Transcranial ultrafast ultrasound localization microscopy of brain vasculature in patients

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Changes in cerebral blood flow are associated with stroke, aneurysms, vascular cognitive impairment, neurodegenerative diseases and other pathologies. Brain angiograms, typically performed via computed tomography or magnetic resonance imaging, are limited to millimetre-scale resolution and are insensitive to blood-flow dynamics. Here we show that ultrafast ultrasound localization microscopy of intravenously injected microbubbles enables transcranial imaging of deep vasculature in the adult human brain at microscopic resolution and the quantification of haemodynamic parameters. Adaptive speckle tracking to correct for micrometric brain-motion artefacts and ultrasonic-wave aberrations induced during transcranial propagation allowed us to map the vascular network of tangled arteries to functionally characterize blood-flow dynamics at a resolution of up to 25 μm and to detect blood vortices in a small deep-seated aneurysm in a patient. Ultrafast ultrasound localization microscopy may facilitate the understanding of brain haemodynamics and of how vascular abnormalities in the brain are related to neurological pathologies.

Obtaining functional information of living organs non-invasively across different scales is a major challenge of medical imaging research, as diseases often start locally at the cellular level before inducing large-scale observable symptoms. Brain imaging adds another layer of complexity, as the organ relies on extremely complex, multiscale and interpenetrated networks of neurons, glial cells and vessels. Assessing the vascular morphology and function of the brain is of course key for the diagnosis and monitoring of intracranial aneurysms, stenosis or arteriovenous malformations as well as the management of acute stroke. It is also of interest for neurological pathologies, such as degenerative diseases, as there is increasing evidence for a close interaction between cerebrovascular diseases and cognitive impairment. In humans, most of this cerebrovascular imaging is performed using expensive, ionizing and/or invasive contrast-injection-dependent techniques, namely computed tomography and magnetic resonance angiography (CTA and MRA, respectively). Furthermore, they are often limited to an anatomical description of the vasculature at a millimetric resolution and fail to capture the blood-flow time dynamics relevant for assessing cerebrovascular function. No in vivo and non-invasive imaging technique has so far been proven capable of capturing anatomical and functional features below the millimetric scale at the whole-brain level in humans. In this brain clinical imaging landscape, contrast-enhanced ultrasound is marginally used. It is mostly performed through transcranial acoustic bone windows (Fig. 1a,b), if present, when the imaging contrast of a regular Doppler examination is too low, to monitor the blood-flow velocities in large cerebral basal arteries in the case of spasms, stenoses, vascular malformations and dissections or after acute ischaemic stroke. It is an easy-to-use, inexpensive and widely available bedside method that shows haemodynamics in real time across the brain. However, its poor spatial resolution and limited sensitivity still prevent contrast-enhanced ultrasound from being a decisive imaging modality for cerebrovascular diseases.

The recent introduction of ultrasound localization microscopy (ULM) solved the conceptual trade-off between spatial resolution and penetration depth while increasing sensitivity due to the joint use of ultrasound contrast agents and ultrafast imaging. ULM relies on the same concept that has been the basis for the development of photo-activated localization microscopy and stochastic optical reconstruction microscopy in optics: even if an imaging modality is diffraction-limited, in the particular case of imaging an isolated object we can hypothesize that this object is at the centre of the diffraction spot. We can therefore localize it with a sub-resolution precision that depends on the signal-to-noise ratio of the imaging device. In the case of ultrasound imaging, these isolated objects consist of diluted ultrasound contrast agents and the method gains from imaging at the fastest rate possible. The unique ability of ULM to perform non-invasive deep microvascular imaging at the microscopic scale using ultrasonic waves has been demonstrated in rodents.

However, although the concept of ULM was introduced almost 10 years ago, no translation of ULM imaging to the human brain has been shown so far. The application of ULM to microvascular brain imaging in humans faces major challenges such as non-invasive brain accessibility, limited acquisition time, transcranial propagation and brain motion. Our group demonstrated brain-flow imaging in humans without contrast using ultrafast Doppler Ultrasound, but never both transcranially and with super-resolution: it was performed either in neonates through the fontanelle or in adults during brain surgery (a result that was reproduced by Soloukey et al. in 2020; ref. 14) using an opened skull flap. Moreover, imaging without a contrast agent does not allow the diffraction limit to be beaten and would result in the case of transcranial imaging to images with a resolution of the order of between one and a few millimetres, two orders of magnitude above ULM. Temp-transversal imaging through the skull imposes the use of a small acoustic aperture, as the acoustic temporal bone window rarely exceeds 20 mm in diameter, and the

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use of a low imaging frequency given that the ultrasound attenuation coefficient in the skull is very strong and increases greatly with frequency—it scales in $f^{2.1}$ around 1 MHz, where 2.1 is the highest frequency exponent of all biological tissues. This large ultrasound attenuation is explained by two mechanisms: irreversible absorption in the bone and theoretically reversible diffraction effects due to the speed-of-sound mismatch between the brain tissue (approximately 1,500 m s$^{-1}$) and bone (approximately 3,000 m s$^{-1}$) that

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**Fig. 1 | Transcranial ULM of deep brain vessels in patients.**

a. Positioning of the ultrasound transducer (phased array XP 5-1, Supersonic Imagine) on the bone temporal window for brain imaging.

b. Typical field of view in transverse (left) and coronal (right) brain sections.

c. Diverging waves cover a wide field of view. Isolated microbubbles backscatter circular wave fronts towards the transducer array, which are distorted by the skull bone.

d. Schematic of the aberration correction. Each transducer records the echoes backscattered from the brain (left); after SVD filtering, wave fronts from individual bubbles are visible and aberration law can be determined (middle); and after time reversal of the estimated aberration delays, the bubbles’ echo returns to an expected hyperbola profile (right).

e. After aberration correction, image reconstruction is performed on a polar (r,θ) grid.

f. Magnified view of the region in the white box in e. After SVD filtering, individual bubbles are localized in successive images at successive time steps $t_i$ to $t_n$, geometric centres of the point-spread function (red crosses) are estimated and tracking of trajectories is realized.

g. After tracking all of the bubbles in the field of view, a density map is reconstructed (an acquisition time of 45 s was used here). Single micrograph.

h. Conventional Doppler image obtained in the same imaging plane as g. The green arrows serve as landmarks for comparison of the two images.

i. Magnified view of the region in the white box in g (15 x 17 mm area). Small penetrating vessels of the mesencephalon (blue arrowheads) and two very close vessels (350 µm apart; white arrow) can be observed.
distorts the acoustic wave front (Fig. 1c), a process called wave aberration. Finally, the influence of motion artefacts escalates greatly in the micrometric resolution range and is of highest importance, as ultrasound is traditionally handled and the brain moves inside the skull cavity.

