4D functional ultrasound imaging of whole-brain activity in rodents

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We extended the capabilities of functional ultrasound to whole-brain four-dimensional (4D) neuroimaging. Our multiplane-wave transmission scheme on matrix arrays at thousands of frames per second provides volumetric recordings of cerebral blood volume changes at high spatiotemporal resolution. We illustrated the approach in rats while providing multi-sensory stimuli, for 4D functional connectivity and during instantaneous tracking of epileptiform events.

Functional neuroimaging has become an invaluable approach in neuroscience, especially to study the brain as a dynamic global network of interacting regions. Neuroimaging modalities such as magnetic resonance imaging (MRI), electroencephalograms and magnetoencephalography offer a wide field of view at the price of either a limited spatiotemporal resolution or sensitivity. Conversely, electrophysiology or optical techniques offer high spatiotemporal resolution but in a limited field of view. Therefore, we still have a fragmented understanding of the brain as a large-scale network. To better understand brain activity, long acquisition times at high frame rates are essential to monitor spontaneous neuronal activities, as is imaging with a large field of view. Photocoustic tomography could fulfill these requirements and has shown promising results on rodents where the penetration depth limited by the acceptable light fluence safety exposure is less critical.

Functional ultrasound (fUS) imaging is an alternative and portable method to image dynamic deep brain activity by directly measuring subtle cerebral blood volume (CBV) changes induced by neurovascular coupling. The technology is characterized by high spatiotemporal resolution and sensitivity and compatible with imaging in a large field of view. fUS imaging relies on plane wave transmissions and highly parallel electronics to achieve fast frame rates (>5,000 frames per s) for sensitive Doppler imaging. This neuroimaging modality is currently limited to acquisitions in a two-dimensional (2D) imaging plane, as electronics for sensitive Doppler imaging relies on a limited number of channels incompatible with the high number of individual elements of matrix array probes required for electronic focusing in three dimensions.

However, following spontaneous or transient activity patterns or measuring functional connectivity (FC) between remote structures requires the simultaneous acquisition of information in the whole brain and therefore extension of the technology to a volumetric acquisition scheme.

Although 2D piezoelectric matrix array transducers are emerging for volumetric imaging, they are hampered by low individual element sensitivity compared to conventional one-dimensional linear probes, or by their integrated sequential pre-beamforming required to lower data transfer rate from the probe to the scanner. These issues become critical for applications that require both fast frame rates and high sensitivity such as in fUS neuroimaging.

We overcome these issues and extend ultrasound neuroimaging to four dimensions (three-dimensional (3D) space + time) whole-brain fUS imaging. Our approach relies on high frequency 2D matrix array transducer technology (8 MHz, 0.3 mm-pitch, Vermon) coupled with a high channel count electronic system for fast 3D imaging. To counterbalance the intrinsically poor sensitivity of matrix elements, we devised a 3D multiplane-wave scheme with 3D spatiotemporal encoding of transmit signals using Hadamard coefficients. For each transmission, the backscattered signals containing mixed echoes from the different plane waves are decoded using the summation of echoes from successive receptions with appropriate Hadamard coefficients. This summation enables the synthetic building of echoes from a virtual individual plane wave transmission with an N-fold higher amplitude (Fig. 1a). Finally, we perform coherent compounding beamforming of decoded echoes to produce 3D ultrasonic images and apply a spatiotemporal clutter filter separating blood flow from tissue motion to compute a power Doppler volume (proportional to the CBV).

While functional ultrasound imaging can be performed through the intact skull in mice or with contrast agents through the intact skull in rats, here we performed a craniotomy surgery to prepare Sprague Dawley rats for imaging and to avoid the attenuating and distorting effect of the skull on the ultrasonic waves. We acquired whole-brain power Doppler volumes (proportional to CBV) using the 3D multiplane-wave sequence at almost 400 compounded volumes per second. Major arteries of the rat brain such as the superior sagittal sinus as well as the Willis circle, located at 6 mm depth, as well as smaller vessels, particularly in the cortex, were visible in the reconstructed 3D volume with a 170 x 170 x 85 μm³ voxel size (Fig. 1b).

