nature biomedical engineering

PEER-REVIEW INFORMATION

Transcranial ultrafast ultrasound localization microscopy of brain vasculature in patients

Corresponding author: Mickael Tanter

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Thu 09/06/2020
Decision on Article nBME-20-1300-T

Dear Prof Tanter,

Thank you again for submitting to *Nature Biomedical Engineering* your Article, "Deep Transcranial Adaptive Ultrasound Localization Microscopy of the Human Brain Vascularization". The manuscript has been seen by three experts, whose reports you will find at the end of this message. You will see that the reviewers have good words for the work, and that they raise a number of technical criticisms that we hope you will be able to address. In particular, we would expect that a revised version of the manuscript provides:

* detailed methodological section, clarifying the degree of technical advances of the work.

* discussion on the limitations of the work, in particular in regards to its feasibility in the clinic.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 25 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

Rosy

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Dr Rosy Favicchio
Senior Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

The authors have achieved microscopic brain angiography by combining ultrafast ultrasound localization microscopy (ULM) of intravenously injected microbubbles both with adaptive corrections of ultrasonic wave aberrations induced during transcranial propagation and micrometric brain motion corrections using speckle tracking. Performance of this highly innovative achievement is illustrated by clinical examples in human brain.

This is a world class advancement in the field of brain imaging. Not only is it done with non-ionizing and available technology, but with better spatial and temporal resolution than conventional and much more expensive methods.
This new technology will have wide application throughout the world. