Transcranial functional ultrasound imaging of the brain using microbubble-enhanced ultrasensitive Doppler

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**ABSTRACT**

Functional ultrasound (fUS) is a novel neuroimaging technique, based on high-sensitivity ultrafast Doppler imaging of cerebral blood volume, capable of measuring brain activation and connectivity in rodents with high spatiotemporal resolution (100 µm, 1 ms). However, the skull attenuates acoustic waves, so fUS in rats currently requires craniotomy or a thinned-skull window. Here we propose a non-invasive approach by enhancing the fUS signal with a contrast agent, inert gas microbubbles. Plane-wave illumination of the brain at high frame rate (500 Hz compounded sequence with three tilted plane waves, PRF = 1500 Hz with a 128 element 15 MHz linear transducer), yields highly-resolved neurovascular maps. We compared fUS imaging performance through the intact skull bone (transcranial fUS) versus a thinned-skull window in the same animal. First, we show that the vascular network of the adult rat brain can be imaged transcranially only after a bolus intravenous injection of microbubbles, which leads to a 9 dB gain in the contrast-to-tissue ratio. Next, we demonstrate that functional increase in the blood volume of the primary sensory cortex after targeted electrical-evoked stimulations of the sciatic nerve is observable transcranially in presence of contrast agents, with high reproducibility (Pearson’s coefficient ρ = 0.7 ± 0.1, p = 0.85). Our work demonstrates that the combination of ultrafast Doppler imaging and injection of contrast agent allows non-invasive functional brain imaging through the intact skull bone in rats. These results should ease non-invasive longitudinal studies in rodents and open a promising perspective for the adoption of highly resolved fUS approaches for the adult human brain.

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**Introduction**

The functional activity of the brain can be followed through measurement of its blood supply, as first proposed by Lavoisier (1920) and demonstrated by Mosso (1881). Today, several functional imaging modalities exploit, as their experimental read-out, local hyperemia to map the functional response of the brain to stimuli. These include functional magnetic resonance imaging (fMRI) (Ogawa et al., 1990; Kim and Uguribil, 2003; Rossi, 2006; Richiardi et al., 2011), and other functional imaging techniques such as optical coherence tomography and multiphoton microscopy (Sheth et al., 2003; Chen et al., 2009). Each one of these techniques has its own specificity, sensitivity and spatiotemporal resolution, and have different limitations. These restrictions currently hinder the broad dissemination of functional imaging approaches in translational and pre-clinical research settings. Therefore, the validation of complementary or alternative methods for in vivo imaging of local hyperemia in rodents, a major pre-clinical animal model, is an important scientific objective.

Recently, very high frame rate ultrasound imaging (10,000 frames per second) (Tanter and Fink, 2014) was shown to enable high-resolution and high-sensitivity power Doppler imaging (Bercoff et al., 2011). Applied to brain imaging, it led to high sensitivity mapping of cerebral blood volume (CBV) and functional ultrasound (fUS) imaging of task-evoked changes in cortical activity in the rat brain (Macé et al., 2011; Mace et al., 2013). JUS is able to detect subtle changes of low blood flow in small cerebral vessels with a very high sensitivity. It consists in replacing the conventional line-by-line scanning of tissue with focused beams by successive transmissions of either an ultrasonic...
plane or diverging waves, to attain frame rates close to 20,000 frames per second at 3 cm depth (Osmanski et al., 2012b). At these frame-rates, JUS can differentiate slow-moving blood (down to 1 mm/s), from surrounding tissue (Mace et al., 2013), and consequently, it is potentially able to detect blood flow changes in the capillary network even if its spatial resolution (~ 100 μm) is not sufficient to individually map these vessels. Such sensitivity is required to detect slight variations in blood flow following sensory stimulation, since red blood cell velocities in small cortical arterioles and capillaries are typically in the 1–10 mm/s range (Schaffer et al., 2006; Shih et al., 2009; Kobat et al., 2011; Shih et al., 2012). This elevated sensitivity allowed JUS to track the spatio-temporal dynamics of the activation within the rat brain following whisker stimulation or during induced epileptic seizures (Macé et al., 2011). It was also shown to be sensitive enough to detect odor-evoked stimulation (Osmanski et al., 2014a) and, remarkably, to map functional connectivity in the living rat brain with a much higher spatio-temporal resolution than fMRI (Osmanski et al., 2014b).

However, albeit ultrasound can propagate deep within tissues, cerebral JUS remains limited by its poor penetration through the skull. An attenuation of 6.9 dB/cm at 15 MHz has been observed in previous studies (Fry and Barger, 1978; Goss et al., 1979; Larrat et al., 2010; Pinton et al., 2012), which renders small variations in blood flow indistinguishable from noise. Therefore, in order to study changes in functional blood volume in microvessels deep in the rat brain both elevated resolution (typically 100 μm resolution in-plane) and penetration (up to 20 mm penetration depth) is required. Surgical procedures involving a craniotomy (Mace et al., 2013) or a bilateral thinned-skull window (Osmanski et al., 2014b) meet such requirements, but their invasiveness restrains large dissemination of this neuroimaging technique. Similarly, clinical application of JUS is limited today to patients with a pre-existing cranial tic cover, and the skin was sutured using 5.0 non-absorbable Ethicon thread. Preliminary experiments showed that this method enabled good quality ultrasound imaging for as long as 1 week after skull thinning.