We then validated 4D fUS imaging in three applications in the rat brain. We first present sensory-evoked activations to the animals using either visual or whisker stimulations (Fig. 1bc and Supplementary Video 1). Second, we estimate 3D FC between remote brain structures during rest using seed-based and correlation matrix analysis (Fig. 2a–c and Supplementary Fig. 1). Finally, we use 4D fUS to map in detail the whole-brain propagation of transient epileptiform events (Fig. 2d and Supplementary Video 2).

During stimulations of the whiskers or of the visual system using a flashing light-emitting diode (LED), we acquired power Doppler images and correlated the temporal evolution of CBV in each pixel to the stimulus patterns to generate 3D correlation maps (Fig. 1bc and Supplementary Video 1). In N = 3 rats, whisker and visual stimulations induced robust activation in several brain areas (Fig. 1).

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In a second experiment, we considered spontaneous low-frequency (0.05–0.3 Hz) brain fluctuations observed in 2D IUS and fMRI in N = 4 rats. Using seed-based analysis, we observed that both right and left hippocampus signals were highly correlated (r = 0.7) (Fig. 2a). This result is in agreement with previous work showing strong anatomical and functional links between the left and right hippocampus, as well as bilateral cortical links, due to cortico-cortical projections. Leveraging whole-brain 4D IUS imaging expands the size of FC matrices in rodents by one order of magnitude compared to recent results.

We then analyzed the pairwise correlations between hundreds of regions of interest to construct a FC matrix. Similar to fMRI, clusters of synchronized activity corresponding to large brain regions can be observed as well as strong bilateral connections (Fig. 2b,c). The connectivity maps were reproducible for all N = 4 animals (Supplementary Fig. 1).

Finally, to demonstrate the acquisition capabilities of 4D IUS imaging for whole-brain tracking of transient and complex phenomena, we induced epileptiform events using a 4AP injection in the cerebral cortex of N = 3 rats and imaged their dynamic propagation through the whole brain. Mapping relative CBV variations, cortical spreading depression waves propagated from the point of injection toward the contralateral cortex and in deeper laminae of the ipsilateral cortex (Fig. 2d and Supplementary Video 2). Using isochronous maps (Supplementary Fig. 2 and Supplementary Video 3), we measured the propagation speed at 3 ± 0.3 mm min⁻¹, which is in

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**Fig. 1 | 4D functional ultrasound imaging during task evoked activity.** a. Schematic representation of the multiplane-wave compounding method, with eight plane waves. At transmit signal no. 1, eight wavefronts tilted with eight different angles are quasi-simultaneously transmitted into the medium. Each plane wave is generated by transmitting a signal \( s_{ij}(t) \) on all elements \((i,j)\) of the array. In order to avoid any overlapping in the multiplane wave transmission, each \( k \)-th tilted plane wave is delayed by a constant delay \( \tau_k \). This transmit is repeated eight times with different polarizations \(+1\) or \(-1\) given by the Hadamard matrix \( H_n \). These coefficients \( h_{ik} \) are then used as weights for the summation (represented by \( \Sigma \)) to retrieve each plane wave individually with an amplitude \( N \). The amplitude increase obtained by this summation of ultrasonic raw signals results in an improvement of the signal-to-noise ratio of the image. b. Example of activation maps (top views and bias views) obtained from the same rat after left whisker stimulation and visual stimulation, respectively. Gray color represents the baseline Doppler signal and activated regions are represented in ‘warm’ colors. Activation maps were obtained by estimating the Pearson correlation coefficient between the power Doppler signal and the stimulus pattern. Color scale is proportional to the correlation coefficient. Activated regions of interest: somatosensory barrel field (S1BF), primary visual cortex (V1), superior colliculus (SC) = 0.82 during visual stimulation = 0.23, somatosensory barrel field (S1BF) = 0.58 during whisker stimulation = 0.68, and ventral posterior medial nucleus (VPM) = 0.40.
Fig. 2 | Application of whole rat brain fUS imaging to FC mapping and 4D tracking of epileptiform events. a, Example of 3D correlation maps obtained in one rat with seed-based analyses. Gray color represents the baseline Doppler signal, and regions that exhibit significant correlation with seeds are displayed in 'warm' colors. Seed positioned within left hippocampus (green square): i, top view and ii, coronal view. Seed positioned within frontal cortex (red square): iii, top view and iv, coronal view. Correlation maps were obtained by computing the normalized Pearson correlation coefficient between the temporal signals of each studied seed and each voxel of the brain. Scale bar, 1 mm. b, Mean (N_{rat} = 4) correlation matrix of 103 cortical and subcortical regions. c, Average connectivity graph (correlation coefficient > 0.6 between two linked regions) illustrating 70 connections during the resting state. The color scale represents the strength of functional connections. d, Three views of propagation of a cortical depression wave in a single rat during an ictal event induced by a cortical injection of a potassium channel blocker (4AP). Power Doppler blood volume increases between 15 and 50% during ictal activity. Scale bars, 1 mm. Wave traveling speed = 3 ± 0.3 mm min⁻¹.
agreement with previous studies in rabbits \(^1\) and rats \(^2\). We detected the propagation of ipsi and contralateral spreading depressions in two animals among \(N=3\) animals (Supplementary Fig. 3).

