Webinar Q&A Report

Functional Ultrasound (fUS) Imaging in the Brain of Awake Behaving Mice

Questions in this Q&A Report were submitted during the live webinar, Functional Ultrasound (fUS) Imaging in the Brain of Awake Behaving Mice.

Answers have been provided by:

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1. What is the temporal resolution of fUS?

J. Ferrier: The typical fUS sequence generally offers by default one high quality image every 400 ms. This can be adapted to meet specific requirements.

2. Can fUS be used for clinical investigation like Doppler ultrasound?

L. Lecointre: At this stage Iconeus-One is not approved for clinical use so Iconeus recommend not to use it on human. Nevertheless, we are aware that some research institutes made the decision to perform for clinical research with Iconeus One after being

granted by their representative authorities. Currently, Iconeus cannot support such use of the system which remains solely under the institute responsibility.

3. **Could you 'register' a fUS hotspot of activity to a location on the skull, and if so, how?**

J. Ferrier: If the issue is to position the probe to image specific brain structures, Iconeus One includes an automatic positioning software for mice based on the Allen Brain Atlas so you are never lost. If needed, we can also use a contrasting beacon manually placed on the skull at a specific location (typically an air bubble would do a great job). We would then be able to see it on the scan. Iconeus One also offers the possibility to switch between fUS sequence (for brain activity) and B-mode ultrasound (classic structural ultrasound imaging) in live view so any artificial physical asperity on the skull could be located with this modality.

4. **Do the commercial systems include the analysis packages to create the functional maps, or just the hardware to acquire the data?**

J. Ferrier: Iconeus One includes the acquisition software as well as an analysis package : activation mapping, resting-state functional connectivity, 3D reconstruction and visualization, 3D tomographic vascular scans, automatic brain atlas registration and ROIs, and automatic probe positioning with our motorized platform.

5. **Do you have experience with retinotopic maps in visual cortex? Do they look similar to what you would get for example from intrinsic imaging in rodents?**

J. Ferrier: Sure, you should check these two papers:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5387157/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6292977/

fUS imaging for retinotopic mapping has also been applied in non-human primates very recently: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7321983/

6. **Does fUS function through the bone table, or are the transducer(s?) placed directly on the brain for maximum sensitivity?**

J. Ferrier: It depends on the animal model used. In mice, using our highly sensitive 15 mHz probes, we can measure brain activation and connectivity through the skin and skull (non-invasively). In rats (and larger animals), the skull needs to be removed (craniotomy) or acutely thinned using a standard dental drill. In the rat, one can also inject microbubbles - ultrasound contrast agents - although experiments are less straightforward due to signal fluctuations in time.

7. **Does FUS work in 3D?**

   J. Ferrier: Yes, at this time, we propose a multi-slice acquisition mode using the included motorized platform that allows to image the whole brain automatically with a tradeoff on temporal resolution. fUS imaging is evolving toward 3D (volume) imaging, but there are still some technical challenges to overcome that we are working on to reach the same quality and sensitivity as for our current linear probes. You can read in detail about the current state of the art of multidimensional fUS imaging in our newsletter here: https://iconeus.com/news/the-path-to-4d-fus-imaging/

8. **Is it possible to coregister fUS data to a rat template?**

   J. Ferrier: Yes, this is currently under active development and should be available soon.

9. **Is it possible to adapt this technique for rats?**

   J. Ferrier: fUS has been extensively used in anesthetized as well as freely moving rats. You may find relevant articles here: https://iconeus.com/science-and-technology/publications/

   However, we cannot do fUS through the intact skull in rats without contrast agent injection.

10. **Dr. Lenkei mentioned fUS is much cheaper than fMRI. How much is it of fUS set-up** approximately?

    L. Lecointre: Please contact us at contact@iconeus.com to request a quotation. We'd be happy to have a quick videocall to discuss application and cost.

