the skin over both hemispheres.

2.1.3. Clean the skull with 1% iodine solution and remove any remaining periosteum. Using the headplate as a template, drill two holes (1 mm diameter) in the skull to position the anchoring screws.

CAUTION: Be careful not to drill completely through the skull to avoid any brain damages or dura inflammation

2.1.4. Position the headplate with the screws. Use dental cement to fix the screws and the headplate in front and in the back of the frame to maintain good grip of the implant.

CAUTION: Be careful to not apply cement inside the frame window as it greatly diminishes signal quality. Cover the skull with a thin layer of surgical glue to protect the bone and seal the wounds on the side of the imaging window.

2.1.5. Remove the animal from the stereotaxic frame after the cement is dry and reverse the anesthesia by a subcutaneous injection of atipamezole at 1 mg/kg. A prophylactic administration of meloxicam (5 mg/kg/day, s.c.) is administered for post-operative pain.

2.1.6. Place the animal in a recovery cage on a heating pad (37 °C). The mouse can return its home cage with littermates within a few hours. Place a magnetic 3D-printed cap (polyactic acid material with

magnet inserts) over the headplate for protection (**Figure 2A**). Leave the mouse to recover for 4 to 6 days before the beginning of the habituation to the mobile home cage (MHC).

NOTE: The total weight of the cap and the headplate is 2.8 g.

2.2. Handling and habituation

2.2.1. On day 1 post-recovery (PR), gently hold the mouse in hand for 5-10 min several times a day.

2.2.2. On day 2 PR, repeat handling as in day 1 and leave the animal for 5-10 min exploring freely MHC.

NOTE: Playing some background music in the room can help reduce animal's stress.

2.2.3. On day 3 PR, let the animal freely explore the MHC for 5-10 min. Afterwards, carefully grab the headplate and gently place it in the clamp, moving manually the carbon cage to accompany the mouse. Habituate the animal in the head-fixed position for 5-10 min. Clean the MHC in between training sessions with 70% ethanol solution and rinse with tap water.

NOTE: Make sure that the MHC receives a sufficient air flow as recommended by the manufacturer. The height of the head clamp needs to be manually adjusted to provide a comfortable position.

2.2.4. On day 4 and 5 PR, repeatedly clamp the mouse MHC and gradually increase the head-fixed time, starting from 5 min and up to 30 min. Apply some saline and ultrasound gel on the imaging window to habituate.

2.2.5. On day 6 PR, repeat the protocol from day 4/5 PR and position the probe above the animal's head following step 3.1.

2.2.6. On the day of the experiment, proceed as described above. Then, humidify the imaging window with saline and apply some ultrasound gel. Start the tracking of the animal and proceed to the probe positioning (see below).

NOTE: Clamping in the MHC may also be done by wrapping the mouse in a rag. In that case, mice need to be habituated to the wrapping procedure before head-fixation. A description of a complete experimental setup for awake imaging is provided in **Figure 2B.**

[Place Figure 2 here]

3. Probe positioning

3.1.1. Start the software (e.g., IcoScan) and create an experiment session. Go to the **Move Probe** menu to adjust the position of the ultrasound probe using the navigation keyboard.

NOTE: The probe should be positioned approximately 1 mm above the animal's head. It is crucial to

ensure that the probe is in contact with ultrasound gel before starting any imaging sequence.

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

NOTE: In awake mice experiments, the aperture size needs to be reduced to avoid artefacts induced by lateral muscles contraction.

4. Angiographic scan and atlas registration

4.1. Open the **Angio 3D** option in the acquisition software. On the preset panel, adjust the scanning parameters (first slice, last slice and step size) in order to scan the whole brain (**Figure 3A, B**), and start the acquisition.

NOTE: When setting up the scan parameters, make sure that the scan will cover the posterior part of the brain

4.2. Leave the acquisition software open and start the software for data analysis and visualization (e.g., IcoStudio), and 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.

4.3. Go to the **Brain Registration Panel**. Load the mouse reference template that will be needed for the registration process. Register the scan on the **Allen Mouse Common Coordinates Framework** using the fully automatic or the manual registration modes (**Figure 3C**).

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 (**Figure 3D**). Save the registration as a .bps file.

NOTE: The registration file can be reused for any other acquisition performed during the same experiment session.

5. Brain Positioning System (BPS)

5.1. In the IcoStudio software, make sure that the angiographic scan and its .bps file (generated in **step 4.4**) are loaded.

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 your scan in the 3-views.

5.3. Visualize the targeted regions in the 3-view panel and choose an imaging plane that overlaps the targeted regions for the experiment. To do so, manually set two markers on the coronal position that

includes the regions of interest.

5.4. Click on **Brain Positioning System** (BPS) to extract the resulting motor coordinates. These coordinates correspond to the probe position which allows to image the targeted plane. Check the preview of the image which is computed from the angio scan.

5.5. In the IcoScan software, enter the **Probe positioning** panel and click on **Enter BPS coordinates**. Apply the coordinates given in **step 5.4**. The probe moves and aligns on the targeted imaging plane.

5.6. Perform a live view acquisition and check that the current imaging plane corresponds to the prediction given in **step 5.4**.

NOTE: It is also possible to select parasagittal/non orthogonal planes.

[Place Figure 3 here]

6. Task-evoked experiment: whisker stimulation

6.1. Predefine the stimulation sequence, including time of stimulation, inter-stimulation time and number of repetitions.

6.2. Run a 3D fUS sequence by defining the total time of acquisition, the number of positions as well as the dead-time between positions. In case of automatic stimulation synchronized with the acquisition system through TTL input, select the **Trig-IN** option before starting the acquisition.

NOTE: For the results presented in this work, stimulation was delivered using cotton swab positioned such as to allow deflection of most of the whiskers in the dorsal/ventral direction. It was fixed on a servo-motor driven by an Arduino UNO card, linked to the Iconeus One system to ensure synchronization. The recommended parameters for stimulation are 30 s ON, 30 s OFF, amplitude of 20° and 4 Hz frequency. Alternatively, the stimulation can also be delivered manually by deflecting the whiskers at the defined times during the acquisition.

6.3. Open the acquisition in IcoStudio software and enter the **Activation map** menu. Fill the activation pattern field with start and end times and compute the activation map. Adjust the display parameters for visualization. Export the activation map as a .h5 file for off-line analysis.

NOTE: Activation is estimated using a generalized linear model (GLM) approach with the stimulus convolved by a default mouse hemodynamic response (HRF). Alternatively, activation can be visualized directly by estimating the Pearson correlation between the stimulation pattern and the hemodynamic signal from each voxel.

7. 4D functional connectivity

7.1. Run a 3D fUS sequence by defining the total time of acquisition, the number of imaging plane positions as well as the dead-time between positions.

NOTE: For 3D functional connectivity, we recommend acquisition time between each volume < 2.5 s (sampling frequency of at least 0.4 Hz) and a total acquisition time of at least 10 min (number of time points > 180).

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 (ROI).

7.3. Enter the **Functional Connectivity** menu and select the desired regions in the ROI manager. Visualize the results as connectivity matrix (supervised analysis) or seed-based correlation map (unsupervised). Select and adjust the bandwidth filters as desired and export correlation results for statistical analysis.

NOTE: In 3D fUS imaging mode, the relative probe positions are set manually. Hence, two types of scans are possible and can be chosen depending on the functional application: dense scans versus sparse scans (**Figure 2C**).

REPRESENTATIVE RESULTS:

This protocol describes the 3D quantification of cerebral hemodynamic variations transcranially in the mouse brain, at rest or in response to sensory stimulation. Whisker stimulation, a standard paradigm to map brain functional activation in rodents, has been selected as an example of sensory stimulation-evoked response. **Figure 4** shows a representative activation map in response to mechanical whisker stimulation in an anesthetized mouse obtained using transcranial fUS imaging. The total trial time was 760 s, with a 60 s baseline (before and after the stimulation), an 80 s stimulation and a 60 s recovery time, repeated 5x. Significant activation was determined with the resolution of a general linear model (GLM) using a default mouse hemodynamic response function (HRF). The activated regions (Z scores with p-value >0.0000006 after stringent Bonferroni correction for multiple comparison) are displayed as color-coded values overlaid onto the Allen common coordinate framework template. Voxel-wise time course of the contralateral primary somatosensory cortex, barrel field region (S1BF) revealed a 15-20% increase of the CBV compared to baseline.

[Place Figure 4 here]

The same paradigm has been applied in a head-fixed behaving mouse in the mobile homecage using the awake preset of IcoScan. **Figure 5** presents the activation map after a multiple whisker stimulation experiment using the experimental setup described in **Figure 2**. A few posterior and caudal whiskers were stimulated with the following pattern: 30 s baseline followed by five consecutive trials of 30 s ON (4 Hz) and 30 s OFF (**Figure 5C**). Stimulation was delivered using a servo-motor driven by an Arduino UNO card triggering the image acquisition sequence for synchronization. Significant activation was determined with the resolution of a general linear model (GLM) using a default mouse hemodynamic response function (HRF). Multiple comparison correction was performed with the Bonferroni method.

Conventional alpha level of 0.05 was normalized by the total number of voxels in the acquisition volume, resulting in a final stringent threshold of 0.000003.

[Place Figure 5 here]

Figure 6 shows the temporal correlations of normalized low-frequency (<0.2 Hz) spontaneous CBV fluctuations between 3D brain regions (identified from registration to the Allen common coordinate framework) in a ketamine-xylazine anesthetized mouse. Total acquisition time was 20 min (1200 s). Atlas-supervised analysis revealed strong interhemispheric connectivity patterns, with resulting correlation coefficient values up to 0.8. Seed-based analysis in the dorsal hippocampus revealed a significant interhemispheric connectivity between right and left hippocampus as well as deep retro-hippocampal regions and piriform cortices. A seed region selected in the S1BF also resulted in a symmetrical (cortico-cortical) correlation pattern, as previously described.

[Place Figure 6 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental setup for anesthetized fUS experiments.
Description of the experimental setup showing all the scientific equipment needed during an anesthetized experiment. **1.** Physiological monitoring : live display of both respiratory and cardiac frequencies. **2.** Four-axis motor module (three translations and one rotation) monitored by Iconeus One system (**9**) and allowing to perform transcranial 3D tomographic scans or 4D acquisitions. **3a.** Servo-Motor driving the whisker stimulator (**3b.**) The servo-motor is controlled by an arduino uno card which is interfaced with the Iconeus One system (**9**) in order to synchronize stimulation patterns with imaging sequences. **4.a.** Syringe pump controller. **4.b.** Syringe holder. **5.a.** Temperature plate monitor controlling the heating plate. **5.b.** Heating plate and rectal thermometer interfaced with the temperature plate monitor (**5.a.**). **6.** Ultrasound gel placed between the animal's head and the ultrasound probe, providing acoustic coupling between them. **7.** 15 MHz ultrasound probe. **8.** Probe holder linking the probe (**7**) to the motor module (**2**). **9.** Iconeus One equipment and software, allowing programing different imaging sequences and controlling the motors module (**2**) driving the probe (**7**).

Figure 2: Experimental setup for awake fUS experiments
A. Schematic illustration of the headplate magnetic cover protecting the imaging window. During imaging sessions (Left), the cover is removed to scan the brain in the large aperture offered by the head plate. **B.** Photograph of the experimental setup for transcranial awake imaging in head-fixed freely-behaving mice. **1.** Iconeus One system and software, allowing to set up different imaging sequences and controlling the motors module. **2.** Four-axis motors module (three translations and one rotation) monitored by Iconeus One system (**1**) and allowing 3D tomographic scans or 4D acquisitions. **3.** Air dispensing table. **4.** Mobile Home Cage (MHC). **5a, 5b.** Photographs showing closer views of the animal's environment inside the MHC. **6.** Head fixation system clamping the head plate. **7.** Probe holder linking the probe to the motor module (2). **8**. 15 MHz ultrasonic probe. **9.** Ultrasound gel placed between the mouse head and the ultrasound probe, providing acoustic coupling between them. **10.** Servo-Motor driving the whisker stimulator. The Servo-Motor is controlled by an Arduino Uno card which is interfaced

with the Iconeus One system through TTL signal (1) in order to synchronize stimulation patterns with imaging sequences. **C.** Different spatial sampling possibilities: in each case, the probe is stepped from the first position to the last one and a Doppler image is recorded at each position to reconstruct the stacked volume. This process is continuously repeated during the whole acquisition time. Dense Scan (left): the step between slices must be small enough (typically 400 µm, which corresponds to the elevation resolution) to allow volumetric imaging. Sparse Scan (right): if distant functional regions are targeted (at different positions), it is also possible to decrease spatial sampling to image different slices that intersect these regions while not compromising the temporal sampling.

Figure 3: Fast transcranial angiographic Scan and Brain registration for precise probe positioning

A. Schematic representation of the mouse brain being scanned transcranially by the ultrasonic probe from the first coronal slice (green) to the last coronal slice (blue) during a fast angiographic scan. The current imaged slice (represented in red) moves step by step from the back (green) to the front (blue) of the brain. **B.** Screenshot of IcoScan acquisition software in the Angio 3D panel. The preset parameters on the right configure the fast scan. The positions in mm of the first slice, the last slice and the step size must be well chosen to scan linearly the whole brain. **C.** Screenshot of the IcoStudio processing software. The fast Angio 3D scan is automatically registered to a reference template of the mouse brain. The three-views (left) shows the superposition of the vasculature and the mouse brain Allen atlas in the coronal, sagittal and axial views. **D.** Linear lay-out (montage) of 16 slices (out of 31) from the 3D angio scan, with the registered Allen reference atlas superimposed onto the vasculature. **E.** Screenshot of the Brain Navigation panel showing the predicted imaging plane corresponding to the motor coordinates computed by the software thanks to the two markers placed at the center of the left and right primary somatosensory cortex, barrel fields region.

Figure 4: Transcranial activation Maps and rCBV time course following whiskers stimulation in ketamine/xylazine anesthetized mouse

A. Activation map showing significantly activated voxels following mechanical stimulation of the right whiskers (80 s ON, 60 s OFF ,5x) under ketamine/xylazine anesthesia. Maps were obtained by computing Z-scores based on general linear model analysis (GLM) with Bonferroni correction for multiple comparison. Z-scores (color coded) are overlaid on the Allen brain 3D template (after registration with the brain positioning system) and displayed in three-views: coronal (left), sagittal (middle) and axial (right). Anatomical regions from the Allen mouse brain common coordinate framework are displayed for reference. Activated voxels are well located inside the left S1BF cortex. Scale bar: 1 mm. Each sample volume was scanned over 2.8 mm (corresponding to 7 slices in the elevation direction) in 3.85 s allowing to record 20 volumic samples during each functional response. **B.** 3D rendering of whisker stimulation-evoked relative cerebral blood volume (rCBV) increase compared to baseline level. The anatomical delineation of the S1BF is indicated in blue. **C.** Time course of CBV variations in the left S1BF (blue) and the corresponding stimulus applied (red).