We hereby demonstrated that 4D fUS imaging can be used for dynamic whole-brain functional imaging in rodent models. Similar to other neuroimaging modalities, fUS imaging has limitations. First, fUS is sensitive to CBV variations and as such measures brain activity only through the prism of the neurovascular coupling. Although fUS is limited by the inherent delay of neurovascular coupling, its 20 ms temporal resolution could benefit the mapping of directional propagation of vascular activity based on lag-correlation mapping as recently demonstrated in behaving nonhuman primates during visual tasks \(^11\).

Second, in contrast to photoacoustics or fMRI, fUS does not provide access to oxygen saturation information. For this reason, it does not measure an initial dip in the functional response to stimuli. In addition, fUS is insensitive to quasi-horizontal vessels. Third, the current spatial resolution, estimated at \(240 \times 240 \times 180\) \(\mu m^3\) in the center of the imaged volume and \(320 \times 320 \times 180\) \(\mu m^3\) at the volume edges (Supplementary Fig. 4) is lower than for 2D fUS imaging. The resolution could be improved by using matrix arrays with a refined one-wavelength spatial pitch leading to high angular directivity and smaller focal spots or by increasing frequency up to 15 MHz. Fourth, due to the limited sensitivity of the array elements, our setup requires a craniotomy for rat brain imaging or possibly 15 MHz. Fourth, due to the limited sensitivity of the array elements, our setup requires a craniotomy for rat brain imaging or possibly an intravenous injection of microbubble contrast agents \(^10\). Fifth, in contrast to optical approaches, voltage or calcium indicators for fUS imaging are not yet available, but genetically encoded gas vesicles as acoustic reporter genes could pave the way to such indicators in the future \(^10\). Finally, although one advantage of 2D fUS imaging is that the probes are small enough to be used with freely moving animals \(^6\), due to the large number of cables and weight of the array, 4D fUS is currently limited to head-fixed animals.

Clinical applications could also directly benefit from our 4D strategy both noninvasively in human neonates through the transfontanellar window \(^5\) and in adults during surgery. 4D clinical fUS imaging would then provide whole functional data in a short time span without the need for a careful positioning of the probe before acquisition. For fundamental research, 4D fUS imaging in combination with other modalities such as electrophysiology or optogenetics could extend our capabilities of whole-brain observation in small animal imaging.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at [https://doi.org/10.1038/s41592-019-0572-y](https://doi.org/10.1038/s41592-019-0572-y).

Received: 28 September 2018; Accepted: 8 August 2019; Published online: 23 September 2019

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**Acknowledgements**

This work was supported by the European Research Council under the European Union’s Seventh Framework Program (no. FP7/2007-2013)/ERC Advanced grant agreement no. 339244-FUSIMAGINE) and by a funding from the Human Brain Project, Project no. FUSIMICE ANR-15-HBPR-0004. This work was also supported by the AXA Research Fund.