Preparation of microbubble ultrasound contrast agents

Perfluorocarbon-filled microbubbles (Bracco, Plan-Les-Ouates, Switzerland) with a diameter ranging between 1 μm and 5 μm were dissolved with 0.9% normal saline solution to a concentration of 2 × 10^6 microbubbles/ml. Two initial 200 μl boluses of the contrast agent were injected in the catheterized jugular vein before starting the hind limb primary sensory cortex (S1HL) activations to ensure that the functional ultrasound acquisition was initialized. Hence, the dynamic time-course changes of the bolus injection were investigated by imaging for 10 min the temporal evolution of the circulating bubbles in the blood stream.

JUS imaging

Animals were anesthetized using an initial intraperitoneal (IP) injection of medetomidine (Domitor®, 0.3 mg · kg⁻¹) and ketamine (Imalgène®, 40 mg · kg⁻¹), followed by hourly IP injections of medetomidine (0.1 mg · kg⁻¹ · h⁻¹) and ketamine (12.5 mg · kg⁻¹ · h⁻¹) in order to keep the animals under stable anesthetized conditions. The jugular vein was catheterized in order to perform the injections of microbubble contrast agents. The thinned skull was rinsed with sterile saline and 1 cm² of ultrasound coupling gel was placed on the window. The animals were then placed in a stereotaxic frame (Stoelting; Chicago, IL, USA).

A 15 MHz linear transducer array (128 elements, 8 mm elevation focus, 80 μm pitch) was directly installed on the head of the animal.
connected to the ultrafast research ultrasound scanner (AixPleror, SuperSonic Imagine, Aix-en-Provence, France) and coupled on the brain via standard degased ultrasound gel. Real time B-mode imaging was initially used to control the placement of the probe on the field of view. The software-based architecture of the scanner enabled Matlab (MathWorks; Natick, Massachusetts, USA) programming of custom transmit/receive ultrasound sequences. Each session began with an anterior-posterior Doppler scan, used to visualize the shape of the blood vessels, and identify the stereotaxic coordinates. fUS acquisitions were achieved at the level of the choroid plexus of the lateral ventricle, corresponding to the anteroposterior coordinate Bregma − 1 mm.

Body temperature was maintained constant using a heating pad (Gaymar Industries; New York, NY, USA). A 1 cm incision was made in the skin and muscle of both hindpaws, above the femur in order to expose and isolate the sciatic nerve. A hook-shaped stimulating electrode was gently inserted on the left and right sciatic nerve (LSN and RSN, respectively) to alternatively induce the electric sciatic stimulations. The nerve was allowed to rest for 10 min between each stimulation sequence. Trains of five fiber stimulations (25 pulses at 5 Hz, 0.2 mA, and 100 μs width) were delivered for 5 s on each sciatic nerve, separated by 20 s OFF period to recover the baseline (Fig. 1A). Our exclusion criterion was based on the physiological condition of the animals. Only animals whose fluctuations of the power Doppler signal baseline (absence of stimulation) were stably <5% were included in the study. Experiments were performed on n = 7 male Sprague-Dawley rats, by using three different protocols: bilateral window (BW, n = 1, protocol A, Fig. 2A), unilateral thinned-skull window (TSW, n = 4, protocol B, Fig. 2B) or full transcranial (IS, n = 2 protocol C, Fig. 2C) imaging. This led respectively to the fUS imaging of n = 12 transcranial and of n = 12 thinned skull hemispheres (Fig. 5).

Transcranial ultrafast Doppler imaging sequence

Functional ultrasound (fUS) imaging was performed using Ultrafast Doppler (Bercoff et al., 2011) based on compounded plane-wave ultrasound imaging (Tanter et al., 2002; Montaldo et al., 2009). This technique has sufficiently high sensitivity to detect subtle hemodynamic changes due to the neurovascular coupling with a high spatio-temporal resolution (1 ms, 100 μm).

First, the vascularization map of the brain was obtained by using a dedicated MATLAB script to move the US probe, fixed on a three-axes microstep motor, 0.5 mm after each ultrafast Doppler acquisition. The scanning sequence consisted in 15 tiled planar ultrasonic waves (− 14° to 14° angles, with a step of two angles 2°) with a pulse repetition frequency (PRF) of 7500 Hz. Once the choroid plexus of the lateral ventricle was identified, the ultrasound probe was fixed in coronal orientation over