Results

In this Article, we demonstrate transcranial deep imaging of human cerebrovascular haemodynamics at the microscopic scale by jointly solving the major challenges of spatial resolution, skull-bone aberration and motion artefacts for transcranial ULM (t-ULM). We show that not only can ULM be performed transcranially in human adults but also that at this low ultrasound frequency, with an expected low SNR and in the presence of motion, we were able to achieve resolutions of the order of 25 µm, far beyond the typical 1 mm resolution of functional ultrasound imaging. First, we used a phased array transducer with an acoustic aperture of 19.2 mm, driven at a central frequency of 2 MHz: this choice corresponds to a trade-off (Supplementary Fig. 1a,b) between emitting as much as possible within the available bandwidth of the transducer (it is 1–5 MHz and the central frequency is 3 MHz) most of the available low-frequency imaging transducers are not designed to efficiently emit ultrasound below 2 MHz), keeping the skull-bone attenuation coefficient as low as possible (10 dB cm\(^{-1}\) at 1 MHz, 40 dB cm\(^{-1}\) at 2 MHz, and 60 dB cm\(^{-1}\) at 3 MHz; Supplementary Fig. 1a), targeting as much as possible an efficient backscatter coefficient for the contrast agent (\(\eta = 3.5 \times 10^{-4} \text{ cm}^{-1} \) at 1 MHz, 8.9 \(\times 10^{-4} \text{ cm}^{-1}\) at 2 MHz and 1.2 \(\times 10^{-4} \text{ cm}^{-1}\) at 3 MHz) and keeping the highest possible resolution (at 2 MHz, axial resolution to 0.82 ± 0.07 mm and a lateral resolution between 1 and 5 mm, depending on the imaging depth; Supplementary Fig. 1c–e). We then implemented ultrafast ultrasound imaging using unfocused diverging waves, a technique that has previously been proposed for ultrafast cardiac imaging and was adapted here to human-brain imaging. It uses the propagation of circular waves before recording the intracranial backscattered echoes. The patient is injected with small boluses (0.2 ml) of contrast agents to attain a diluted concentration of microbubbles in the circulating blood. We chose a bolus dose that provided sufficient dilution of the microbubbles for individual identification but a sufficiently high concentration for acquisition in a short time compared with what is described in the ULM literature, would allow for multiple injections in the case of probe repositioning and would remain below the maximum recommended total dose of 2.4 ml.

The individual detection of each of these highly reflecting gas bubbles was exploited both for the correction of skull-bone aberrations on the reflected wave fronts (Fig. 1c,d, detailed in Supplementary Fig. 2) and for subsequent super-resolution imaging of blood vessels. The recorded raw data corresponded to a two-dimensional (2D) spatio–temporal speckle pattern (schematic in Fig. 1d, left), resulting from the interference of ultrasonic waves backscattered from a random set of scatterers. In these data, the tissue signal has a completely different spatial coherence signature compared with the signal of moving microbubbles. Using a spatio–temporal singular-value-decomposition (SVD) filter on these data enables us to retain only the contribution of individual microbubbles (Fig. 1d, middle). Therefore, assuming the acoustic sound speed to be constant in the brain, the backscattered echo of an isolated bubble will appear as a hyperbolic wave front in this particular space–time representation, with a slight aberration due to the propagation through the skull bone. The aberration law is modelled as a near-field phase screen introducing time delays on each element of the phased array. These aberration delays are determined for typically \(1 \times 10^{10} \text{ bubbles s}^{-1}\), enabling averaging and local tuning (the same aberration law is suited for a determined area of the image due to the isoplanatism approximation; Supplementary Fig. 2g), and used to correct for the aberration (Fig. 1d, right and Supplementary Fig. 2h) before beamforming the raw data into images on a polar grid (Fig. 1e). Such adaptive beamforming increases the number of microbubbles detected (increase of 5–13% if considering bubbles whose track length are >10 frames, and up to 43% when considering bubbles whose track length are >30 frames; Supplementary Fig. 2j and Supplementary Fig. 2a–f for a visual comparison). The stack of resulting images is then filtered using a spatio–temporal SVD filter to keep only the microbubble signal (Supplementary Video 1) and the sub-pixel position of the microbubbles is determined using a parabolic fit of local maxima for each image (Fig. 1f and Supplementary Video 1). Trajectories are obtained using a particle-tracking algorithm, the microbubble positions are corrected taking into account motion artefacts estimated using phase correlation of ultrafast ultrasound data (Supplementary Fig. 3) before building an image out of the positions of the microbubbles in the entire field of view: the trajectories are interpolated so that each pixel of the high-resolution grid on the bubble trajectory is lightened up before taking into account the uncertainty of the bubble position by convolving this ‘bubble-presence grid’ with a Gaussian kernel (whose spatial extent is a function of the resolution). This produces an image for which the contrast can be interpreted as proportional to the probability density function of the microbubble distribution and is given a value between zero and one (Fig. 1g). A 2D image revealing vascular structures with unrivalled sharpness can be obtained in a 45-s acquisition. For example, it shows part of the Willis polygon and the posterior cerebral arteries with high precision, which can generally be observed with much lower resolution using a conventional clinical ultrasound scanner (Fig. 1h, green arrows). However, most importantly, a very large number of small calibre vessels, such as small (diameter <200 µm) arteries penetrating the menencephalon, can be visualized (Fig. 1i and Supplementary Video 1).