**Author contributions**

M.T. and T.D. conceived the study. C.R., V.F., M.P. and M.C. developed sequence acquisitions. C.R. and S.P. acquired data. C.R., T.D. and M.T. performed data processing. M.T. and T.D. conceived the study. C.R., V.F., M.P. and M.C. developed sequence acquisitions. C.R. and S.P. acquired data. C.R., T.D. and M.T. performed data processing. C.R., S.P., T.D. and M.T. interpreted the results. C.R. and M.T. wrote the first draft of the manuscript with substantial contribution from T.D., S.P. and M.C. All authors edited and approved the final version of the manuscript.

**Competing interests**

T.D., M.P. and M.T are co-founders and shareholders of Iconeus company commercializing ultrasound neuroimaging scanners.

**Additional information**

Supplementary information is available for this paper at [https://doi.org/10.1038/s41592-019-0572-y](https://doi.org/10.1038/s41592-019-0572-y).

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**Peer review information**

Nina Vogt was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Methods

Animal preparation. All animals received humane care in compliance with the European Communities Council Directive of 2010/63/EU, and the study was approved by the institutional and regional committees for animal care (Comité d’Éthique pour l’Expérimentation Animale no. 59—’Paris Centre et Sud’ Protocole no. 2017-23). Sprague Dawley male rats were used in this study (6-8 weeks old, 250-350g). N=10 animals were used for this study. 4D fUS imaging with visual and/or whisker stimuli was performed in N=3 animals. Imaging of ictal events was performed in N=3 rats. 3D FC mapping was performed in N=4 rats. A craniotomy was performed to avoid the effect of the skull, which can produce notable attenuation and distortion of ultrasound signals at the employed frequencies.

During the surgery and the subsequent imaging session, the animals were anesthetized using an initial intraperitoneal injection of medetomidine (Dormitor, 0.33 mg kg\(^{-1}\)) and ketamine (Imalgene, 40 mg kg\(^{-1}\)), followed by hourly intraperitoneal injections of medetomidine (Dormitor, 0.1 mg kg\(^{-1}\)) and ketamine (Imalgene, 12.5 mg kg\(^{-1}\)). The animal head was placed in a stereotaxic frame and a 1 x 1 cm skull window was removed by drilling (Foredom) at low speed using a micro drill steel burr (Burr number 19007-07, Fine Science Tools). Care was taken not to damage the dura to prevent inflammatory processes in the brain. After surgery, the brain was rinsed with sterile saline and ultrasound coupling gel was placed on the window. The 2D matrix array was positioned directly above the cranial window before acquisition.

During both the craniotomy and the imaging session, the body temperature was maintained constant using a heating pad (Physitemp), and heart and respiratory rates were monitored (Mount’s Plus, Star Life Science Corp.). The eyes of the animal were covered with black masking tape during the surgery so that the light from the scalpel lamp would not saturate the ocular receptors of the animals and produce visual stimulation.

3D fast system with multiplane-wave encoding. A 3D fast scanner\(^{21}\) (1,024/512 transmit/receive channels, 60 MHz sampling rate) was used to drive a 32 x 32 piezoelectric matrix array transducer (0.3 mm element size, 8 MHz center frequency, Vermont). Each individual element has a 0.3 x 0.3 mm\(^2\) size (corresponding to 4\(^2\), much smaller than the typical 20\(^2\) of conventional linear probes, \(\lambda\) being the ultrasonic wavelength). The system has been used in past studies with compounded plane wave transmissions to allow 3D fast volumetric acquisition at thousands of volumes per second to perform 3D shear wave elastography in tissue mimicking phantoms.

To reach a mandatory high signal-to-noise ratio for 4D whole-brain fUS imaging, a transmission sequence based on the emission of a burst composed of multiple tilted plane waves with Hadamard amplitude encoding was implemented. Briefly, multiplane-wave imaging enables an increased signal-to-noise ratio for ultrafast images without compromising either the frame rate or resolution. It also regains the sensitivity loss due to the small element size of the 2D matrix array. This concept was tested for 2D fast imaging\(^{9}\) but never implemented for 3D imaging. Multiplane-wave imaging relies on coded excitations: each emission transmits a burst of successive N plane waves with different angle transmits with each plane wave having a given coded polarity (−1 or +1). N successive transmits are then fired with the same set of plane waves but different polarity combinations (Supplementary Fig. 5). By using different polarities, one can then reconstruct individual plane waves independently with linear operations on receive buffers using linear formation.