![Fig. 1. Schematic view of the fUS imaging setup and fUS imaging protocol. A: The 15 MHz probe was placed on the top of the animals’ heads and coupled via ultrasound gel. A stereotaxic frame immobilized the skull. An isolated constant current stimulator delivered five electrical stimuli alternatively on the left and right sciatic nerves (LSN and RSN, respectively). The panel presents the stimulation parameters. B: The continuous-fUS insonification sequence consisted in three tilted plane waves (− 3°, 0° and 3°). The frame rate was set to 500 Hz to sample the blood flow without aliasing. The resulting three low quality images were coherently summed to obtain a single high quality B-mode image of the brain. The sequence was repeated 374 times leading to 150 s acquisition time to stack a set of 200 high quality images per each sequence. The power Doppler images were acquired over 150 s in order to fully recover the electrical-evoked activation of the brain.](image-url)
the hind limb primary sensory cortex (S1HL) at the anterior-posterior co-
ordinate of Bregma — 1 mm. Over the thinned-skull window, the align-
ment was performed without the addition of contrast agent. In the full
transcranial protocol, an initial injection of 150 μl microbubbles was neces-
sary to highlight the blood vessels and achieve a correct alignment of
the probe over the reference coordinates. Activation maps of the S1HL
cortex were collected with a continuous-fUS activation sequence consisting in a compounded plane wave sequence at 500 Hz frame rate
using three tilted plane waves (3°, 0° and 3°, PRF = 1500Hz).

The backscattered echoes were recorded, beamformed and coher-
ently added to produce a stack of high quality B-mode echographic im-
ages (Fig. 1B). As the intensity of the power Doppler images is propor-
tional to the cerebral blood volume (CBV), we implemented an incoherent mean of the blood signal to cancel blood pulsatility and ob-
tain high quality ultrafast Doppler images. We have previously demon-
strated that this incoherent temporal mean is equivalent to a filter with a cut-off frequency of 75 Hz to further remove tissue and motion artifact coming from the skull. The Doppler signal was origi-

The activation maps were computed from the temporal correlation
between the stimuli and the increase in CBV. We observed a contralat-
eral activation of the brain in response to the evoked task. The activated
pixels were considered significant for a correlation ρ > 2σ, where ρ is the spatial correlation of the activation maps and σ their spatial standard
deviation. The Doppler signal was averaged over time in the region of interest (corresponding to activated areas) to retrieve step by step the
time course of the entire electrical stimulation pattern. Its amplitude
was represented as a percentage of change relative to the baselines of the activated areas ± standard deviation. Furthermore, the hemody-
namic signals from the ROI (activated areas) were extracted to estimate the temporal correlation between the evoked task and the measured in-
crease in blood volume. The reproducibility of the activation was evalu-
ated, with and without the presence of contrast agent, using the Pearson correlation coefficient (R).

Results

Ultrafast Doppler scan through the thinned-skull window or through the intact skull

First, we compared three different ultrasound imaging configurations: imaging through bilateral and unilateral thinned-skull windows (TSW) and full transcranial imaging through the intact skull (IS), with and without intravenous injection of microbubbles (Fig. 2A, B, C). As demonstrated previously (Macé et al., 2011; Osmanski et al., 2014a), the vascular network of the rat cortex could be mapped with ultrafast Doppler imaging through a TSW (Fig. 2D1, F1). Here, the choroid plexus of the lateral ventricle was used as a reference point for the localization of the imaging plane (Bregma — 1 mm), which contains the hind limb primary sensory cortex (S1HL) (Paxinos and Watson, 2006).
The intact skull attenuated the high-frequency ultrasonic waves (15 MHz emitting frequency) hindering the acquisition of the vascular map (Fig. 2E1, F1). Strikingly, after the injection of microbubbles, this loss of signal was fully compensated and blood vessels as deep as 12.5 mm could be visualized (Fig. 2E2). For better visualization of the microbubble-induced signal amplification, we compared the signal obtained in the two hemispheres of a unilateral TSW configuration (Fig. 2F2). A typical example of microbubble-enhanced fUS rostrocaudal scan through the intact skull is presented in Fig. 3, showing that a large part of the rat brain can be visualized up to 12.5 mm depth using micro-bubbles as a contrast agent. We have calculated the contrast-to-tissue ratio (CTR), defined as the ratio of the scattered power from the blood echo (combining red blood cells and microbubbles) and that from the surrounding tissue. In presence of microbubbles, we calculated a gain of 9 dB in the Power Doppler pre-stimulus baseline prior to normalization. Hence, the use of contrast agent reduces the relative contribution of the noise in the Power Doppler estimates as the fUS signal is generally close to the noise floor of the electronic system. These results show that by using inert gas microbubbles as a contrast agent, significant fUS signal amplification can be achieved, allowing mapping of the cerebral vasculature through the intact rat skull. The vascularization maps obtained through the IS vs. TSW were comparable (Fig. 2D–F). Hence, in spite of the presence of the bone, we were able to perform functional imaging of the brain without using aberration correction methods.

Microbubble-enhanced imaging of functional hyperemia through the intact skull

The second step of the study was to investigate whether contrast enhancement by microbubble contrast agents also enables the measurement of functional hyperemia through the intact skull. The activation maps shown in Fig. 4 display the spatial distribution of the hemodynamic response in the S1HL to the electrical stimulation of the sciatic nerve. The grayscale background image shows the vascular network detected by ultrafast ultrasound imaging, through the intact skull or a thinned-skull window, with or without an injection of microbubbles in single representative animals. The overlaid correlation map shows the temporal correlation between the five stimuli and the evoked change in the Power Doppler (PD) signal. As a reference, we first performed the activation of the sensory cortex on the bilateral TSW protocol without the injection of contrast agents (Fig. 4A). The set of five stimuli were repeated up to a maximum of three times on the left or right sciatic nerves (LSN and RSN, respectively) on a total of \( n = 12 \) IS hemispheres and \( n = 12 \) TSW hemispheres (Fig. 5).