Compared with typical clinical contrast computed tomography (CT) imaging (Fig. 2a,e), t-ULM shows increased sensitivity and resolution (Fig. 2b) at all depths (up to 120 mm here, which extends to the contralateral cortex; Fig. 2d). Note that subtle anatomical differences may appear when comparing the two images as the CT image represents a slab with a constant thickness of 6 mm, whereas the thickness of the ultrasound image varies in space due to elevation focalization. Our images have better quality near to 60–70 mm depth, as this corresponds to the elevation focus of the probe, and are degraded at large depth due to the loss of this elevation focalization and therefore of larger out-of-plane uncertainty on the localization of the microbubbles. Attenuation is of course also a factor but we can see in Fig. 2d that sensitivity is still good at 110 mm (vessels are visible), while the resolution is degraded (vessels are blurry). The biggest vessels, such as the middle cerebral artery (MCA) and the anterior cerebral artery (red arrows), as well as smaller branches (blue arrows) are visible in both modalities but many structures that are faintly captured by CT (green arrows) become clearly visible on t-ULM (Fig. 2e), with at least two more degrees of vessel branching delineated (Supplementary Fig. 4). At first glance, the increase in resolution compared with conventional ultrasound exceeds at least one order of magnitude, as neighbouring vessels that are estimated to be 214 µm apart at 60 mm depth are evident in this image (Fig. 2f). However, quantification of the spatial resolution by resolving two nearest small vessels is not an adapted approach as it is very unlikely to track a sufficient number of microbubbles flowing in two extremely close microvessels in the 45-s acquisition time. Therefore, the proper estimation of t-ULM spatial resolution will be detailed further in the paper based on a functional rather than an anatomical definition.

The sub-resolution positions can indeed be used to build an anatomical t-ULM image but, more interestingly, these positions are part of a trajectory that provides each microbubble with an instantaneous 2D speed vector (Fig. 3a) and reveal the flow dynamics...
(Supplementary Video 2). This enables us to build vector field maps of the flow velocity inside vessels with a diameter of 1 mm or less (Fig. 3b). In Fig. 3c, one can deduce from the very sharp change in direction that two overlapping but unconnected vessels are present, which was not obvious on the anatomical-only image. In addition, Fig. 3d shows turbulent flow in the turn of the U-shaped vessel, with a large variety of flow-speed magnitudes across the vessel section due to the strong change in direction. We quantified the functional t-ULM resolution on a cross-section of the vessel in Fig. 3c at three different scales (see ‘Flow quantification and functional resolution estimation’ in Methods). When taking bubble speeds in 188-µm-wide bins along this cross-section with a width of 1 mm, it exhibits a typical parabolic blood-flow profile with important speed differences (Fig. 3e). Furthermore, this cross-section flow-speed analysis can also be conducted at different moments in the cardiac cycle due to the very high temporal resolution of ultrafast imaging (see ‘Motion detection and correction’ in Methods), showing an increase of the central median speed from 24.9 ± 6.2 cm s⁻¹ in diastole (Fig. 3e, left) to 33.1 ± 7.8 cm s⁻¹ in systole (Fig. 3e, right). When looking at the vessel edge where the speed gradient is the sharpest (Fig. 3f), it is possible to exhibit important differences on bins that are 62µm wide both in systole and diastole, corresponding to λ/12 (equivalent to conventional axial resolution ÷ 12 and conventional lateral resolution ÷ 24 at this depth (38 mm), with a numerical aperture of two; see Supplementary Fig. 1), where λ is the axial dimension. When taking 25-µm-wide bins, it is still possible to exhibit important speed differences near the edge of the vessel but only in diastole, probably due to a more regular flow and a higher number of detected bubbles (Supplementary Fig. 5). The maximum achievable resolution in the experimental conditions of the present study is therefore 25µm but might be improved with a longer acquisition time or more efficient aberration corrections and motion-compensation techniques.