Figure 5: Activation Maps and rCBV time course following whiskers stimulation in awake behaving mouse

A. Activation map showing significantly activated voxels following mechanical stimulation of the right whiskers (30 s ON, 30 s OFF, 5x) in an awake mouse in the mobile homecage. Maps were obtained by computing Z-scores based on general linear model analysis (GLM) with Bonferroni correction for multiple

comparison (normalization by the total number of voxels). Z-scores (color coded) are overlaid on the Allen brain 3D template (after registration with the Brain Positioning System) and displayed in three-views: coronal (left), sagittal (middle) and axial (right). Anatomical regions from the Allen Mouse Brain Common Coordinate Framework are displayed for reference. Activated voxels are well located inside the left S1BF cortex. Scales bars, 1 mm. Each sample volume was scanned over 1.6 mm (corresponding to 3 slices in the elevation direction) in 3.85 s allowing to record 17 volumic samples during each functional response. **B.** 3D rendering of whisker stimulation-evoked relative Cerebral Blood Volume (rCBV) increase compared to baseline level. The anatomical delineation of the S1BF is indicated in blue. **C.** Illustration of the mouse in the mobile homecage during the right whisker stimulation experiment, during which five 30 s trials were performed for a total acquisition time of 330 s. **D.** Instantaneous relative CBV time course extracted inside the activated area (blue), with the corresponding stimulus superimposed (red).

Figure 6: Transcranial volumetric resting-state functional connectivity of the mouse brain under ketamine/xylazine anesthesia assessed on a 20 min 3D fUS acquisition.

A. Correlation matrix based on 3D regions of the Allen common coordinate framework registered on the transcranial functional acquisition. The matrix is obtained by computing the normalized Pearson's correlation of spontaneous low-frequency fluctuations (<0.1 Hz) of the average time signals from all the voxels included in each identified ROI after slice timing correction. Each sampled volume was scanned over 1.6 mm in the elevation direction (corresponding to 4 slices) acquired over 2.2 s. **B**. Seed-based analysis projected onto a 3D template. The seed was selected within the right dorsal hippocampus at β – 2.1 mm. Correlation map is obtained by computing the Pearson Correlation coefficient between the temporal signals of the seed and each voxel of the whole acquisition after slice timing correction. **C.** 3D correlation map based on seed-based analysis with seed region selected within the S1BF at β – 2.1 mm. Scale bars: 1 mm.

DISCUSSION:

Whole brain imaging methods are crucial tools to better understand brain physiology and pathology. The method described here allows the precise quantification of hemodynamic signals in the living brain directly at the bench. The unmatched sensitivity and spatio-temporal resolution of functional ultrasound is particularly well suited for the mouse physiology. Functional responses and resting-state networks can be mapped within short acquisition times, longitudinally and without having to average trials or subjects to obtain a reliable measure. The relevant combination of high sensitivity ultrasonic linear probes and fast motorized setups enables one to perform transcranial volumetric fUS imaging in mice within reasonable acquisition times. This protocol can be performed either on anesthetized or awake mice using a mobile home cage.

Whisker stimulation, the sensory stimulus used as an illustrating example in this manuscript, is a standard functional activation paradigm in rodents and a reliable read-out to study sensory processing, neurovascular coupling and their alterations[5,6,10, 11]. While coarse manual brushing of the whiskers may be preferred for its ease of use, this method lacks spatial and temporal precision. The use of an automatic stimulator, such as the one described here triggered with the fUS imaging scanner, allows for a better control of several parameters including the time of onset, the amplitude displacement, the frequency as well as the angle of the Q-tip/comb, resulting in a better inter-animal reproducibility. Additionally, a more precise timing of stimulation enables the modeling of the Hemodynamic Response Function (HRF)

by determining the time to onset and time to peak parameters[12,13]. To ensure better precision on the number of whiskers deflected during the stimulation (and thus the area of the activated region), more sophisticated stimulators can be adapted to this protocol. Many other stimuli such as light[8], sound[14] or odor presentation[15] can be implemented using the same protocol.

The compatibility of functional ultrasound with awake and behaving animals is an important advantage compared to other neuroimaging techniques, enabling functional activation mapping without the anesthesia bias. Using an air-lifted mobile homecage is a good alternative to other existing head-fixed apparatus such as linear or spherical treadmills. While being firmly head-fixed, the motion of the homecage gives the mouse the illusion to navigate the environment, allowing a wide-range of behavioral testings to be coupled to fUS imaging[16]. However, the habituation procedure to head-fixing constitutes an important step to decrease stress, especially for experiments where it can be considered a confounding factor. The procedure detailed here (6-days of handling and habituation to head fixation) gives robust results for sensory stimulation and resting-state functional connectivity. However, it might be necessary to extend the habituation period for more refined behavioral tests[17].

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DISCLOSURES:

Jeremy Ferrier and Bruno Osmanski are employees of Iconeus. Thomas Deffieux, Zsolt Lenkei and Mickael Tanter are co-founders and shareholders of Iconeus.

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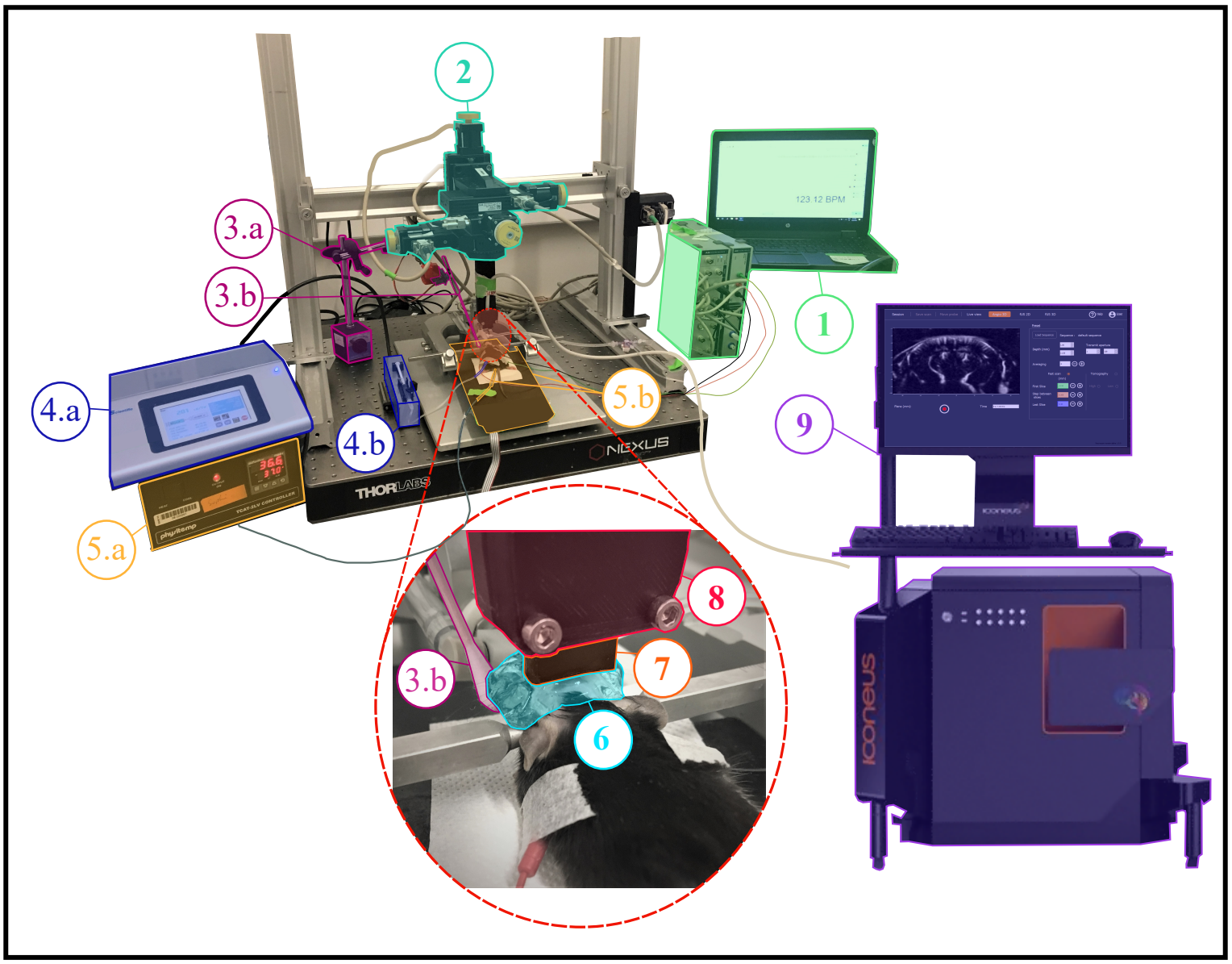
Figure 1