As previously described (Osmanski et al., 2014a), using a thinned-skull window in absence of micro-bubbles, the stimulation of the right and left sciatic nerve induce an evoked hemodynamic response in the contralateral area of the S1HL (Fig. 4A2, A3, D2). However, in absence of microbubbles, such evoked hemodynamic response cannot be observed through the intact skull (Fig. 4B1, B2, B3, D3). Strikingly, intravenous injection of microbubbles allowed the observation of the brain vasculature through the intact skull (Fig. 4C1), allowing the measurement of stimulation-induced hemodynamic changes in the right and left S1HL (Fig. 4C2, C3, E2, E3). Comparison of the activation maps retrieved from the thinned-skull hemispheres without injection of microbubbles with those acquired transcranially with the use of contrast agents showed that transcranial Ultrafast Doppler imaging in combination with ultrasound contrast agents could detect reproducibly (Pearson correlation coefficient of 0.7 ± 0.1, \( p = 0.85 \)) and non-invasively functional changes deep in the rat brain.

Sensitivity and robustness of contrast-agent enhanced transcranial Doppler imaging

In order to further characterize the performance of contrast agent enhanced transcranial imaging, we observed the time-resolved power Doppler (PD) signal in the activated area with a high-frame rate imaging sequence (500 frames per second), in the TSW and IS configurations without and with contrast agent injection. As shown in Fig. 6A, this allowed the characterization of the temporal evolution of the hemodynamic response to the evoked task, i.e. the set of five stimuli. For each hemisphere, the intensity of the fUS signal was normalized to the pre-stimulus baseline and was represented as % changes of the Power Doppler signal. The PD increase corresponding to the vascular response was consequently higher after the first stimulation than after the following four, corresponding to around 2% diminution in peak-height between the first and the fifth peaks, in the thinned-skull configuration without contrast agent. This gradual diminution of peak amplitude, most likely due to the physiological adaptation to the electrical stimulations, was further enhanced with the use of contrast agents, because of the gradual elimination of the microbubble bolus from the circulation (Fig. 6B). We could correct for this second source of signal diminution by continuously measuring the power Doppler intensity in the sagittal sinus and by adjusting the temporal evolution of the hemodynamic response function by the slope of this reference intensity. The resulting corrected curve (blue on Fig. 6B) was comparable to the curve obtained with the thinned-skull configuration in absence of microbubbles, where no correction was needed. After these corrections, the mean temporal hemodynamic parameters were retrieved by averaging the five electrical stimuli to obtain a typical hemodynamic

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**Fig. 3.** Antero-posterior fUS scans allow to map the vasculature of the rat brain through the intact skull (IS) after an intravenous injection of microbubbles. Injection of 150 μl of contrast agent led to a clear ultrasound signal at all different coronal imaging planes, at full depth. The stereotaxic coordinates (antero-posterior distances to Bregma) are reported on the top of each Doppler acquisition. Scale bar: 2 mm.
response profile in correspondence to the evoked tasks (for individual values see Table 1). In Fig. 6C, we report the mean values, the standard errors across the different trials and across subjects for the three investigated experimental configurations. An unpaired Student’s t-test was performed aiming to compare the control case (TSW no injection of contrast agents), with the other case studies in presence of microbubbles. As shown in Fig. 6D, we measured a $4 \pm 1\%$ stimulation-induced increase of the peak amplitude (PA) with the control imaging protocol (i.e. TSW without control agent).

Strikingly, for both protocols involving the injection of microbubbles, the peak amplitude displayed a highly significant increase, leading to $12 \pm 2\% (p < 0.0001)$ increase in PA for the thinned-skull and $9 \pm 2\% (p < 0.0001)$ PA increase in the transcranial configuration, as compared to the control imaging protocol (unpaired Student's t-test). In the time-domain, the increase in blood volume was strongly correlated with the evoked task (thinned hemispheres without bubbles: $\rho = 0.7 \pm 0.1$, thinned hemispheres with bubbles: $\rho = 0.7 \pm 0.1$, transcranial hemispheres with bubbles: $\rho = 0.6 \pm 0.2$). Finally, dynamic parameters of the hemodynamic response, such as the peak-to-peak and time-to-peak values were roughly equivalent in the TSW or IS with microbubbles imaging protocols (Table 1).

Onset times, assessed as the time needed to reach 10% of the maximum dilation (Hall et al. 2014) were $2–3$ s for each condition (Table 1). Taken together, these results show that microbubble contrast agents allow robust and highly sensitive dynamic tracking of activation-evoked hemodynamic changes through the intact skull, significantly surpassing in performance the current state-of-the-art thinned-skull imaging configuration.