11. **How are the probes installed? What anesthetic(s) would you recommend?**

    J. Ferrier: The probes are included in the package and can be plugged/unplugged easily to the Iconeus One system. The placement on the head of animal depends on the experimental configuration (anesthetized, head-fixed awake, freely moving) and employs classical ultrasound gel. In terms of anesthetics, we usually recommend a mixture of ketamine/xylazine and discourage the use of isoflurane because of its strong vasodilatory effects that deflects the neurovascular coupling.

12. **In the images presented during the webinar we are able to see cortex and hippocampus. Is it possible to image deeper structures like the thalamus?**

    J. Ferrier: In mice and rats we can image the whole brain in depth. In Ferrier et al., 2020,

PNAS, we deliberately focused on cortical structures and the figures only show the upper part of the brain, indeed.

13. **Is it possible to make measurements from more than one animal in the same arena simultaneously?**

L. Lecointre: it is indeed technically feasible, and a dual-channel solution can be developed. Feel free to contact us at contact@iconeus.com for more information.

14. **Is the skull still intact for the head mount imaging?**

J. Ferrier: Yes, the skull is intact in mice, with the exception of two small screws to fix the head plate. In Ferrier et al., the authors removed the skin for a better anchoring of the head stage. In anesthetized mice, we can obtain high quality fUS images in a totally non-invasive way through the skull and the skin.

15. **Is it possible to perform fUS on larger animals (e.g. non-human primates) or alternatively without opening the skull?**

J. Ferrier: Most of the studies published in larger animals either used a craniotomy or thinned-skull window for fUS. For transcranial imaging in large animals such as NHP, we could use US contrast agents (microbubbles). You can see this reference for a nice demonstration in rats: https://pubmed.ncbi.nlm.nih.gov/26416649/

16. **Is this possible in large animals focusing on specific regions of the brain?**

J. Ferrier Yes if a craniotomy can be performed in the vicinity, and depending on the depth, we might need to adapt the probe. This has been done for instance in awake ferrets focusing on the auditory cortex (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6039176/) and non-human primates focusing on the supplementary eye field: (https://pubmed.ncbi.nlm.nih.gov/30923310/)

17. **With regards to adding in electrophysiology or EEG recording during fUS - does this generate artifacts in the data?**

J. Ferrier: No, fUS is completely compatible with LFP or EEG recordings. You can find more information in this article published in Nature Methods: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4671306/

18. **What behavioral testing is common/possible use with fUS? (looking in combination with pharmacological and stimulation effects on anxious behaviors)**

    J. Ferrier: As fUS is compatible with conscious behaving animals, all behavioral tests are conceivable. Regarding pharmacological effect on brain activity, it is important to note that direct effect on vasomotion can be a confounding effect.

19. **What is the best spatial coverage that fUS can achieve now? In what temporal sampling rate?**

    J. Ferrier: Our typical 15 mHz probes have a lateral aperture of 13 mm and an imaging depth down to 20 mm. Other configurations can be achieved if required.

20. **What is the spatial resolution of fUS? For example, in comparison with the size of a typical beta-amyloid plaque.**

    J. Ferrier: Our 15 mHz probes yield a spatial resolution of a 100 µm in-plane. We can provide higher frequency probes (30 mHz) to increase the in-plane resolution (50µm). However, high frequency ultrasound waves are highly attenuated by tissue so only the first few millimeters would be visible. There is a tradeoff between spatial resolution and spatial coverage.

21. **What is the technical basis of the functional vs. structural mode of fUS?**

    J. Ferrier: fUS measures blood flow using the Doppler effect. We use this parameter as a surrogate for brain activity thanks to the neurovascular coupling. Structural ultrasound (B-mode for instance) measures the echogenicity of the medium to display anatomical information.

22. **With fUS you are collecting coronal slices – is it possible to mount 2 devices to get coronal recordings from different brain regions simultaneously, or is there not enough space on a mouse head for this?**

    J. Ferrier: Our fUS probes are designed to be stackable so it would be possible.

    The best option is to use our neuronavigation tool which allows discretionary planes (such as tilted planes) to be acquired easily.

23. **For pharmaco-fUS what is the sensitivity of detection if we apply a small dose of agonist or antagonist?**

A. Shatillo: In our experience, sensitivity of fUS is higher than in fMRI, and interpretation of the signal change is more straightforward, however it depends a lot on the specific experiment design, types and doses of the compounds applied.