In a 79-year-old patient (Supplementary Fig. 6) presenting with an aneurysm on the distal part of the right MCA, t-ULM using an acquisition time of 24 s (Fig. 4a) showed an increased sensitivity and resolution compared with both CTA (Fig. 4b) and time-of-flight MRA (Fig. 4c). Common features, such as the communicating posterior cerebral artery and part of the Willis polygon, can be seen (blue arrows) but numerous additional vessels that were invisible on those two major clinical modalities were unveiled, despite a very short acquisition time. The aneurysm is clearly visible under the form of an excrescence at the bifurcation of the right MCA (Fig. 4a, green arrow) both in clinical images and on the t-ULM image. However, t-ULM provides an exhaustive functional comprehension of the blood-flow pattern inside this aneurysm, whereas CT and magnetic resonance images only give its anatomical description. The t-ULM revealed a small vortex inside the left part of this aneurysm whose blood-flow dynamics can be characterized due to this 25-µm resolution (Fig. 4d–f and Supplementary Video 4). The potential for such imaging is hard to predict but it is possible that the degree of clotting or the risk of rupture of a given aneurysm...
Fig. 3 | ULM characterizes haemodynamics and discriminates diastolic and systolic flow. a, The x and z components of the speed vector (left and right, respectively) extracted from the bubble trajectories overlaid on the density map. Single micrograph. x and y scale bars, 3 mm. b, Quantitative representation of the flow vector fields inside 1-mm-wide cerebral vessels (Fig. 2d) is enabled through the combination of these two components. The two ROIs, labelled (i) and (ii), are further magnified in c and d, respectively. c, A 6 × 6 mm ROI of the speed vector representation shows two superimposed vessels (top; crossing without junction). The two bottom vessels, with lower flow speeds, are joining veins. The black arrows quantify the local flow speed (the direction of the arrow corresponds to the flow direction and the arrow amplitude corresponds to the blood-flow amplitude). d, A 5 × 5 mm ROI showing a sharp turn in an artery. The velocity profile across the vessel is very asymmetric due to the tortuosity of the vessel. e, Quantitative assessment of the velocity profile across the whole 1-mm section of the artery in c (blue line in the inset). Bubble velocities are gathered in 188-µm-wide bins (λ/4 ≡ diffraction-limited lateral resolution = 8 at this depth). This quantitative velocity assessment was performed during diastole (left) and systole (right), and shows greatly increased bubble speeds during systole. The mean ± variance (in mm s⁻¹) and sample numbers (bubble positions) of the bins (188 µm), as well as P values are (from left to right): 180 ± 78 (n = 714), 215 ± 86 (n = 2,162), 235 ± 83 (n = 2,593), 239 ± 90 (n = 2,081), 228 ± 83 (n = 1,194) and 208 ± 79 (n = 670) during diastole (P = 1.1 × 10⁻⁸, 7.0 × 10⁻⁸, 0.013 and 1.3 × 10⁻⁸); and 254 ± 95 (n = 579), 284 ± 103 (n = 1,575), 312 ± 106 (n = 2,619), 322 ± 100 (n = 2,410), 303 ± 92 (n = 1,206) and 276 ± 99 (n = 547) during systole (P < 0.01, 3.7 × 10⁻⁵ and 5.7 × 10⁻⁴). f, The same quantitative assessment performed on a 250-µm-wide cross-section (inset) on the extreme border of the vessel. The bins are 62.5 µm wide (λ/12 ≡ diffraction-limited lateral resolution = 24 at this depth) and still show important speed differences where the speed changes are the sharpest. The mean ± variance and sample numbers (bubble positions) of the bins (62.5 µm), as well as P values are (from left to right): 185 ± 70 (n = 213), 188 ± 71 (n = 301), 209 ± 76 (n = 401), 225 ± 88 (n = 510) and 233 ± 87 (n = 525) during diastole (P = 5 × 10⁻⁸, 0.010, 0.044 (third versus fourth) and 9.5 × 10⁻⁵ (third versus fifth)) and 243 ± 90 (n = 173), 267 ± 88 (n = 206), 294 ± 95 (n = 230), 302 ± 102 (n = 364) and 299 ± 101 (n = 528) during systole (P = 2.1 × 10⁻⁵, 0.04 (second versus third), 5.0 × 10⁻⁴ (second versus fourth) and 0.01 (second versus fifth)). e,f, The red horizontal line indicates the median; the boxes show the 25th and 75th percentiles; the whisker gaps represent ±1.26σ corresponding to 80% coverage in the Gaussian hypothesis; the blue cross indicates the mean value. Significant differences between the mean values were calculated using analysis of variance with a post-hoc t-test and Bonferroni’s correction; *P < 0.05, **P < 0.01, ***P < 0.001.
might be influenced by the local fluid mechanics and blood-flow speeds reached in the aneurysm. Numerous studies have tried to link local flow mechanics, wall shear stress and health of the vessel wall\(^\text{28–30}\). Therefore, such unique functional information could be of clinical relevance for making the decision regarding surgical resection or coiling.

In a 63-year-old patient (Supplementary Fig. 7) presenting with chronic cerebral hypoperfusion of the right posterior cerebral artery of fetal origin and an early occlusion of the right MCA that led to the development of a complex local network of collateral arteries (Moyamoya-like disease\(^\text{31}\)), t-ULM (Fig. 4g) using an acquisition time of 24 s largely surpassed the CTA image (Fig. 3).

**Fig. 4 | Clinical relevance of t-ULM for a deep-seated aneurysm.**

- **a**, ULM (24 s of acquisition) of a tilted axial brain section of a patient diagnosed with an aneurysm (green arrow) in the right MCA. The blue arrows depict vascular landmarks visible on CT and magnetic resonance images for orientation. The three white boxes of decreasing size are enlarged in **d–f**, respectively. Representative micrograph of three images. **b**, Maximum intensity projection of a 6-mm-thick CTA slab (in HUs) corresponding to the ULM imaging plane. **c**, Maximum intensity projection of a 7-mm-thick MRA slab (in time of flight (TOF) units) corresponding to the ULM imaging plane. **d**, Magnified view of the 12 x 12 mm ROI of the MCA aneurysm in **a** (outer white box) depicting the local flow vector field. The longest arrows correspond to a speed of 20 cm s\(^{-1}\); values above 20 cm s\(^{-1}\) were clipped. **e**, Magnified view of the 2.5 x 2.5 mm ROI on a flow vortex inside the aneurysm in **d** (outer white box). **f**, Further zoom on an 800 x 800 µm ROI centred on the vortex (white box in **e**), showing almost null velocity at the centre. The longest arrows correspond to a speed of 5 cm s\(^{-1}\); values above 5 cm s\(^{-1}\) were clipped. **g**, ULM (24 s of acquisition) of a brain section of a patient presenting with an occlusion of the right MCA resulting in the abnormal development of a large number of collateral arteries (white boxes). The contralateral side is included for visual comparison. Single micrograph. **h**, Maximum intensity projection of a 10-mm-thick CTA slab corresponding to the ULM imaging plane in **g**, where these collateral arteries are faintly observed and without functional information (flow). **i, j**, Magnified views of the ROIs (10 x 10 mm) of the collateral arteries (i) and (ii) in **g** (i and j, respectively) depicting the direction and magnitude of the flow, stop points and bifurcations, enabling functional quantification. The longest arrows correspond to a speed of 20 cm s\(^{-1}\).
in terms of sensitivity (white boxes). Although it is difficult to assess the functional efficiency of each of these collaterals on a CT or magnetic resonance image, it becomes very easy to understand where the flow is distributed with t-ULM due to the capture of both flow speed and directions in all these vessels at microscopic scales (Fig. 4i) and Supplementary Video 3).