Click here to access/download;Figure;Figure1.pdf



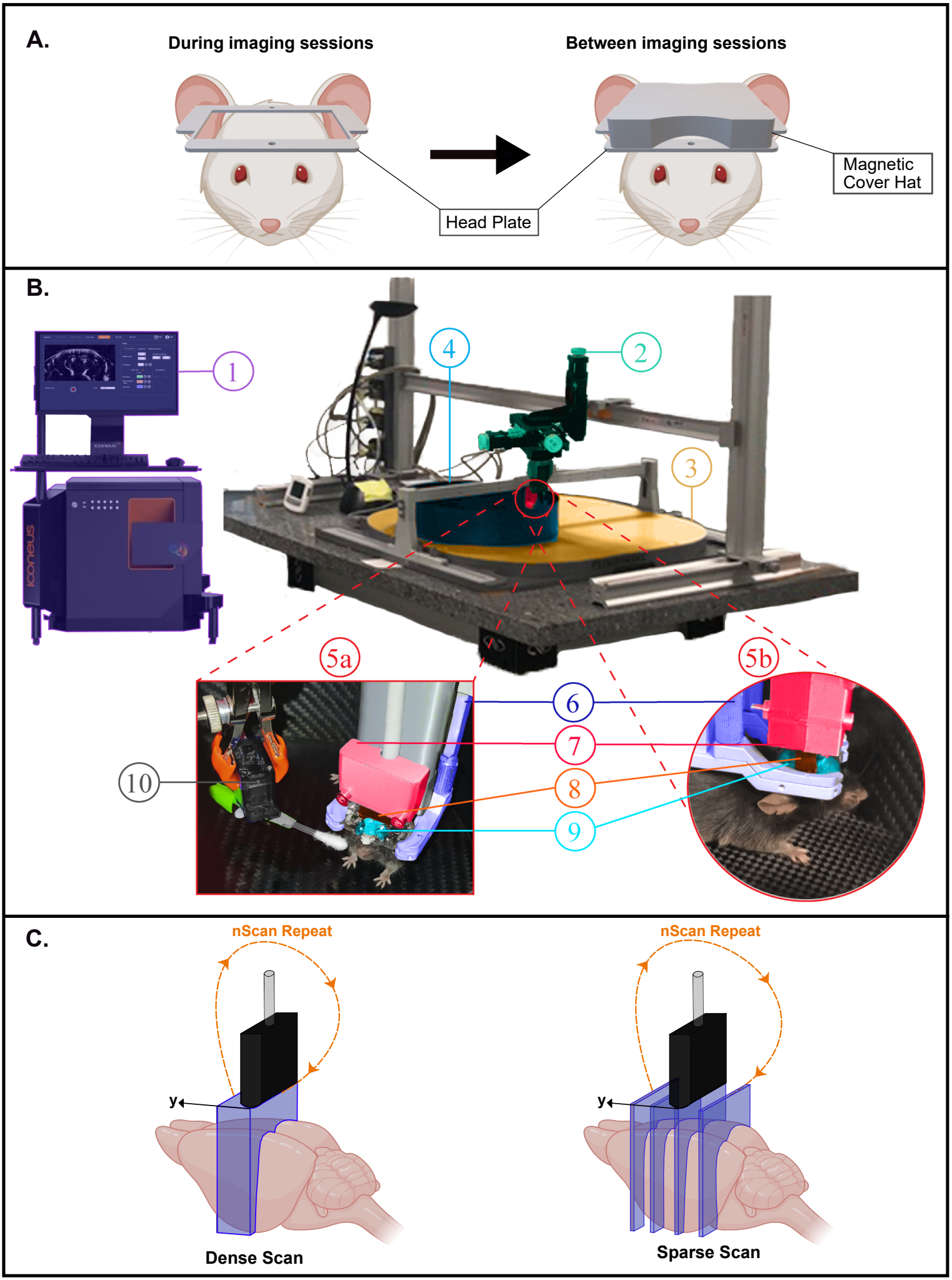


Figure 3

Click here to access/download;Figure;Figure3.pdf

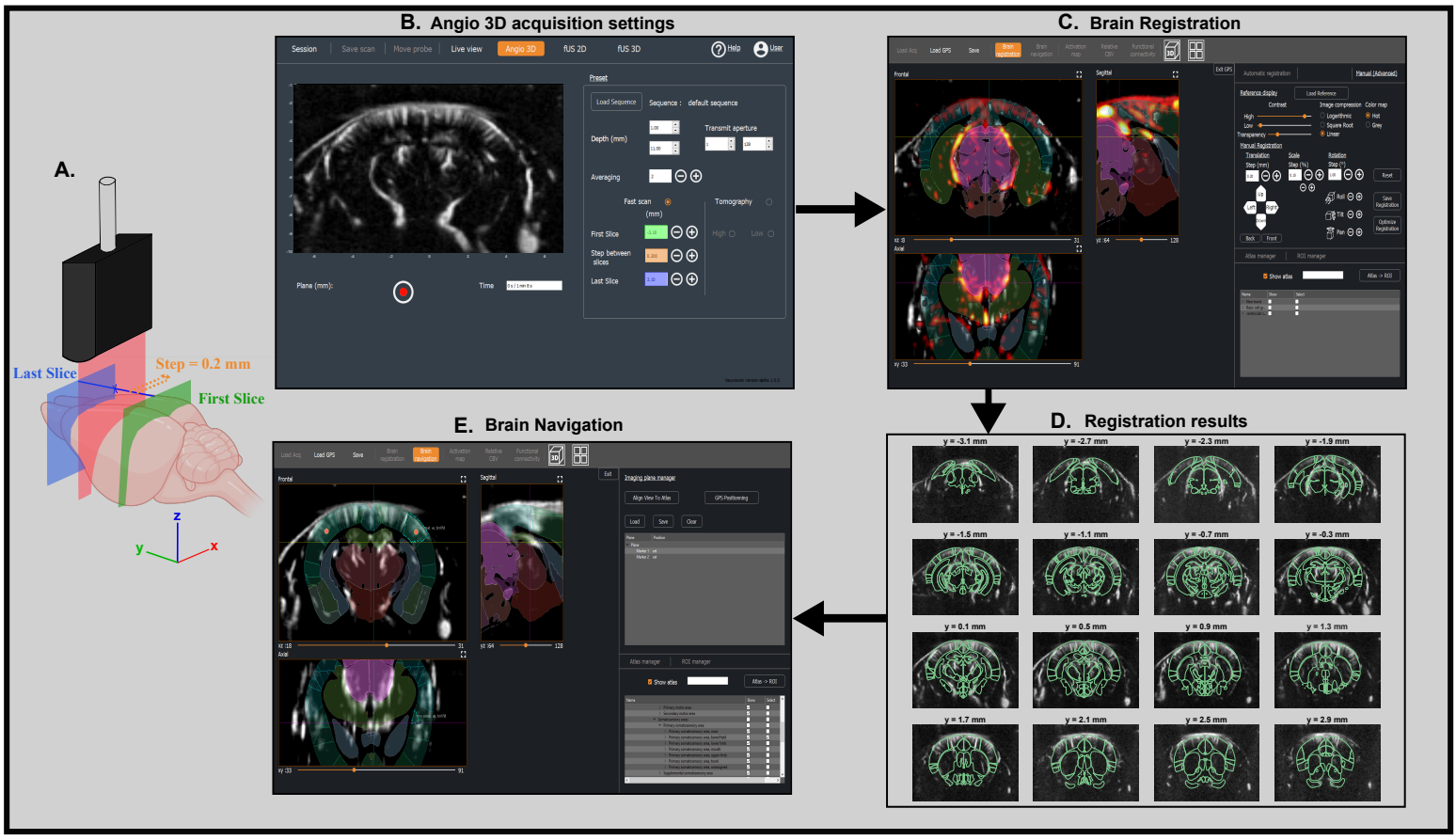
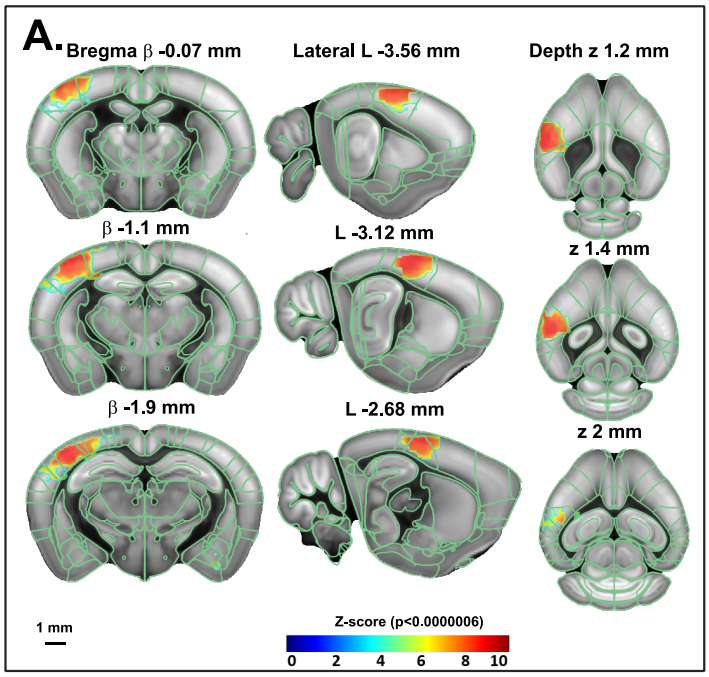
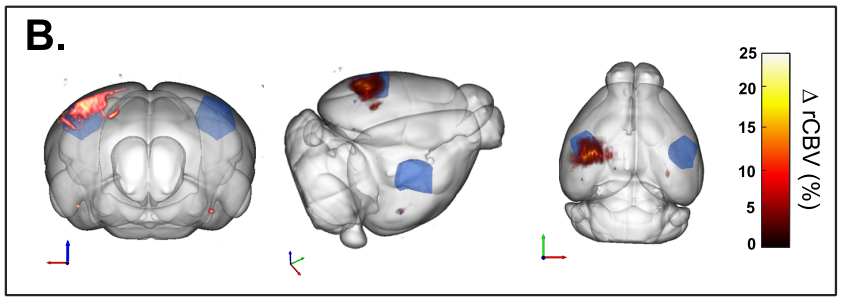