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Fig. 4. Microbubble contrast agents allow fUS imaging of cortical hyperemia evoked by electrical stimulation of the sciatic nerve, through the intact skull (IS). A–E, The left column shows the vascularization maps obtained by power Doppler imaging of the investigated coronal plane at Bregma $-1$ mm in representative animals for each imaging condition. The middle and the right columns show the hemodynamic responses evoked by stimulation of either the right (RSN) or the left sciatic nerves (LSN). The correlation coefficient shown in the color bar represents the temporal correlation between the evoked task and the hemodynamic response recorded in the S1HL. Scale bar: 2 mm.

Fig. 5. Summary of successful trials on each animal for the different fUS imaging configurations. Functional activations of the S1HL were performed on $n = 7$ animals. A distinction between transcranial hemispheres and thinned skull hemispheres led to a total of $n = 12$ transcranial S1HL activation through the intact skull when boluses of microbubbles were delivered in the bloodstream and $n = 12$ activations on the thinned skull windows ($n = 6$ without microbubbles and $n = 6$ with injection of contrast agent), fUS imaging through the intact skull in absence of microbubbles was not applicable (NA).
Discussion

In the past few years, functional ultrasound (fUS) has been validated as a novel neuroimaging modality in rodents. However, its application have been limited by the presence of the skull which attenuates the ultrasonic waves. The objective of our work was to combine the high spatiotemporal resolution and sensitivity of fUS with contrast-agent induced signal enhancement for transcranial applicability. Our results show that after intravenous microbubble injection, vascular structure and hemodynamics can be imaged with excellent spatiotemporal resolution through the intact rat skull. Consequently, the vascular map could be obtained over several parallel slices and the choroid plexus of the lateral ventricles could be identified as an orientation landmark. Microbubbles-aided transcranial fUS was able to detect the variation of the power Doppler intensity in the primary sensory cortex (S1HL), in strong temporal correlation with the stimulation of the sciatic nerve. Therefore, in presence of contrast agents, the sensitivity of ultrafast Doppler can greatly benefit from the strong enhancement of raw ultrasonic signals (gain of 9 dB in the contrast-to-tissue ratio), overcoming the attenuation of ultrasonic waves by the cranium. We have already successfully imaged the vascular dynamics of the rat brain with an ultrafast Doppler acquisition on craniotomized animals without contrast agents by delivering whisker stimuli (Macé et al., 2011) as well as odor evoked ones (Osmanski et al., 2014a). Compared to the sciatic nerve stimulation used in the present study, these stimulations are more relevant physiologically; however the detection of blood flow changes was facilitated by the absence of the skull bone. In the current study, the injection of microbubbles drastically improved the SNR by completely separating the fUS signal from the noise floor. Hence, we foresee this technique to be sensitive enough to measure the CBV changes in the brain also after physiologically evoked tasks.

In our study, similar or better results could be attained transcranially than by using the previous TSW protocol, after injecting a bolus of microbubbles that increase the intensity of the blood signal with respect to the tissue. These results show that the signal enhancement created by contrast agents could compensate the attenuation induced by the skull. In our work, we noticed that, at 15 MHz imaging frequency, no aberration correction was needed neither on the beamforming process, nor on the Doppler images. Indeed, as it can be noticed in Fig. 2(D–E), the vascularization maps relative to the intact skull vs. thinned-skull

![Fig. 6. Sensitivity and robustness of contrast-agent enhanced transcranial Doppler imaging. A: Averaged hemodynamic response function (n = 6, black curve) evaluated on the thinned-skull hemispheres without injection of microbubbles (control imaging protocol). The stimulation pattern is shown in red. Due to physiological adaptation to the electrical stimulations, the peak amplitude displays a decrease of ~2% from the first to the last activation. B: Time evolution of the hemodynamic responses of a representative animal with the transcranial imaging protocol (blue curve). The red curve shows the US signal profile corrected for the signal diminution, due to the dissolution of the injected boluses of contrast agent. C: Mean evolved hemodynamic response function of the three imaging protocols: thinned-skull window (TSW) without (black curve, n = 6) and with (blue curve, n = 6) microbubbles and intact skull (IS) transcranial hemispheres with microbubbles (red curve, n = 12). The fUS signal in response to the neuronal stimulation is represented as the mean ± standard deviation across trials and across subjects. D: Peak enhancement of the three imaging protocols: TSW hemispheres with microbubbles (blue, n = 6); 2, transcranial (IS) hemispheres with microbubbles (red, n = 12); 3, TSW hemispheres without microbubbles (black, n = 6). Statistics: paired Student’s t-test, compared to the control condition (TSW no bubbles). P values: ****P < 0.0001.