Discussion

By revealing both the human cerebral vascular anatomy and flow dynamics at microscopic scales, t-ULM is very likely to become of major importance for the management of cerebrovascular diseases in the future. Its low cost, ease of use, sensitivity and quantification capabilities in combination with an improvement of almost two orders of magnitude in resolution compared with current clinical modalities could transform the workflow of cerebrovascular patient management. One main limitation of this present study is the lack of real-time processing for positioning the probe, given that this crucial positioning is performed using only the guidance of the B-Mode and conventional colour Doppler. With our ultrasound machine, raw data are saved to disk for off-line processing due to limited computational capabilities and fine tuning of the image reconstruction parameters. At present, calculations are performed on a regular computer (Intel Xeon CPU E5-2630 at 2.40 GHz, NVidia GeForce GTX Titan X) with limited optimization (mostly MATLAB code), and the typical post processing time for 1 s of acquisition is: beamforming and aberration correction, 8 s + 45 s + 8 s; filtering, 0.5 s; localization, 45 s; and bubble tracking, 1 min. The two latter operations are non-optimized MATLAB-based calculations that can be heavily accelerated. In particular, localization is heavily parallelizable as it is image independent and bubble tracking can be done in parallel on image sub-patches, which would drastically reduce the calculation time given that it is nonlinear with the number of bubbles considered. Real-time t-ULM displays (or at least partial displays) would enable the investigation of less obvious cerebrovascular pathologies—such as very small aneurysms, stenoses or defects in small vessels. These aspects are only due to the computational power available on our scanner and real-time displays will be achievable in the next few years.

It is difficult to infer the success rate of t-ULM compared with colour Doppler imaging based on our present study, as we need a larger cohort. In Europe, SonoVue is routinely used in neurosonology to image difficult patients (absence of temporal bone window), with a good success rate, but it is difficult to foresee how the precision of t-ULM will be patient-dependent. SonoVue has been approved in more than 40 countries and is widely used in Europe for various imaging applications. It is routinely used for transcranial ultrasound Doppler imaging in several European countries—such as Germany, Switzerland, Portugal and France. It has been approved in the USA under the name LUMASON for echocardiographic and liver-imaging applications both in adults and children, underlining its innocuousness. As transcranial colour-coded ultrasound is only performed in a very few specialized medical centres in the USA, SonoVue/LUMASON is not registered for this application. Nevertheless, the concept of ULM should be applicable to any other approved contrast agents (Option, Levovist and Definity).

One important limitation of our work is the limited access provided by the temporal sutures through which sound can enter the brain. This was a deliberate choice so as to use an ultrasonic probe and a hardware set-up that was as close as possible to what is already available in the clinics. The images obtained show very good delineation of the central cerebrovascular structures but we could potentially, with a slightly lower central frequency and deeper elevation focus, envision good delineation of the cortical structures from the contralateral temporal window and thus whole-brain imaging using both temporal windows. However, we could also imagine lifting the temporal access constraint by an important technological development involving much larger transcranial matrix probes working at lower frequency. By taking the skull-bone attenuation coefficient and the backscatter coefficient of SonoVue into account (Supplementary Fig. 1a), there could be a huge advantage to developing large ultrasonic helmets working at a low central frequency of 1 MHz or less. Simulating the relative receive-signal amplitude when using SonoVue microbubbles and propagating back and forth through 1 cm of skull bone, there is a typical 25 dB difference between working at 2 MHz and 1 MHz (ref. 40). There will of course be a small loss in resolution, but that might be largely compensated for by the much larger apertures, as the temporal window would no longer be a constraint. In addition, working on contrast agents with a peak of backscatter coefficient at lower frequency might be of interest.

Three-dimensional (3D) t-ULM imaging would also be of major interest for screening in particular. There is no impediment to it as proofs-of-concept of 3D ULM in flow phantoms were recently published. Three-dimensional t-ULM imaging would probably reduce the examination time, operator dependence and injected doses of microbubbles, thereby improving the workflow of the clinicians. Beyond clinical applications, the ability of t-ULM to reveal human cerebrovascular dynamics at microscopic scales will profoundly expand our fundamental understanding of the brain vascular function and dysfunction.

Methods

Patient management and safety of imaging protocols. All experiments were in strict compliance with the ethical principles for medical research involving human subjects of the World Medical Association’s Declaration of Helsinki. One healthy volunteer and three patients were recruited from the ambulatory consultation unit and the stroke unit/neurovascular ward after giving informed and written consent according to the imaging protocol approved by the ethical committee of Geneva (GUER 2017-00353). The three patients presented in Figs. 1, 2 and 4 are different individuals. By reducing the dose of injected contrast agent to the minimum enabling ULM and minimizing the amplitude and duration of the ultrasound exposures, the imaging sessions followed the ALARA principle. These were largely below the FDA recommendations (AUIM/NEMA 2004, Track 3) for ultrasound imaging, with a maximum mechanical index of 0.46 (the FDA recommended maximum value is 1.9), a maximum derated spatial peak temporal average intensity (ISPTA) of 64.3 mW cm−2 (FDA recommended maximum value is 720 mW cm−2) and a maximum thermal cranial index (TIC) of 1.99 (no recommendation, but FDA regulations ask for an explanation for values above 1). The overall aim of the study and procedures were explained to the patients before procurement of the informed and written consent. An intravenous cannula was placed in the medial cubital vein for subsequent injection of the ultrasound contrast agents (SonoVue, Bracco). SonoVue (8 µl ml−1 powder and solvent for dispersion for injection) is an ultrasonic contrast agent consisting of a suspension of tiny microbubbles (most microbubbles were between 2 and 9 µm) with a unique structure made of an inert gas, sulfur hexafluoride and a phospholipid shell that provides stability and prevents microbubble coalescence. When injected intravenously, SonoVue strongly enhances the echogenicity of blood, which results in an improved signal-to-noise ratio. It is commonly used in many European countries when the temporal acoustic window does not allow good visualization of the intracranial vessels. It is quickly eliminated from the bloodstream in the lungs (EMA/502204/2015 and EMEA/H/C/000303).