Figure 4





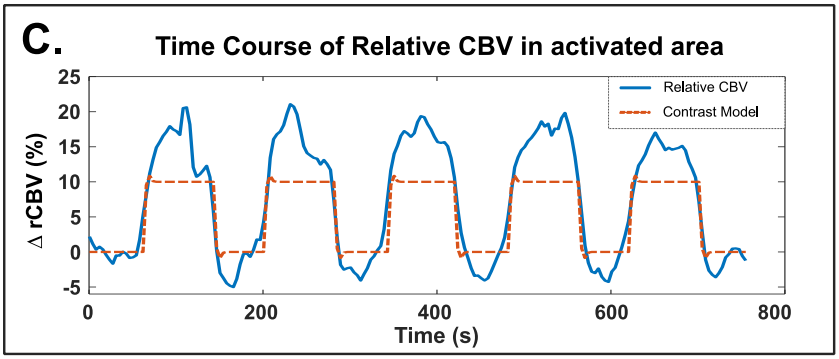
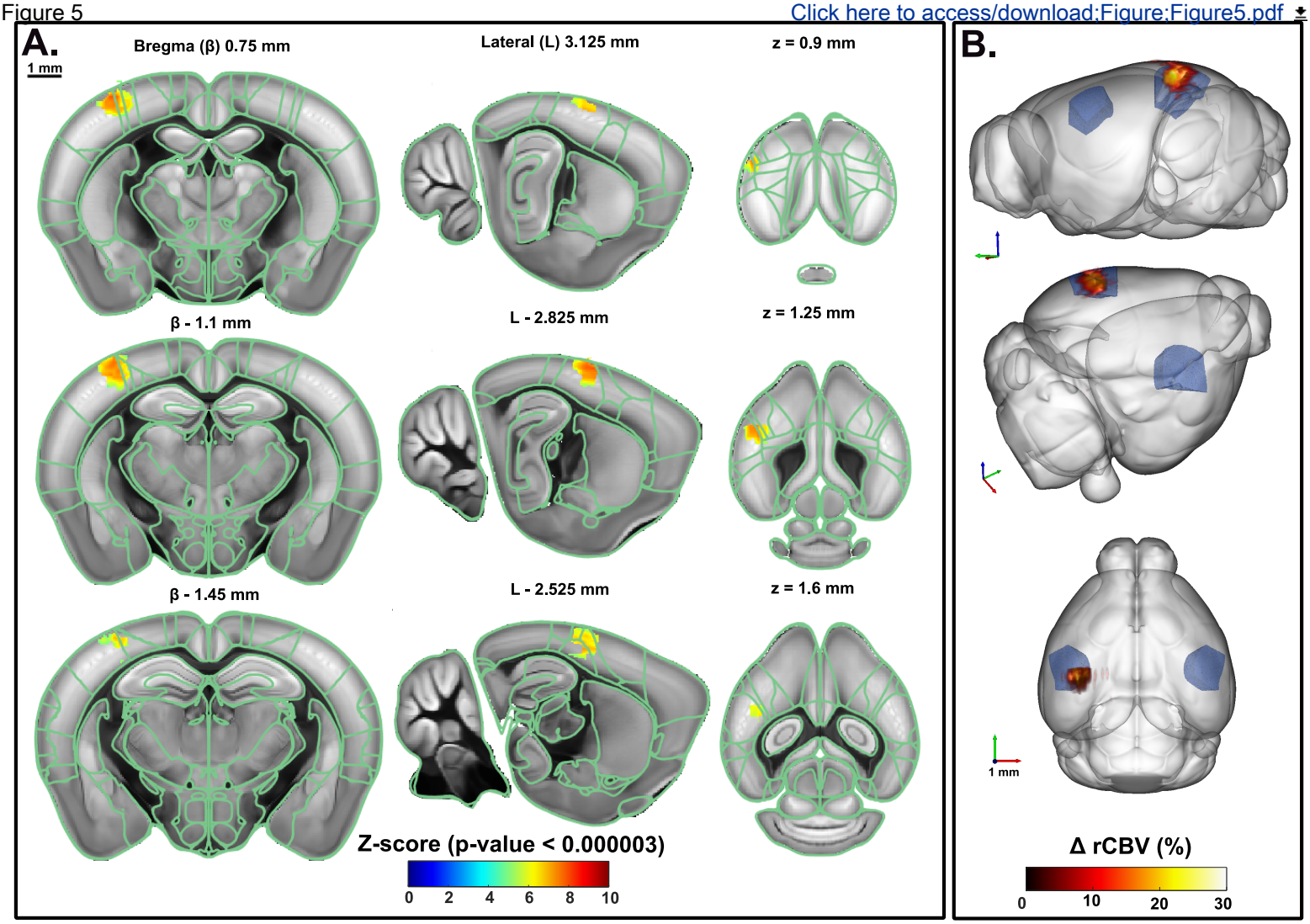
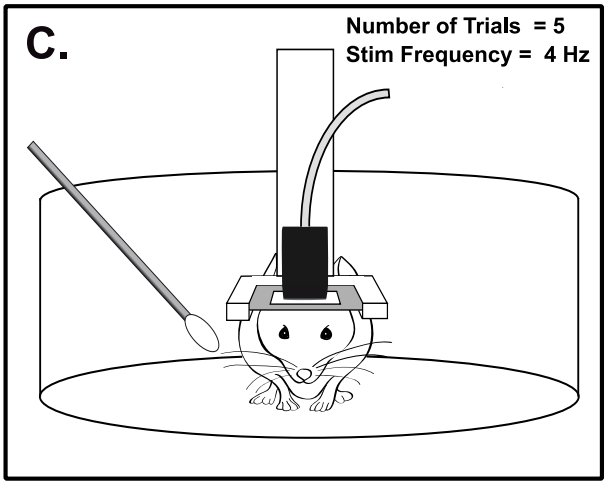