Table 1

|                      | Thinned-skull (TSW) hemispheres | Transcranial (IS) hemispheres | Thinned-skull (TSW) hemispheres |
|----------------------|---------------------------------|-----------------------------|---------------------------------|
|                      | With bubbles                    | No bubbles                  |                                 |
| Peak-to-peak (s)     | 25.0 ± 0.3                      | 25.0 ± 0.3                  | 25.0 ± 0.5                      |
| Peak amplitude (%)    | 12 ± 2 (p < 0.0001)             | 9 ± 2 (p < 0.0001)          | 4 ± 1                           |
| Onset time (s)       | 2.5 ± 0.8 (p < 0.2)             | 2.0 ± 0.6                   | 2.0 ± 0.3                       |
| Time-to-peak (s)     | 6.0 ± 1.0 (p < 0.07)            | 6.0 ± 0.2 (p < 0.003)       | 5.0 ± 0.7                       |
window can be comparable. However, if strong phase distortions would have appeared on our Doppler images, we could have implemented an already existing aberration correction method (Osmanski et al., 2012a) by directly using the information contained in the Doppler images.

Understanding which physiological parameter is measured by the fUS sequence is crucial for the interpretation of functional imaging data. Power Doppler imaging without contrast agents injections is known to be directly proportional to the number of moving red blood cells in the sample volume, i.e. to the local blood volume (Shung et al., 1976; Rubin et al., 1995; Rubin et al., 1997), provided that backscattering properties do not vary over time. Without contrast agents, this assumption is valid as backscattering properties of red blood cells, such as hematocrit and shear rate, remain time-invariant, which is highly probable (Shung et al., 1992; Cloutier and Qin, 1997). In microbubble-enhanced fUS, such direct proportionality of the power Doppler signal with the CBV is compromised, since the backscattering property of the blood depends on the temporal variation of contrast agents concentration. In order to ensure a direct proportionality of the fUS signal with CBV during the experiment, we corrected for the transient decrease of the backscattering signal, due to the elimination of the bolus, by using the power Doppler intensity within the large sinus vessels as reference value to normalize the measured functional signals. This correction of time-varying backscattering properties enabled compensation for the progressive decrease of the estimated stimului responses. After normalization, the remaining decrease in the response between the five successive stimulations was comparable to that obtained without contrast agent injection, interpreted as a physiological adaptation to the stimuli. Thus, the measurement of the hemodynamic response with fUS appears robust even though microbubbles introduce an important variability in the power Doppler intensity curve (as shown in Fig. 6C, the standard deviation of the normalized curve in the thinned hemisphere configuration, is three times higher in presence of micro-bubbles than in the two other protocols). The high fluctuations in the hemodynamic response function (HRF) curves in presence of microbubbles are explained by fluctuations of the backscattering properties as the number of bubbles recruited in each voxel is stochastically fluctuating in the region of interest as compared to the red blood cells that are continuously perfusing the vessels. The spatial pattern of the contralateral transcranial activations of the sensory cortex detected with microbubbles was similar to the pattern obtained during control activations through the thinned-skull window, without contrast agents. Repeated stimulations gave highly reproducible responses. However, it has been demonstrated that the effect of the anesthesia could interfere with the neuronal coupling (Masamoto and Kanno, 2012). Hence in the present study we excluded animals whose fluctuation of the power Doppler baseline (without stimulation) was higher than 5%, putatively indicating unstable physiological conditions.

Brief stimuli are ideal to investigate the central layers of the somatosensory cortex, which is highly filled with capillaries, however they would not be long enough to achieve vascular delayed compliance (Siegel et al., 2003). Most parameters obtained from the hemodynamic response, namely the peak-to-peak, the onset time and the time-to-peak were equivalent when measured through a thinned-skull window and through the skull with microbubbles and are consistent with previous reports (van Raaij et al., 2011) and other imaging modalities (Silva et al., 2007; Tian et al., 2010; Goloshvsky et al., 2011; Hirano et al., 2011; Yu et al., 2012). Very recently, in vivo 2-photon microscopy directly showed that capillary vasodilatation is not a passive response to arteriole dilation but on the contrary, neural activity first dilate capillaries by actively relaxing pericytes, temporally preceding arteriole dilation (Hall et al., 2014). In that study, during a 15-s long whisker-pad stimuli the onset time was −2.7 s for the capillary response (average vessel diameter d = 4.4 μm), whereas it was −3.7 s in the penetrating arterioles (average d = 12.8 μm). In our imaging conditions, measured onset times were relatively short, falling between 2 and 3 s (Table 1), suggesting that the hyperemic signal measured by fUS is dominated by the capillary response. In conclusion, we demonstrate highly sensitive functional ultrasound imaging through the intact skull of adult rats after intravenous injection of microbubble contrast agents. Hence, this novel technique combines the high spatial and temporal resolution of fUS, with minimal invasiveness. The use of contrast agents for fUS should allow easier longitudinal studies in animals and paves the way to clinical transcranial f-ultrasound of the adult human brain. Notwithstanding, the translation to a clinical application of transcranial imaging of the human brain vasculature will be more challenging as the thickness of the skull bone is in the range of several millimeters. Thus, we expect an attenuation much higher signal attenuation compared to a small animal as well as an important phase distortion of the ultrasonic waves passing through the cranium (Pinton et al., 2012). To apply transcranial fUS on patients, lower frequencies should be preferred (1 MHz), which would reduce resolution, but would exploit microbubbles echo closer to their resonance frequency range (1–5 MHz).