To optimize those polarity combinations for N emissions of the N tilted plane waves \(p_1, p_2, \ldots, p_N\), a Hadamard matrix was used to obtain independent vectors with +1 and −1 components from each of its columns. Note that the multiplication of \(H_N\) (Nth-order Hadamard matrix) by its transpose is equal to N times the identity matrix \(\sum_{i=1}^{N-1} H_N \times H_N^T = N I\). Thus, the backscattered echoes of each independent plane wave can be reconstructed individually by a dedicated coherent summation of the successive receive signals.

Finally, by coherently summing the N initial receive buffers of the N successive transmissions with the right coefficients given by the Hadamard matrix lines, we can reconstruct N new buffers corresponding to the backscattered echoes of individual plane waves only, but with a virtual amplitude N times greater.

As an example, for \(N=2\), the rows and columns of Hadamard matrix
\[
H_2 = \begin{bmatrix}
1 & 1 \\
1 & -1
\end{bmatrix}
\]
are used, respectively, for the coding and decoding of two successive multiplane-wave transmissions. The first and second transmissions correspond, respectively, to the combined emission of \(p_1+p_2\) and \(p_1-p_2\), based on \(H_2\) \(H_1 p_1 \rightarrow \{p_1 p_2\} = \{p_1 p_2\} - \{p_1 p_2\}\). By multiplying the resulting backscattered signal by \(H_2\) one can code the echoes and synthetically reconstruct the signals virtually transmitted by \(2p_2\), as \(H_2^T \{p_1 p_2\} = H_2^T \times H_1 \times p_1 = (2p_2, 0p_2)\).

The order of a Hadamard matrix (and thus the number of tilted plane waves) must be two or a multiple of four. In our sequence, we used \(N=8\) plane waves (MP eight-angles transmit sequence) (Supplementary Table 1 for angles of tilted plane waves and Supplementary Fig. 5), enabling an important gain \((\sqrt{8})\) of signal-to-noise ratio without compromising the frame rate (390 Hz). Using such coded excitations, we managed to achieve high quality Doppler volumes thanks to an artificial increase in the amplitude of the transmit signal. To estimate the improvement achieved by multiplane transmit imaging, we performed one multiplane transmit scheme (Sequence A) lasting 350 ms followed by a conventional plane wave compounding sequence with \(N=8\) successive individual compounded plane wave transmissions lasting 350 ms. The 9 dB signal-to-noise ratio increase obtained by multiplane-wave transmit compared to conventional plane wave compounding permits to image many vessels that were hidden in the conventional compounding sequence (Supplementary Fig. 6).

4D functional ultrasound sequences. Different sequences were implemented for the 4D functional ultrasound experiments:

- **Sequence A**: sensory-evoked led. A multiplane-wave eight-angle (MP eight-angles) transmit sequence (3,120 Hz transmission rate) is repeated at 390 Hz for 350 ms to average approximately two cardiac cycles. This enables the constructive interference of the compounded ultrafast volumes, which form a power Doppler volume when squared and averaged. Each power Doppler imaging block is repeated every 1.5 s (1.15 s pause time) during 180 s. Several successive acquisitions were performed in a row and concatenated.
- **Sequence B**: FC experiment. The same multiplane-wave eight-angle transmit sequence in Sequence A (repeated at 390 Hz for 350 ms) is used. Imaging blocks also are repeated every 1.3 s (1.15 s dead time) for 360 s. This long acquisition time is adapted to measure FC.
- **Sequence C**: epileptiform activity experiment. The same multiplane-wave eight-angle transmit sequence in Sequence A (repeated at 390 Hz for 350 ms) is used but repeated every 3 s (2.65 s dead time) for 360 s.

A pause between imaging blocks as well as sequence repetitions was initially chosen as a compromise between the frame rate (to ensure adequate blood volume variations sampling) and a conservative dead time needed both for the continuous storage of raw data and to prevent any possible damage of our prototype probe due to any internal accumulated heating. In the future, we expect faster storage units, as well as a less conservative approach toward probe safety to reduce the dead time as much as possible. This would boost temporal sampling to achieve continuous blood volume monitoring, as is currently possible with 2D functional ultrasound imaging.