Figure 5





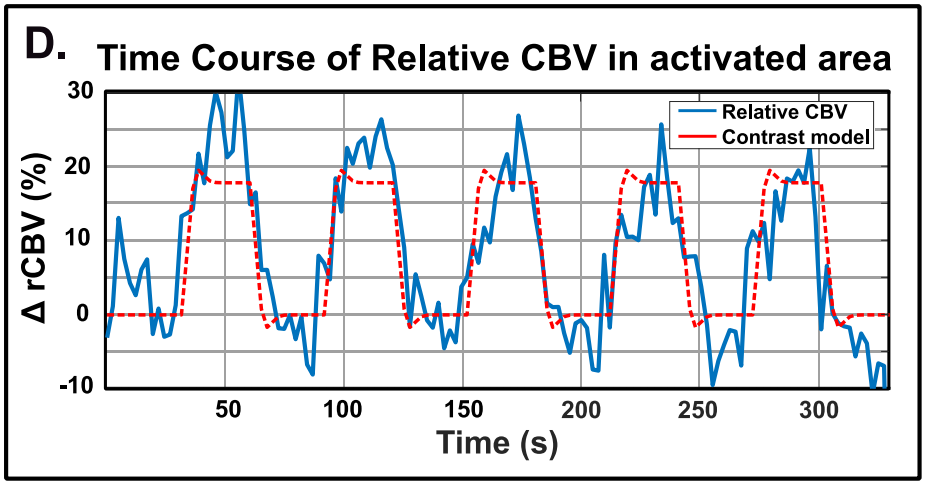
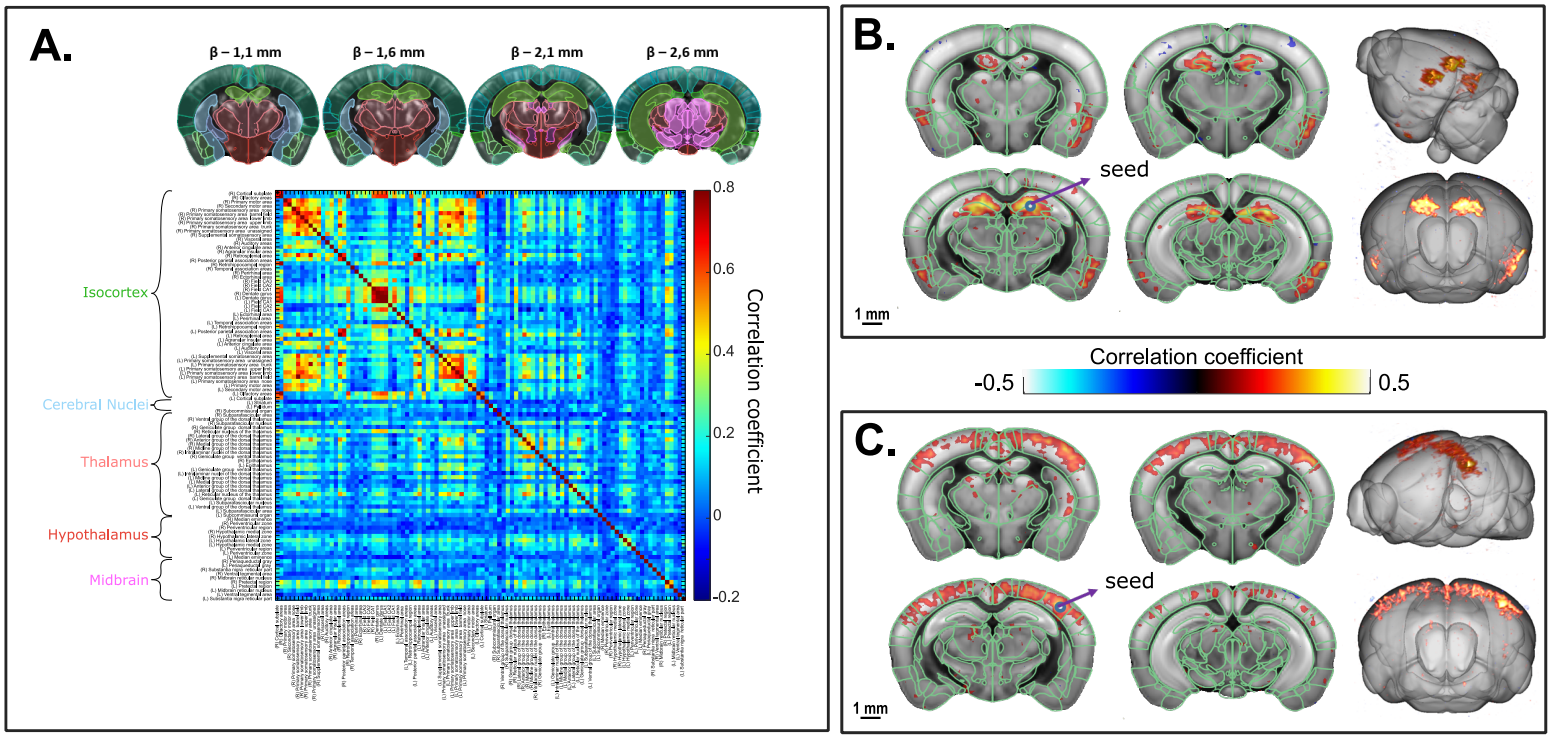


Figure 6

Click here to access/download;Figure;Figure6.pdf



Materials

Click here to access/download;Table of Materials;JoVE_Materials_fUS_2.xlsx

Name of Material/ Equipment	Company	Catalog Number
BD Plastipak 1 mL syringes	Dutscher, France	303172
BD Microlance 26 Gauge needles	Dutscher, France	303800
Animal Temperature Controller (heating Plate	Physitemp	TCAT-2DF
Arduino	Arduino	Arduino Uno-Rev3
Atipamezole	Orion Pharma, France	Antisedan®
Dental Ciment	Sun Médical, Shiga, japan	Superbond C&B
Depilatory cream	Klorane	N/A
Eye Ointment	TVM, UK	Ocry-gel
Hair trimmer	Wella Profesionnals	N/A
Head plates	Neurotar, Finland	Model 14
Iconeus One standard package for fUS	Iconeus, France	Iconeus One
IcoScan acquisition software (v1.0)	Iconeus, France	IcoScan
IcoStudio analysis software (v1.0)	Iconeus, France	IcoStudio
Isoflurane Anesthesia station	Minerve, Esternay, France	
Ketamine	Virbac, France	Ketamine1000
Lidocaine	Vetoquinol	Lurocaine®
Medetomidine	Orion Pharma, France	Domitor®
Meloxicam	Boehringer lingelheim	Metacam®
Mobile HomeCage Large with tracking capability	Neurotar, Finland	MHC-L-T-V4
Monitoring of ECG and breathing rate	AD Systems, (USA) and LabChart software	
Servomotor	Feetech	FT90B
Stereotaxic frame	David Kopf (Tujunga, USA)	900-WA
Surgical glue	3M, USA	Vetbond
Syringe Pump	KD Scientific, USA	Legato® 130, Cat# 788130
Ultrasound gel	DREXCO medical, France	Medi'Gel
Xylazine 2%	Bayer, France	Rompun®

	Comments/Description
5 mg/ml injectable solution	
100 mg/ml injectable solution	
20 mg/ml injectable solution	
1 mg/ml injectable solution	
0.5 mg/ml injectable solution	
Using Mouse Adaptor (Ref: 922) and Non-Rupture Ear Bars (ref: 922)	
20 mg/ml injectable solution	

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.