Z. Lenkei: In our recent experience, low doses of agonists, just above the threshold of inducing measurable behavioral changes, led to statistically significant connectivity changes in cohorts of 10 awake mice.

24. Is fUS imaging performed through a 'window' in the skull, or rather through a layer of bone?

Z. Lenkei: In most strains of mice we tested, fUS imaging can be performed until up to 1 years of age through the intact skull, and if the animal is anesthetized, through the intact bone and skin. However, in some cases in mice and in most of cases in rats or larger animals we found that skull thinning is necessary to get good signal. For chronic imaging over up to one week, a craniotomized window covered with acoustically transparent plastic may be necessary, as described now in several publications from fUS teams in Paris.

25. Could you show us a video of this technology in action?

Z. Lenkei: We are currently preparing several educational videos, stay tuned!

26. Can we use functional ultrasound imaging while using a general anesthetic drug?

A. Shatillo: Yes, it is possible. We have used several anesthetics in fUS, starting from low dose of isoflurane to medetomidine or medetomidine-ketamine mixture. Choice and dose of the anesthesia depends on the research question and may affect the outcome of the measurements quite heavily.

27. Can you use isoflurane instead of medetomidine?

Z. Lenkei: We know from the fMRI literature that isoflurane anesthesia is not optimal for imaging functional connectivity and our own anecdotical evidence confirms is. However, you might use isoflurane anesthesia for functional imaging, such as whisker stimulation.

28. What does negative correlation mean in the context of resting state functional connectivity?

Z. Lenkei: This is a good question and it is not easy to give a short answer. I advise to look first at the abundant fMRI literature about the relevance of negative correlations in functional connectivity. In fUS, similarly to fMRI, we typically see negative correlations between cortical brain regions that are known to have functionally opposed roles, such as the midline Default Mode Network hubs and the more lateral somatomotor cortex areas.

29. Is fUS easy to implement without MRI experience?

A. Shatillo: Yes. Imaging expertise helps but is absolutely not required for fUS.

30. Will fUS replace MRI?

A. Shatillo: No, not at all. MRI provides unmatched brain tissue contrast and plethora of structural information. On the functional side, fMRI gives full brain coverage that allows measurements without prior knowledge or hypothesis of the localization of responses and of course true functional connectivity mapping. Full brain imaging is still under development for fUS, so meanwhile it remains a big fMRI advantage. I believe both methods complement each other well.

31. How do we learn more about the equipment and its cost?

L. Khirug: Contact us at mhc-support@neurotar.com. We can also help to establish compatibility with your imaging set-up, select the best suitable head plates and cage, and share air source requirements and (if you don't have a suitable air source) tips for portable air pumps. If you decide to buy, we shall also share surgery tutorials (video), mouse handling and training recommendations. Visit our support page for the FAQs and the list of resources available online: https://www.neurotar.com/research-instruments/support-for-experiments-in-awake-head-fixed-mice/

L. Lecointre: [answer] Please visit our site at www.iconeus.com to learn more about the first commercial fUS system and use the contact links to get in direct discussion with us.

32. Dr. Lenkei mentioned fUS is much cheaper than fMRI. How much is it of fUS set-up approximately?

L. Lecointre: The cost of Iconeus One depends on the options but it is in the usual range of significant lab equipment, such as advanced microscopy. It has almost no impact on running budgets as there is no consumables, no maintenance, and there is no need for a dedicated specialist to run it, since this can be done by any scientist. Added up, this may make a huge difference as compared to fMRI.

For details about the price please contact us: contact@iconeus.com

33. In awake animals, how do you differentiate the blood signal from brain movement signals? Asking this because the underlying measurement is based on Doppler effect.

L. Khirug: In functional ultrasound imaging, the spatiotemporal filtering helps remove "echoes" (reflections from surrounding tissues) and minimize motion artifacts. This is critical for imaging awake animals (Mace et al., 2018).