Beamforming and ultrasensitive Doppler processing. The first step of the beamforming process is the decoding and coherent compounding of raw channel data as described previously. The received range-compensated time-gate transmits with the coefficients given by the Hadamard matrix. As a result, we obtained, for each sequence of N transmission containing N-angle multiplane waves, N receive buffers after synthetic decoding. Each of them virtually resulted from a single tilted 2D plane wave transmission with an N-fold higher amplitude. We then compensated each time delay \(\Delta t\) and the resulting N buffers were coherently summed to obtain one final buffer, as is equivalent to a coherent compounding method\(^{4}\).

Volume beamforming was then performed using a delay-and-sum processing implemented on a graphical processing unit (K6000, Nvidia) to reconstruct a volumetric dataset.

The clutter filter then removed all static or quasi-static information corresponding to slowly moving tissue to keep only the signals coming from blood scatterers. We implemented a multidimensional spatiotemporal filtering based on a singular value decomposition\(^{7}\), which was extended to 4D data (time + 3D space). The algorithm allowed the discrimination of tissue motion (coherent motion within the volume) from blood flow (incoherent motion within the volume) by eliminating the coherent subspace corresponding to the first singular vectors (N=30) of the data decomposition.

After a clutter filter and for each voxel, the energy of the temporal signal was integrated over the whole imaging block (corresponding to 350 ms) to obtain one ultrasound power Doppler volume. The block duration was chosen so that the CBV was averaged over approximately two cardiac cycles to remove the influence of the pulsatility. Power Doppler images have been shown to be quasi-proportional to blood volume\(^{1}\).

To estimate the spatial resolution of 4D fUS imaging using the 9 MHz matrix array and multiplane-wave transmit sequence, we imaged a 80 μm diameter nylon wire in water by applying the 3D decoding and beamforming with an isotropic 85 × 85 × 85 voxel size. This static wire was placed at different locations both in depth and lateral directions to estimate the spatial resolution in the center and in the periphery of the imaged volume. We found a 240 × 240 × 180 μm\(^3\) spatial resolution in the middle of the three-dimensionally imaged volume. This spatial resolution was slightly and continuously decreasing toward the lateral borders of the imaged volume up to a 320 × 240 × 180 μm\(^3\) at the edges.

For all the in vivo data, considering the spatial resolution and the time of beamforming inversely proportional to the size of the reconstructing voxel, we chose to beamform with a 170 × 170 × 85 μm\(^3\) voxel. This corresponds to approximately \(\lambda \times \lambda \times 1/2\).

Although fUS imaging is able to detect very slow blood flow (1 mm/s\(^{-1}\)) values occurring in tiny vessels\(^{22}\), it permits to image only vessels with diameter comparable or higher than the spatial resolution. For this reason, only arterioles or venules with a diameter larger than 100 μm are visible and delineated in fUS.
images. In the brain cortex, such large vessels are mainly perpendicular to the brain surface. Nevertheless, the fUS signal is also able to image large arterioles and venules with angles ranging between 0° and more than 70° compared to the ultrasonic beam axis as demonstrated in ref. 1. As an example, vessels such as the superior sagittal sinus or inferior sagittal sinus are visible in Figs. 1 and 2.

A guide for implementation, Hadamard coding and decoding and data examples are provided in the Supplementary Software package. Step-by-step instructions are provided in the Readme pdf document.

**Sensory-evoked 3D functional imaging.** We performed (on the same rat) two different stimulations following each other: whisker stimulation and visual stimulation.

**Whisker stimulation.** The whole (left) pad of whiskers was stimulated manually using a cotton swab using the following parameters: 15 s on period, 5–7 Hz, 1 cm amplitude, separated by a 15 s off period (starting with the off period). The stimulation pattern was repeated six times for a total acquisition duration of 180 s.

**Visual stimulation.** Visual stimuli were delivered using a green LED (532 nm wavelength) positioned at 3 cm in front of the right or left eye of rats. At this distance, the light luminance was of 18 lux when the light was on and of 0.01 lux when the light was off. Stimulation runs consisted of periodic flickering of the green LED using the following parameters: 15 s of rest followed by 15 s of a flicker repeated six times for a total duration of 180 s. Between stimuli presentation sessions, the rats were kept in a dark environment.