The manuscript has been proofread for spelling and grammar mistakes.

2. Please define all abbreviations before use. E.g. Lines 36,48: fMRI, Line 135: AD system etc.

All abbreviations have been defined throughout the manuscript.

3. Abbreviate all SI units as: L, mL, µL, cm, kg, h, min, s, etc., and include a space between all numbers and the corresponding unit: 50 mg, 100 mL, 37 °C, 60 s, etc.

Done

4. Consider re-numbering the protocol in the following manner to increase the clarity and follow the JoVE style.
a) Remove the "1" for the "Animals" step, as this is a description of the ethics statement and animals, rather than the actual protocol.
b) Designate the "Anesthesia and…imaging" as step 1, and the included sub-steps as 1.1, 1.2 etc.
c) Line 97: Instead of providing alternate protocols with their (separate) monitoring procedures, specifically present only the anesthesia (and subsequent monitoring) protocol which was used.

d) Line 147. The details outlined in this step (1.2) reflect more of the preparation for imaging rather than the actual imaging itself. Consider rephrasing the title of this step

e) Also add a single line space between successive steps of the protocol.

The manuscript has been modified accordingly. The second anesthesia protocol (isoflurane/medetomidine) has been removed from the manuscript.

5. Line 104: Which syringe, needle is used? (also in line 155)

Precisions have been added to the manuscript (" using a 26 gauge needle and 1 mL disposable syringe"). References have been added to the material list.

6. Line 120-121: "..(0.13 mg/kg/hr) through an automated.." instead of "..(0.13 mg/kg/hr) an automated.." .

This line has been deleted from the protocol following comment 4.c)

7. Line 137: Which ointment? Contents? Dosage?

This information has been added to the manuscript

8. Line 140: How are these inserted? Positions?

Positions have been specified

9. Line 141: Centrifuged at what speed?

Added: 1500 rpm, 5 min

10. Line 160: Concentration of iodine solution?

Added: 1%

11. Line 170: To avoid mention of commercial names, use generic terms . e.g. "surgical glue" instead of "Vetbond".

Done.

12. Line 176: Details about the 3D printed cap? Material etc.?

Added: Polyactic acid material with magnet inserts.

13. Line 180: Please specify what "Day1" indicates to avoid misinterpretation. E.g. "Day 1 post-recovery.."

Done.

14. Line 199: Please provide details about the probe and its installation.

Done.

15. Line 275: How was the whisker-stimulation performed? Please provide more details.

More details have been added to the protocol: "The cotton swab is positioned such as to allow deflection of most of the whiskers in the dorsal/ventral direction.

NOTE 2: Alternatively, the stimulation can also be delivered manually by deflecting the whiskers at the defined times during the acquisition."

16. Please add appropriate scale bars to Figures 4B, 5B.

The figures have been modified accordingly.

17. Please sort the Materials Table alphabetically by the name of the material.

Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper describes protocols for whole-brain functional ultrasound imaging in anesthetized and awake mice using the Iconeus fUS system. The methods described could certainly be of interest to the scientific community, given the growing interest around functional ultrasound imaging of brain activity. However, I have some major concerns on the manuscript in its current state as discussed below.

The manuscript is essentially showcasing a commercial product (for which the authors correctly disclose their conflicts of interest), and it reads more like a user manual than a scientific methods journal. The paper would have certainly been interesting, were the same imaging protocols described using off-the-shelf components and a general-purpose scanner (such as Verasonics or similar). Indeed, such components/systems are more widely available

to the scientific community and would allow broader reproducibility of equivalent results. For the same reasons, it is impossible to review all the details of the imaging protocol starting from Sec. 2, given that these details are currently known only to the few labs having access to an Iconeus system.

We thank the reviewer for his fruitful comments and review of the manuscript. We made our best efforts to improve the quality of the manuscript following his/her advice.

Nevertheless, regarding the general comment about the use of a commercial product for the fUS imaging protocol, we respectfully disagree with the reviewer for several reasons:

First of all, the protocol presented here is not dependent on the ultrasound imaging system used. The aim of this article is to propose a detailed and stand-alone protocol for fUS imaging to the wide community of neuroscientists who do not have a strong expertise in ultrasound. The detailed description is focused on the animal preparation, handling, anesthesia, positioning, acquisition and treatment of data. The goal of this manuscript is not to explain how to develop a scanner for fUS imaging.

Secondly, the only commercial neuroimaging scanner available today is precisely the one used in the current protocol. This system, officially introduced to the neuroscience community at the SfN Meeting in Chicago in 2019, is now widely available with dozens of user labs worldwide and this users community is fast growing. In the neuroscientific community, the Iconeus scanner is today more widely available than do-it-yourself solutions and it is the first off-the-shell ultrasound neuroimaging system available for everyone.

Thirdly, the general-purpose scanners mentioned by the reviewer (Verasonics or similar) have been designed for the community of ultrasound researchers and not for the neuroscientific community. The potential use of such a general-purpose scanner combined with off-the-shelf components is not relevant here as it requires skills in ultrasound imaging and ultrasound programming, which is definitely not the expertise of the neuroscience community. The Iconeus system used for the manuscript has been designed by physicists, with decades of expertise in ultrasound imaging technologies, in close collaboration with neuroscientists, as shown by co-signed patents and dozens of collaborative articles in top-tier journals, in order to provide the best possible and reproducible data and make it available for the neuroscience community. The use of research platforms such as the Verasonics research platform based on open programming and DIY components is even a counter-argument when it comes to the reproducibility of results from different groups. It is not possible to ensure such reproducibility when the low level programming of ultrasonic sequences, raw data signal processing, transmit signals, hardware components can be used or modified completely differently from one group to another.

Nevertheless, in order to satisfy readers who would be willing to have more in-depth information on the technology, we have provided more information on the ultrasonic acquisition sequences used and the signal processing pipeline in the revised manuscript.

Please find below some specific comments:

<u>Minor Concerns:</u>

1. Sec. 1.1.3: "They can be maintained in the desired range by subtle changes in the

rate of anesthesia perfusion". This paragraph should discuss how to intervene on the anesthesia perfusion to maintain the prescribed temperature, heart rate and respiratory rate.

This section has been removed from the protocol to improve clarity following the editor's recommendation.

2. Sec. 1.2.1: The surgical protocol should also be included in the video as it is critical to reproduce the experiment correctly.

The surgical protocol has been included in the video script.

3. The organization of Sec. 1.1 is somewhat confusing. Two anesthesia regimes are first introduced (which are only used for the imaging session), and then a third one is described for the surgery. This can be confusing to the reader. I recommend reorganizing the section, perhaps using two different subsections: one including the preparatory surgery and one for all the imaging protocols.

The protocol has been modified accordingly. The isoflurane/medetomidine protocol has been removed from the manuscript to ensure clarity. The title of the sections have also been refined to avoid confusion.

4. P1-L67-68: "fUS bench fully compatible…" Please rephrase this sentence as it is not clear.

The sentence has been rephrased as following: "Furthermore, it is fully compatible with conscious and behaving animals"

5. P2-L97: adapted to fUS imaging

We thank the reviewer for pointing out this typo. The manuscript has been corrected.

Reviewer #2:

Manuscript Summary:

The paper presents a simple and robust protocol for studying task-based brain activation and functional connectivity using fUS imaging in mice, both in anesthetized and awake animals. This paper will be very useful for specific users of the commercial fUS system presented here, but also more generally provides a complete method from animal preparation to data analysis that can be used by the community in order to improve reproducibility across fUS studies. I have a few major or minor comments to improve the quality of the article.