Safety measurement procedure. Ultrasonic amplitudes and durations were evaluated using certified Acoustic Measurement Tank and its software (Acerta) following the provided calibration procedures. After probe alignment in the depth direction, a 2-D scan was performed in the centre of the aperture to obtain the pressure profile. Raster scans were then performed in three depth positions: Z=0.5, 1 and 6.5 cm, corresponding to measurements in the near field at the skull location, just behind the skull and at the probe elevational focus, respectively. The regulation indices (mechanical index, ISPTA and TIC) were then measured at each depth position (including measurement of the maximum peak negative pressure in the radiated ultrasonic field for the mechanical index and ISPTA calculations, and integration of the ultrasonic energy over the ultrasonic field spatial extent at each depth for the TIC calculations) before using the maximum value over the three depths for each regulation index. The maximum mechanical index was 0.465 at 0.5 cm (0.202 at 6.5 cm; measured in water; the values will be strongly mitigated.
by the presence of the skull (typically divided by a factor 5.6 if we consider a 15 dB attenuation due to 0.5 cm of skull bone); the FDA recommendation is <1.9) and the maximum derated (−3 dB·cm−1) ISPTA was 64.3 mW cm−2 (the FDA recommendation is <720 mW cm−2). Those phases, which were stored in water, are probably much lower when imaging a patient, due to skull-bone attenuation. The thermal indices (TI) were also calculated and reached maximum values of 1.49 (soft-tissue TI), 1.9 (bone TI) and 1.99 (TIC)—well below the limit attenuation. The thermal indices (TI) were also calculated and reached maximum of four that is recommended by the FDA (values above four are possible but should be explained). The TI is defined as $T = W_p/W_{max}$ where $W_p$ is the relevant (attenuated) acoustic power at the depth of interest and $W_{max}$ is the estimated power necessary to raise the tissue equilibrium temperature by 1 °C according to a chosen specific tissue model35,36. Therefore, a TI value of two would correspond to an increase in equilibrium temperature of 2 °C, assuming a worst-case scenario. The most important index in our set-up is the TIC, which largely overestimates the temperature rise given that it is designed for ultrasound imaging and, in our case, ultrasound is on for only half the duration of the examination (1 s acquisition, 1 s pause).

Ultrasonic sequences. Ultrasonic acquisitions were performed using an ultrafast programmable scanner (Aixplorer, SuperSonic Imagine) and a phased array ultrasound probe (XP 5-1, SuperSonic Imagine; central frequency, 2.93 MHz; 90% bandwidth at −6 dB; pitch, 0.2 mm; and 96 elements). Ultrafast ultrasound imaging sequences consisted of the emission of diverging waves coming from four virtual sources that were regularly spaced (every 5.2 mm) and placed 11.44 mm behind the transducer (giving an 80° angular aperture of view). The central frequency was based on an optimization of three parameters (the evolution versus frequency of which is given in Supplementary Fig. 1a): available transducer bandwidth (given by the transducer constructor), skull-attenuation coefficient $\alpha_{skull}$ (adapted from Bamber et al.37 and SonoVue backscatter coefficient γ (adapted from Schneider and colleagues38). Supplementary Fig. 1b shows in black the curve combining the available bandwidth (squared as the temporal filter is applied through the transducer at emission and at reception), $\alpha_{skull}$ is calculated for a total distance of skull propagation of 1 cm (0.5 cm thickness back and forth; this length does not influence the position of the peak), the backscatter coefficient γ is taken as a square root as it comes from a power measurement and we are considering the amplitude of the received signal). Supplementary Fig. 1b shows in red the spectrum of the 2 MHz two cycles-square electric pulse sent from the ultrasound scanner to the ultrasound transducer: it fits nicely above the black curve; we chose a slightly higher frequency to maximize the resolution as much as possible and it is a squared signal as this is the kind of signal that ultrasound pulsers are able to emit. Therefore, ultrasonic pulses consisted of two cycles centred at 2 MHz, fired at the maximum pulse repetition frequency allowed by the desired imaging depth (typically, 4,900 Hz) by groups of four pulses (for the four sources), repeated at a ‘frame rate’ of 800 Hz (frame rate refers to the frequency at which compound frames made of the four different virtual sources of sonification are outputted) during 1 s. For each pulse, backscattered echoes were recorded by the transducer array, digitalized at 200% bandwidth (that is, four samples per wavelength) and stored in a so-called radiofrequency (RF) data matrix. These RF data matrices are the starting point of aberration correction and image reconstruction. This operation was repeated every 2 s, which is the fastest sequence rate achievable by our system at this point that enables saving on the enormous amount of data to store (second-order processing). Therefore, all RF data were acquired over 1 s and both pulsing diverging waves at 4,900 Hz and acquiring data, the ultrasound is then off for 1 s and saving the RF data that were just acquired to disk, and this process loops during a desired duration (45 s for the patient in Fig. 1, 2 min 15 s for the patient in Figs. 2 and 3, and 24 s for the patients in Fig. 4).

Aberration correction and beamforming procedures. The correction of skull-bone aberrations induced during transcranial propagation can be done in a two-step approach as already discussed ex vivo23–25. First, we analyse the wave front reflected by individual microbubbles acting as point-like sources in the medium. This reflected wave front corresponds to the exact Green’s function related to the sound velocity to each element of the ultrasonic array and can be used to measure the skull aberration. Second, the time-reversed version of this aberration law (corresponding to the temporal delays induced by the skull bone during the backward propagation) is introduced as a time-shift correction on each element of the array before reprocesing the beamforming of the raw ultrasonic data and produce corrected ultrasonic images. To remove tissue signal and isolate wave fronts coming from individual microbubbles, SVD clutter filtering was performed on the RF data matrices. Uncorrected images were reconstructed from these filtered RF matrices using delay-and-sum beamforming on a polar $(r, \theta)$ grid with classical spherical delays, and bubble positions corresponding to the isolated wave fronts were stored, along with the forward and return spherical delay laws associated with these positions. For each considered bubble, the forward delay law was used to virtually focus the filtered RF signals from the four diverging wave emissions on the bubble location and the return delay law was then used to flatten the isolated wave front. The phase-aberration profile was calculated via cross-correlation, as the remaining relative delays appeared in the wave front across the transducer elements. Measurement of the spatial coherence of the wave front was used as a metric for convergence, and only the bubble positions that encountered an increase of this coherence through this iterative process were kept until the end.

Phase-aberration laws were compared over the whole image to determine isoplanatic regions where within the aberration could be considered constant (variations across bubbles <1/8 with T being the period of the ultrasonic signal). Individual bubble aberration laws were averaged in each of these regions and corrected images were reconstructed from raw RF data using regionally corrected delay laws in a delay-and-sum beamforming on a polar $(r, \theta)$ grid.