**Activation maps.** Correlation maps were computed individually from the normalized correlation between each voxel temporal signal with the different stimulus patterns (Pearson’s product moment) using MATLAB (MathWorks). Example MATLAB codes for all post-processing steps are included in Supplementary Software.

We chose a level of significance of \( z > 3.1 \) by applying the Fisher’s transform (\( P < 0.001, \) one-tailed test), which corresponds to \( r > 0.193 \). The map after thresholding for the same example is shown in Fig. 1b. Brain structures (S1BF, VPM) were identified using a rat brain atlas by performing a rigid registration of the corresponding coronal slice.

**Doppler volumes, relative CBV and correlation volumes were then imported into a 3D software for visualization (Amira v.6.0.1 software, Visualization Sciences Group).**

**3D FC matrix.** We used established conditions to obtain a 3D FC\(^{11} \). Briefly, we kept the animal under medetomidine (Domitor, 0.3 mg kg\(^{-1} \)) and ketamine (Imalgene, 40 mg kg\(^{-1} \)) for at least 2 h before the acquisition to obtain stable and reproducible results. Eight acquisitions of 90 s each were acquired and then concatenated.

Datasets were filtered independently with a high-pass filter with a cutoff frequency of 0.05 Hz. We first selected two seeds located in the left hippocampus and the left frontal cortex. Seed-based correlation maps were formed by computing the normalized Pearson correlation coefficient between the average signal of those two seeds and each individual voxel of the dataset.

Second, using the ultrasonic Doppler volumes, the acquisitions were manually registered (rigid transformation) using known vascular landmarks to two brain atlases: the Papp atlas for subcortical structures\(^{24} \) and Valdes-Hernandez atlas for cortical structures\(^{25} \). Structure labels were subsequently hierarchized using the Neuronames ontology\(^{26} \) to group them in six larger regions (neocortex, olfactory bulb, midbrain, interbrain). Corresponding brain regions for each number label (103 in total) are listed in Supplementary Table 2. The spatially averaged and temporally filtered signals were extracted from each of the base regions of interest, normalized and correlated with each other to form the correlation matrix using the MATLAB software. A mean correlation matrix (\( N_{5,8} = 4 \)) reflecting inter-regional connectivity is presented in Fig. 2b (individual matrices presented in Supplementary Fig. 1). We chose to measure the 3D FC in four rats to prove the robustness of our modality to detect functional patterns and to minimize the numbers of used animals.

A visual representation of the brain connectivity networks was constructed from the connectivity matrix by creating color-coded plots using the Pearson correlation value and linking the barycenter of each region of interest in three dimensions.

**Induction of epileptiform events.** Epileptiform ictal events were induced by the cortical injection of 1 μl of 4-aminoypyridine (4AP, Sigma-Aldrich) solution at a concentration of 18 mmol l\(^{-1} \) using a 150-μm-diameter Hamilton needle, 1 mm deep into the cortex of the anesthetized animal with continuous injections of medetomidine (Domitor, 0.1 mg kg\(^{-1} \) h\(^{-1} \)) and ketamine (Imalgene, 12.5 mg kg\(^{-1} \) h\(^{-1} \)).

It should be noted that our 4AP injection model was not designed to provide new insights into seizure dynamics as such events are distinct from the spreading depressions we tracked in our experiments.

In this set of experiments, a multiplane-wave eight-angle transmit sequence (390-Hz pulse repetition frequency, for 350 ms, 136 compounded volumes) repeated every 3 s during 360 s was used to follow the crisis propagation.

As we were able to detect the propagation of spreading depression waves in two rats, we did not use more animals for this proof of concept to minimize the number of animals used.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data supporting the findings of this study associated with figures are available upon request. Example data can be downloaded in the Supplementary Information.

**Code availability**

Example encoding and post-processing codes associated with figures are provided as Supplementary Software. Step-by-step instructions are available in a Readme pdf document to guide the user. A binary executable file for GPU-based 3D beamforming is included to process decoded sample radio frequency data. The low-level beamforming code is available in the framework of an official collaboration between academic institutions.