Conclusion

Neuro-imaging systems are invaluable tools in the understanding of the brain both for fundamental research and clinical diagnosis. Our results show how functional ultrasound (fUS) could become a novel neuroimaging technique to perform high resolution transcranial imaging of the brain. Until today, invasive surgery was necessary to create anatomical acoustic windows in the skull to image the microvasculature and its functional response. Here, by combining the sensitivity of ultrafast Doppler imaging with microbubbles to detect blood flow under the skull, stimuli-induced changes in the cerebral blood volume are evaluated deep within the cortex. It leads to non-invasive functional imaging of the whole brain at typical 100 μm resolution, facilitating longitudinal studies and paving the way for future clinical applications.

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Appendix A. Processing of ultrasonic signals

Computing ultrasound images

The use of ultrasound imaging array allows the transmission of ultrasonic waves and the reception of the backscattered echoes on the same transducer. In our study, as reported in the Material and Methods, we used a custom-built array with 128 elements and a central frequency of 20.3 MHz (pitch = 0.08 mm, elevation focus = 10 mm). Its 15.4 MHz bandwidth allowed the use of this probe at a frequency of 15 MHz. Once the medium was insonified, the ultrasonic echoes were recollected and beamformed to obtain a complex ultrasonic image s(x, z) using the method described in Montaldo (2009), x and z refer respectively to the lateral position and depth of a spatial pixel. This operation was repeated every 2 ms (we used a 500-Hz frame rate) and we obtained a 3D matrix s(x, z, t) where t described the time at which the image was computed.

Extracting ultrasound power Doppler signal

The principle of Doppler ultrasound imaging relies on the detection of the moving red blood cells. In fact, this stack of ultrasound images is
the summation of the backscatter echoes recorded from the red blood cells as well as those coming from the tissue. In our study, we will have to consider also the presence of contrast agents. Ultrasound images $s(x, z, t)$ can be decomposed as:

$$s(x, z, t) = S_{\text{tissue}}(x, z, t) + S_{\text{RBC}}(x, z, t) + S_{\text{microbubbles}}(x, z, t)$$

where $S_{\text{tissue}}(x, z, t), S_{\text{RBC}}(x, z, t)$ and $S_{\text{microbubbles}}(x, z, t)$ are respectively the signals coming from the tissue, the red blood cells and the microbubbles.

We also would like to point out that ultrasound contrast agents as micro bubbles are blood pool agents and they can only be distinguished from the red blood cells and tissue if non-linear imaging is performed. In our study, we preferred fundamental imaging to harmonic imaging, since the transmitting ultrasonic frequency at 15 MHz was far from the resonance frequency range of the microbubbles contrast agents, and thus we would not have been able to benefit from their nonlinear properties. The signal backscattered from the blood $s_{\text{blood}}(x, z, t)$ can then be written as:

$$s_{\text{blood}}(x, z, t) = S_{\text{RBC}}(x, z, t) + S_{\text{microbubbles}}(x, z, t).$$

In order to separate the blood signals from the tissue, we implemented a spatiotemporal filter based on the singular value decomposition (SVD) of the stack of the f-US images. Such method is described in Demene et al. (2015).

Subsequently, the ultrasound signal $s(x, z, t)$ can be decomposed along with its eigenvalues $\lambda_i$, its spatial eigenvectors $u_i(x, z)$ and its temporal eigenvectors $T_i(t)$, $s(x, z, t)$ can be written as

$$s(x, z, t) = \sum_{i} \lambda_i u_i(x, z) T_i(t)$$

It is also demonstrated in Demene et al. (2015) that the tissue signal is contained in the first elements of this sum. $s_{\text{blood}}(x, z, t)$ can be obtained by withdrawing the tissue first components of this sum.

$$s_{\text{blood}}(x, z, t) = \sum_{i \neq \text{tissue}} \lambda_i u_i(x, z) T_i(t)$$

Moreover, in order to further remove any motion artifact at the micrometric scale coming from the hindlimb stimulation of the animal, we decided to apply also a numerical Butterworth high-pass temporal filter with a cut-off frequency of 75 Hz.

Eventually, we evaluated the power Doppler signal as the modulus of the Doppler signal at each pixel.

$$PD(x, z, t) = ||s_{\text{blood}}(x, z, t)||$$

Computing the activation map

To obtain the activation map to detect the augmentation due to the sensory-motor activations with respect to the baseline, the Power Doppler signal was normalized:

$$PD_{\text{norm}}(x, z, t) = \frac{PD(x, z, t) - \frac{1}{n_{\text{act}}} \sum_{i=1}^{n_{\text{act}}} PD(x, z, t)}{\sqrt{\left(\frac{1}{n_{\text{act}}} \sum_{i=1}^{n_{\text{act}}} PD(x, z, t) - \frac{1}{n_{\text{act}}} \sum_{i=1}^{n_{\text{act}}} PD(x, z, t)\right)^2}}$$

Then we evaluated the spatial correlation between the temporal evolutions of the power Doppler signal, $PD_{\text{norm}}(x, z, t)$ with a reference function $ref(t)$ representing the delivered stimulations.

$$ref(t) = \prod_{i} (T_{i} \delta(t - nT_{0})$$

with $\prod(t)$ being a rectangular function with a width $T_{i}$, $\delta$ being a Dirac function and $\otimes$ stands for convolution. $T_{0}$ and $T_{1}$ respectively refer to the period and the duration of the evoked tasks and $n$ is the number of repetition of the stimuli.