Motion detection and correction. Tissue motion within a 1-s stack of images was estimated using cross-correlation techniques already described for ultrasonic tissue displacement or strain estimations26. Briefly, estimation of the inter-frame axial displacement was obtained by multiplying the in-phase-quadrature content of one pixel of the cross-correlation of the ultrasound images with the same pixel in the next frame. This was done for all pixels; a 3 × 3 pixel averaging filter was used before estimating the phase of the complex in each pixel, being proportional to the inter-frame displacement. This displacement was also used to detect the systole time point, as the tissue motion will be larger and exhibit a distinct peak during the systole. Therefore, the spatial position of the maximum was accompanied by a time position relative to the systole position, enabling discrimination between systole and diastole. This was used for further intra-cardiac cycle speed analysis. Motion between 1-s blocks was estimated as a twofold process: first, by calculating the affine transformation matrix between the corresponding Power Doppler images and, second, by computing the fine transformation matrix on dense images after localization microscopy processing of those images that were already pre-registered. The effect of the motion correction can be appreciated on Supplementary Fig. 3.

Processing of the localization microscopy data. Images beamformed in phase and quadrature data were filtered using the spatio–temporal SVD filter described by Demené et al.21 (between 35 and 60 singular values were removed, depending on the level of tissue motion) to remove tissue signals and keep only the signals from the moving scatterers (the microbubbles). The magnitude of the filtered in-phase and quadrature signal was kept and formed a stack of images depicting the individual temporal motion of bubbles. A binary mask was built based on the vascularity filtering28 of this stack of images. The vascularity filter belongs to a particular family of image processing filters optimized to improve the contrast of vascular structures in 2D or 3D that are particularly suited for improvement of vascular imaging of the retina (2D) or improvement of 3D CT angiography, for example. They rely on the Eigen decomposition of the Hessian matrix. The Hessian matrix is defined as the convolution between the image intensity (can be 3D) and the second derivative of a Gaussian kernel—therefore, it approximates a second-order derivative at a certain scale22. Taking into account the relative sign and magnitude of the Eigen values of this Hessian matrix within an ‘enhancement function’ enables the identification of certain types of structures (namely plate-like, tubular and blob-like). The vascularity filter is based on a certain formulation of this enhancement function, with several formulations having been proposed in the past—such as, Frangi, Sato, Li and so on2. This type of filter is efficient in our case as bubbles moving in time will appear in a 3D matrix (space × time) as tubular (vessel-like) structures and will therefore be enhanced using a vascularity filter. Both the image and mask stacks were interpolated (Fourier-space-based interpolation for the image stack and nearest-neighbour interpolation for the binary mask) down to a radial resolution $d_R = 6/6$ and an angular resolution of $d_\theta = 0.5^\circ$. Local-maxima detection was then performed within the masked area for each 2D image of the image stack. These local maxima are locally correlated with a typical point-spread function (imaging response of an isolated microbubble) modelled as a Gaussian spot of axial dimension $\lambda$ and lateral (angular) dimension of arctan($\lambda$)/$d_R$ the transducer aperture, and local maxima with a weak correlation (<0.6) were discarded (local spatial speckle fluctuation can generate local maxima but are not like a point-spread function). Sub-pixel maxima localization was then performed using a fast local (5 × 5 pixel neighbourhood) second-order polynomial of the maximum of the array of maxima returned from polar to Cartesian coordinates. Tracking of the positions of the maxima was performed using a classical particle-tracking algorithm26 with no gap filling and maximal distance linking of 1 mm (corresponding to a bubble maximum speed of 80 cm s−1). Bubble tracks shorter than the detected positions were removed, which was based on the idea that microbubbles should be observed in several consecutive frames, enabling us to drastically reduce the level of false bubble detection. The successive positions gathered in one track were used to compute the inter-frame bubble velocity vector components $V_x$ and $V_y$, as well as the velocity magnitude $V$. The successive positions were also used to compute a curvilinear abscissa along their trajectory to interpolate both positions and speeds at a smaller space step (0.01 mm).

Density and velocity display. Compared with ULM maps obtained by others in animals in very controlled conditions and non-transcranially24–26, the data obtained in our paper were acquired in a very short time (between 24 s and 2 min 15 s) to minimize discomfort for the patient and in challenging conditions.
Flow quantification and functional resolution estimation. For quantification on cross-axis speed profiles, the speeds of individual bubbles were binned along a transverse section of the vessel (bubbles inside a 1-mm longitudinal extent) before statistical testing (analysis of variance with post-hoc t-test with Bonferroni’s correction). The bins were 188, 62.5 (Fig. 3) and 25 mm (Supplementary Fig. 4) wide. Bubbles with trajectories within 1 mm on either side of the cross-section were retained in order to be recorded in systole when they were within 0.5 mm of the vessel wall. Bubbles with trajectories within 1 mm on either side of the cross-section were recorded in systole when they were within 0.5 mm of the vessel wall.

Velocity vector fields were obtained on a regular grid via bilinear interpolation of the irregularly distributed vector speeds of the microbubbles. These vector fields were also spatially filtered using a Gaussian kernel (σ = 62 mm). This enabled us to display the velocity components of Fig. 3b and subsequent vector field images.

Animated flow rendering. Computed-generated imagery animation (Supplementary Video 1) was created using the software Houdini 17.5.360 (SideFX) to help visualize the functional information extracted from the bubble positions and tracking information. The density map and velocity vector components \( V_x \), \( V_y \), \( V_z \) were imported into Houdini. A particle distribution was initialized with a particle density proportional to the local bubble density. Next, particle position, size, colour and light duration were updated for each frame based on the velocity vector field and local density. Depending on the field of view of the highlighted area, particles were constantly emitted at a rate of 1 × 10^6–1 × 10^7 particles per second, with a lifetime of 3 s. Particle size was made proportional to the measured local microbubble density for better visualization, meaning that a particle passing by a region with high local density seems bigger than a particle moving through a less dense area. The colours of the particles were based on either the density colour map (black-to-red-to-yellow colour map) or velocity colour map (blue-to-green-to-yellow-to-red map). Finally, animation was rendered using the ‘3D render’ Mantra library of the Houdini software after setting up an additional dome-light effect.