J. Ferrier: Ultrasounds detect very well brain movements, so we have developed and implemented several ad hoc filters for separating those from blood flow (see Demene et al., 2015 from our collaborators https://pubmed.ncbi.nlm.nih.gov/25955583/). Iconeus has further refined and developed proprietary technologies for this purpose.

34. Is it possible to adapt this technique for rats?

L. Khirug: Both the standard size Mobile HomeCage and the Mobile HomeCage Large are designed for experiments in mice and juvenile rats (under 70 g).

J. Ferrier: There are several high-level publications from Paris teams where fUS was used in rats, both anesthetized (Osmanski et al., 2014) or awake and freely moving (Sieu et al., 2015; Bergel et al., 2018).

35. Is it possible to make measurements from more than one animal in the same arena simultaneously?

L. Khirug: It is possible to place two mice into the same cage and observe their interactions. However, it is possible to make measurements only from one (head-fixed) mouse at a time.

L. Lecointre: With the current Iconeus One system you can measure one animal at a time but we can discuss about the possibility of simultaneous acquisitions as a custom -based project.

36. My research looks at the effects of pharmacological stimulation on behavior. What behavioral testing is possible for use with fUS?

L. Khirug: The following behavioral read-outs have been validated for the Mobile HomeCage Large: open field, novel object recognition, spatial learning and memory, social memory and social interactions, mating behavior (limited to courting, no copulation), escape response to a looming visual stimulus, the resident-intruder paradigm (modified). We are working on expanding this list. Contact us about your test of interest.

J. Ferrier: Virtually all behavioral tests can be considered compatible with fUS imaging (even natural sleep). If you are thinking about a specific paradigm, feel free to contact us to discuss with our application specialists.

37. How do you correct motion artifacts when a mouse is moving?

Z. Lenkei: There are several ways to do it. First, ultrasound is very well suited to detect motion artefacts and to filter it at the raw data level. But if you suspect residual motion artifacts in your data, you can also select motion-free periods by using video recordings or the impressive built-in tracking system in Mobile HomeCage. In a recent study (Rabut et al., 2020), we used the baseline CBV recording by fUS to select motion-free periods for intrinsic connectivity imaging in completely freely moving mice.

L. Khirug: The Mobile HomeCage ensures excellent image stability by keeping the skull (and brain) of the mouse firmly fixed in place. Even during running, the mouse' brain tissue shows lateral displacement values that do not exceed 1-1.5 micrometers. This is sufficient for stable two-photon and fUS imaging, as well as for single-cell electrophysiological recording.

38. Does this technique cause stress to animals and thus interfere with their responses?

L. Khirug: Due to its flat floor and presence of the walls that provide natural tactile stimulation for mice, as well as due to the mouse's being in control of its speed and trajectory of movement, the Mobile HomeCage is currently the least stressful head-fixation system for mice. However, it requires proper handling and training of mice to achieve the maximal stress reduction.

39. Is fUS performed similarly to neuronal calcium transient imaging using 2-photon microscopy?

L. Khirug: fUS and 2P imaging modalities share some similarities, as both allow functional imaging in the brain of awake or anesthetized mice. However, there are several differences between these two modalities: i) penetration depth: fUS allows imaging at any depth throughout the mouse' brain, while 2P is limited to 600-1000 micrometers in depth; ii) imaging resolution: 2P allows sub-cellular resolution of 0.3-1 micrometer, whereas fUS offers lower resolution in the range of tens to hundreds of micrometers; iii) field of view: fUS enables exceptionally broad field of view covering several centimeters (i.e., the whole brain of a mouse), as compared to a more limited field of view of 2P (typically <1 mm in diameter); iv) contrast generation: fUS is a label-free technique that derives image contrast from endogenous blood flow dynamics, while 2P imaging relies on an exogenous contrast agent such as GFP or FITC (with the exception of autofluorescence imaging applications which are rather limited for 2P microscopy).

Contact Information

If you have additional questions for Artem Shatillo, Ludovic Lecointre or Leonard Khirug regarding content from this webinar, or if you would like to receive additional information about this technology, please contact them at:

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