Major Concerns:

1. Although the use of a motorized system enables to perform 3D fUS imaging, it is unclear to me if the entire brain of the mice can be imaged without compromising too much the temporal resolution. Therefore the title stating that it is a "whole-brain" study may be misleading. In this regard, important precisions are lacking in the manuscript: for the figures 4 to 6, the authors should state the total size of the volume that was scanned, with the dead time between each slice and temporal resolution. It would also help future readers and users to address this important question of volume size versus temporal resolution in the discussion: from the authors' experience, what should be the minimal sampling rate for task-based CBV changes, and why ? (a value of 2.5s is given for functional connectivity, please explain why). Finally, it would also be useful to state the minimal dead time between slices depending on the speed of the motors and the distance between slices of interest.

We thank the reviewer for this insightful and very important comment. Indeed, there is a trade-off between the extent of the area scanned and the temporal resolution. However, we do not believe that "whole-brain" is an overstatement. As stated in the introduction, whole-brain fUS imaging has already been described in [Gesnik et al., 2017] and [Macé et al., 2018] with acquisition times in the range of 3-6 h per animal. Here, we propose a refined methodology using a fast linear motor to step the ultrasound transducer during the acquisition, reducing the acquisition time to a few tens of minutes. In the case of task-based CBV changes, as long as the task can be repeated multiple times, the sampling rate can be lowered down depending on the duration of the stimulation and the number of repetitions. For instance, a 60 sec whisker stimulation can be sampled 5-6 times with a sampling rate of 10 sec/volume which is enough to cover the whole mouse brain. Alternatively, the whole brain can be scanned progressively by acquiring successive portions of a few millimeters. However, Functional connectivity is characterized by very slow CBV oscillations (typically around 0.1 Hz). To sample these oscillations properly, we recommend a volume rate superior to 0.4 Hz. According to the MRI litterature (N.Huotari et al; doi: 10.3389/fnins.2019.00279), we could even lower the volume rate until 0.33 Hz without compromising functional connectivity maps.

The following details have been added to the figure legends:

The deadtime between two planes due to the motor movement is of 150 milliseconds for figure 4 and 5 and 200 milliseconds for figure 5. As the time of acquisition for one image is of 400 milliseconds, the temporal resolution per slice is 600 milliseconds. The step between two slices is set to the slice thickness which is equal to 400 microns (corresponding to the size of the pressure field in the elevation direction).

In figure 4: "Each sampled volume was scanned over 2.8 mm in the elevation direction (corresponding to 7 slices) in 3.85 s. For this experiment, the stimulation duration was set to 80s, allowing to record 20 volumic samples during each functional response."

In figure 5:"Each sampled volume was scanned over 1.2 mm in the elevation direction (corresponding to 3 slices) in 1.8 s. Here, the stimulation duration was reduced to 30 s, providing 17 volumic samples each functional response. "

For the figure 6: "Each sampled volume was scanned over 1.6 mm in the elevation direction (corresponding to 4 slices) in 2.2 s".

As illustrated in figure 2.c. all these volumes could also be scanned with the sparse approach.

2. The power and sensitivity of the technique can be clearly appreciated in the figures that show results from single experiments. However, it is not possible to evaluate the variability of the measurements. The authors should add another figure to display results from a group of different animals (ideally both task-based stimulation and functional connectivity). Additionally, showing results of several experiments from the same animal would also be informative to evaluate the test-retest reliability.

We thank the reviewer for this comment. Variability is an important issue in neuroimaging, but we believe that this issue is outside of the scope of this protocol. In this work we aim to share an imaging protocol that has been developed for several years, and that already led to several publications reporting statistically solid functional connectivity measurements performed on mice cohorts: Ferrier et al; Rabut et al. Here, we wanted to give a precise and exhaustive description of each step of our protocols, and we also wanted to present the type of measurements that could be performed with a functional Ultrasound scanner such as

Iconeus One system. We believe that such protocol standardization across labs will undoubtedly lead to a better reliability and reduced group variability.

Minor Concerns:

3. The adviced dose of ketamine/xylazine seems quite high (see for instance this review: 10.1186/2191-219X-2-44), please justify.

Ketamine/Xylazine is considered a reliable combination for mouse anesthesia with a wide safety margin. The recommended dose varies: 100-200 mg/kg for Ketamine and 5-16 mg/kg for Xylazine. The most widely used dose of Ketamine/Xylazine for mouse surgery is 100 mg/kg and 10 mg/kg body weight, respectively (Flecknell 1993), for a duration of around 30-40min. Duration of the effect may be extended by increasing the proportion of Xylazine or by an additional dose. In our protocol, we chose to increase the initial dose of Xylazine to increase stability over time and total duration of the sedation (up to 1h30 without redosing) without compromising the safety of the protocol.

4. conversely, 2% isoflurane for induction seems low compared to the usual 3-4%, please justify/modify.

Thank you for noticing. The percentage of isoflurane may have to be closer to 4% depending on the vaporizer used. Following reviewer 1 comments, the isoflurane/medetomidine anesthesia protocol has been removed from the manuscript for further clarity.

5. line 177: what is the total weight of the headplate + the cap, and consequently what should be the minimum age/weight of the animals for awake fUS experiments?

The total weight of the headplate + the cap is approximately 2.8 g. This information has been added to the protocol.

As detailed in the manuscript, this protocol was developed on 2-3 months old mice (young adults). The weight of the setup might be an issue at younger ages.

6. line 292: please justify why the total acquisition time should be >10 min for functional connectivity. What should be the minimal time window for reliable measurements?

Unpublished data from our group suggest that a reliable measurement of functional connectivity can be obtained with 3 min of acquisition at a sampling rate of 1Hz. When using this protocol, since the sampling rate is decreased to acquire multiple slices, we recommend to extend the measurement time to at least 10 min to have (at least) the same amount of samples/slice.

The protocol for Functional Connectivity now includes: "NOTE: for 3D functional connectivity, we recommend acquisition time between each volume < 2.5 s (sampling frequency of at least 0.4 Hz) and a total acquisition time of at least 10 min (number of time points > 180)."

7. line 299: what kind of filters are implemented in this software for the functional connectivity analysis? and what should be the optimal cut-off values?

Functional connectivity analysis includes signal detrending and low-pass filter. Optimal cut-off values for the low-pass filter is hard to estimate, as a wide-range can be found in the literature (from 0.08 to 0.25 Hz). In our analysis, we chose a cut-off value of 0.1 Hz as it led

to a better regional specificity of the obtained correlation. We added this information to the Figure 6 legend.

8. figures 4 and 5: please explain how was the GLM analysis performed; is this the activation map analysis from line 284?

Activation Maps were generated by the embedded software using the Generalized Linear Model approach, widely used in fMRI studies.

Briefly, a design matrix X was created with a constant baseline and the stimulus signal convolved by a default Hemodynamic Response function (found by fitting several activation datasets on mice and defined by a mix of two gamma functions). No Nuisance signals were used for these experiments. A contrast of +1 was set for the stimulus signal and a pseudo inverse was computed for each pixel to compute the weighting matrix Beta and residual error E so that Y=Beta X + E. The z-score, p-value and rCBV maps were then estimated from the weighting matrix and residual error.

A Bonferroni correction, where the number of tests corresponds to the number of voxels in the brain area, was then used to correct the p-value threshold for significance in the context of multiple comparison tests.

The section now includes : "NOTE : Activation can be visualized by computing the Pearson correlation between the stimulation pattern and the hemodynamic signal from each voxel. Alternatively, a default mouse Hemodynamic Response Function (HRF, obtained from several previous activation datasets) can be used using a Generalized Linear Model (GLM) approach."

9. same figures: for further clarity, maybe the authors should change the color of the ROI in the 3D rendering because readers could misinterprete the blue spots for a CBV decrease (as stated by the color scale).

The colorbar range has been modified to avoid any misinterpretation.