Clinical imaging. The clinical image in Fig. 2a–c is a mean intensity projection reconstruction from a cerebral multi-detector computed tomography cerebral angiogram with contrast injection. The clinical images in Fig. 4b and Supplementary Fig. 6 (top) are from cerebral multi-detector computed tomography with contrast injection illustrating the function of the right and left MCA and local density. The image of the right and left MCA and local density. The dominant right A1 segment and fetal origin of left PCA served as normal variants.

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

Data availability

Data examples are available from the repository with the identifier https://zenodo.org/record/4048550#X6GPVBGCJPY. All raw and analysed data used in this study are available on request.

Code availability

Data samples and MATLAB codes regarding the most important steps of the processing routine are available from the Zenodo repository at https://zenodo.org/record/4048550#X6GPVBGCJPY. Codes for the loading and management of data are available on request.

Received: 20 May 2020; Accepted: 5 February 2021; Published online: 15 March 2021

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**Acknowledgements**

This work was supported by a research grant from the European Research Council (ERC) under the European Union’s Seventh Framework Program (FP7/2007–2013)/ERC Advanced grant agreement no. 339244-FUSIMAGINE (M.T.), by the Swiss National Foundation (Fond National Suisse) grant no. CR3213_150654 (FP) and the Fondation Bettencourt–Schueller. We thank L. Puke for her help with patient recruitment. We thank the NVIDIA Corporation for their support through the NVIDIA GPU Grant program and the donation of a Titan Xp GPU used for this research.

**Author contributions**

C.D., F.P. and M.T. conceived the study. C.D., J.R. and M.P. developed the sequence acquisition and beamforming software. C.D., J.R. and F.P. acquired data. C.D., J.R., B.H., A.D., M.P. and M.T. developed data-processing algorithms. A.D. and C.D. developed the visualization algorithms. C.D., F.P. and M.T. interpreted the results. C.D. and M.T. wrote the first draft of the manuscript with substantial contribution from F.P. M.T. and F.P. co-directed the work. All authors edited and approved the final version of the manuscript.

**Competing interests**

M.P. and M.T. are co-founders and shareholders of the Iconeus company commercializing ultrasound neuroimaging scanners. M.T. is co-inventor of the patent WO201208614A1 on the ultrasound localization microscopy method filed on 16 December 2010 and licenced to the Iconeus company. All other authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at [https://doi.org/10.1038/s41551-021-00697-x](https://doi.org/10.1038/s41551-021-00697-x).

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**Peer review information** *Nature Biomedical Engineering* thanks James Greenleaf and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Software and code

Policy information about availability of computer code

Data collection

Custom codes were used to collect the data. These codes will be made available to readers upon request.

Data analysis

For data analysis, a custom code was used for the filtering of tissue signals (published in Demene, C. et al. Spatiotemporal clutter filtering of ultrafast ultrasound data highly increases Doppler and ultrasound sensitivity. IEEE Trans. Med. Imaging  PP, 1–1 (2015)) available upon request. A custom code was developed for aberration corrections, motion correction and localization of the microbubbles.

A binary mask was built based on the vesselsness filtering of this stack of images. The implementation of vesselsness filtering is available on Mathworks file exchange, ©Dirk-Jan Kroon 2009, and © Tim Jerman, 2017.

Tracking of the maxima positions was performed using a classical particle tracking algorithm (simpletracker.m available on mathworks ©Jean-Yves Tinevez, 2019, wrapping matlab munkres algorithm implementation of ©Yi Cao 2009).

For statistical analysis, we used the commercial software MATLAB R2018a (MathWorks, Cambridge, MA, USA).

Superresolution images and movies were obtained using a 3D software for visualization (Houdini 17.5.360, SideFX, Toronto, Canada)

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Software codes for the loading of data and for the management of data will be available. Step-by-step instructions are available in a Readme.pdf document to guide the user.
Data samples and codes (matlab) regarding the most important steps of the processing routine are publicly available without restrictions at https://zenodo.org/record/4048850#.X6GPf9RcdPy. In this data and code package, examples are provided for:

- Beamforming (image formation): we supply raw RF data and the Matlab based beamforming routine.
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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 transcerebral Superresolution ultrasound was successful in all N=4 patients. Case examples of vascular pathologies (N=2) were presented and compared with CT Angio and MR Angio data |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data exclusion |
| Replication | For these proof of concept experiments, all attempts of replication of the experiment were successful. Beamforming processing was validated in ultrasonic phantoms before clinical experiments. The implemented ultrasonic sequences and all these processing steps were fully automated and the software containing these features 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 ultrasound to map dynamically the brain vascular flow up to microscopic resolution. For each patient, the experiment was successful. |
| Blinding | Blinding was not relevant for our study as it consists of a technical proof of concept study for a new clinical superresolution angiography technology. Acquisitions were performed by the authors blindly and postprocessing of data was revealing the brain vascular maps at microscopic levels and compared to other modalities. |

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Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging|

Human research participants

Policy information about studies involving human research participants

Population characteristics

Healthy volunteers and patients were recruited from the ambulatory consultation unit and from the stroke unit/neurovascular ward after giving informed and written consent according to the imaging protocol approved by the ethical committee of Geneva (CCER 2017-00353).

Recruitment

Healthy volunteers and patients were recruited from the ambulatory consultation unit and from the stroke unit/neurovascular ward after giving informed and written consent according to the imaging protocol approved by the ethical committee of Geneva (CCER 2017-00353).

Patient 1: female, 27 years old, healthy volunteer (Figure 1)
Patient 2: female, 25 years old, no cerebrovascular pathology targeted (Figure 2 and 3)
Patient 3: male, 79 years old, aneurysm on the distal part of the right MCA (Figure 4 a-f)
Patient 4: male, 63 years old, chronic cerebral hypoperfusion, right posterior cerebral artery of foetal origin and an early occlusion of the right MCA that led to the development of a complex local network of collateral arteries (Figure 4 g-j)

Ethics oversight

The imaging protocol was approved by the ethical committee of Geneva (CCER 2017-00353).

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