The activation map $Actmap(x, z)$ can then be computed using the formula:

$$Actmap(x, z) = \max\left\{ \sum_{t} ref_{\text{norm}}(t - \tau) PD_{\text{norm}}(x, z, t) \right\}$$

with

$$ref_{\text{norm}}(t) = \frac{ref(t) - \frac{1}{n_{\text{act}}} \sum_{i=1}^{n_{\text{act}}} ref(t)}{\sqrt{\sum_{i=1}^{n_{\text{act}}} ref(t) - \frac{1}{n_{\text{act}}} \sum_{i=1}^{n_{\text{act}}} ref(t)^{2}}}$$

Thresholding the activation map

The goal of this subsection is to determine if/where a reliable activation is present on the activation map. To this extent we have to find a threshold over which the activation can be considered significant. The activated pixels are defined with the formula:

$$Actmap(x, z) > \text{threshold}$$

The threshold can be computed by studying the fluctuations of activation map $Actmap_{\text{baseline}}(x, z)$ computed using equation XX from baseline acquisitions where no electrical stimulus was delivered.

$$\text{threshold} = 2 \sqrt{\text{Var}(Actmap_{\text{baseline}}(x, z))}$$

with Var standing for the spatial variance.

Eventually, a spatial Pearson’s coefficient was evaluated to retrieve the likelihood of the activations of the three configurations under investigation.

References

Bercoff, J., Montaldo, G., Loupas, T., Savery, D., Meziere, F., Fink, M., Tanter, M., 2011. Ultrafast contrast doppler imaging: providing full blood flow characterization. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 58, 134–147. http://dx.doi.org/10.1109/TUFFC.2011.1780.

Burns, P.N., Wilson, S.R., 2006. Microbubble contrast for radiological imaging: 1. Principles. Ultrasound Q. 22, 5–13.

Chen, Y., Agarre, A.D., Rovinskaya, L., Devor, A., Boas, D.A., Fujimoto, J.G., 2009. Optical coherence tomography (OCT) reveals depth-resolved dynamics during functional brain activation. J. Neurosci. Methods 178, 162–173. http://dx.doi.org/10.1016/j.jneumeth.2008.11.026.

Cloutier, G., Qin, Z., 1997. Ultrasound backscattering from non-aggregating and aggregating erythrocytes—a review. Biorheology 34, 443–470.

Couture, O., Bannouf, S., Montaldo, G., Aubry, J.-F., Fink, M., Tanter, M., 2009. Ultrasound imaging of ultrasonic contrast agents. Ultrasound Med. Biol. 35, 1908–1916. http://dx.doi.org/10.1016/j.ultrasmedbio.2009.05.020.

Couture, O., Fink, M., Tanter, M., 2012. Ultrasound contrast plane wave imaging. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 59, 2676–2683. http://dx.doi.org/10.1109/TUFFC.2012.2508.

Demene, C., Pernot, M., Biran, V., Alison, M., Fink, M., Baud, O., Tanter, M., 2014. Ultrasound Doppler reveals the mapping of cerebral vascular restitutivity in neonates. J. Cereb Blood Flow Metab 34, 1009–1017. http://dx.doi.org/10.1038/jcbfm.2014.49.

Demene, C., Deffieux, T., Pernot, M., Osmanski, B.F., Biran, V., Genisson, J.-C., Sieu, L.-A., Bergel, A., Franqui, S., Correas, J.M., Cohen, I., Baud, O., Tanter, M., 2015. Spatiotemporal clutter filtering of ultrafast ultrasound data highly increases Doppler and f-ultrasound sensitivity. IEEE Trans. Med. Imaging 35.

Desai, Y., Couture, O., Fink, M., Tanter, M., 2013. Sonically activated ultrasound localization microscopy. Appl. Phys. Lett. 103, 174107. http://dx.doi.org/10.1063/1.4826597.

Fringk, P.J., Bouakaz, A., Kirkhorn, J., Cate, F.J., Ten, de Jong, N., 2000. Ultrasound contrast imaging: current and new potential methods. Ultrasound Med. Biol. 26, 965–975. http://dx.doi.org/10.1016/S0301-5629(00)00229-5.

Fry, F.J., Barger, J.E., 1978. Acoustical properties of the human skull. J. Acoust. Soc. Am. 63, 1576–1590.

Goertz, D.E., Needles, A., Burns, P.N., Foster, F.S., 2005. High-frequency, nonlinear imaging of microbubble contrast agents. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 52, 495–502. http://dx.doi.org/10.1109/TUFFC.2005.1417273.

Goloshevsky, A.G., Wu, C.W.-H., Dodd, S.J., Koretsky, A.P., 2011. Mapping cortical representations of the rodent forepaw and hindpaw with BOLD fMRI reveals two spatial