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Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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\textit{Our web collection on statistics for biologists contains articles on many of the points above.}

Software and code

Policy information about availability of computer code

**Data collection**

Custom codes were used to collect the data. These codes are available in the supplementary software files provided with the manuscript [supplementarysoftware.zip - scripts 1 to 4]. In addition, a binary executable file for GPU-based 3D beamforming is included to process decoded sample RF data. The low level beamforming code is available in the framework of an official collaboration between academic institutions.

**Data analysis**

For data analysis, custom codes were used for the correlation of acquired data with the stimuli. These codes are available in the supplementary software files provided with the manuscript [supplementarysoftware.zip - script 5]. For the analysis of functional connectivity, custom codes (already published in Osmanski et al, Nature comm, 2015) were used. These codes are available in the supplementary software files provided with the manuscript [supplementarysoftware.zip - script 6].

For statistical analysis, we used the commercial software MATLAB R2016a (MathWorks, Cambridge, MA, USA). Ultrasonic Doppler volumes, relative cerebral blood volume and correlation volumes were then imported in a 3D software for visualization (Amira 6.0.1 software, Visualization Sciences Group, Burlington, MA).

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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- A list of figures that have associated raw data
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All data supporting the findings of this study associated with figures (listed below) are available upon request. Example data can be downloaded in the
supplementary materials of the publications on the Nature Methods website.
- Figure 1.b and 1.c, Figure 2, Supplementary Figure 1, Supplementary Figure 2, Supplementary Figure 3, Supplementary Figure 4 and Supplementary Figure 6 have associated raw data

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No sample size calculation was performed as the proof of concept of functional ultrasound activation during stimuli was successful in all N=3 animals.
For epilepsy study, fUS imaging was successful in all N=3 animals in tracking the propagation of spreading depression waves. As we were able to detect the propagation of spreading depression waves in 2 rats, we didn’t use more animals for this proof of concept in order to minimize the number of used animals.
For functional connectivity experiments, fUS imaging in all N=4 animals successfully exhibited left-right functional connectivity. We arbitrarily chose N=4 rats to demonstrate the robustness of the method. We didn’t use more animals for this proof of concept in order to minimize the number of used animals.

Data exclusions
No data were excluded

Replication
For these proof of concept experiments, all attempts of replication of the experiment were successful. 4D Beamforming processing was validated in ultrasonic phantoms and ultrafast Doppler imaging of the rat brain vasculature was validated successfully in all animals. 4D mapping of the brain activity was also successfully validated in all experiments. The implemented ultrasonic sequences and all these processing steps were fully automated and the software containing these features and used for functional imaging was not modified by the operator ensuring that all experiments were performed exactly the same way as described in the manuscript.

Randomization
Randomization was not relevant for our study as it is a proof of concept study demonstrating the ability of functional ultrasound to map dynamically in 3D the brain activity. For each proof of concept experiment (task evoked stimulus, functional connectivity at rest and spreading depression tracking), animals were randomly chosen and the experiment was successful for all animals.

Blinding
Blinding was not relevant for our study as it consists of a technical proof of concept study for a new 4D neuroimaging technology. Acquisitions were performed by the authors blindly and postprocessing of data was revealing the 3D activation maps of the stimuli.
For epileptic seizures experiments and functional connectivity acquisitions, the experimental acquisition was also blind for the investigators and seizure propagation tracking was revealed only in postprocessing.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

Methods

n/a Involved in the study
☑ n/a Involved in the study
☐ Antibodies ☐ ChIP-seq
☐ Eukaryotic cell lines ☐ Flow cytometry
☐ Palaeontology ☐ MRI-based neuroimaging
☐ Animals and other organisms
☐ Human research participants
☑ Clinical data

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Sprague Dawley male rats were used in this study (6-8 weeks old, 250-350 g).

Wild animals
The study did not involve wild animals
| Field-collected samples | The study did not involve samples collected from the field |
|-------------------------|----------------------------------------------------------|
| Ethics oversight        | All animals received humane care in compliance with the European Communities Council Directive of 2010 (2010/63/EU), and the study was approved by the institutional and regional committees for animal care: CEEA (Comité d’Ethique pour l’Expérimentation Animale) numéro 59 - “Paris Centre et Sud” Protocole # 2017-23. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.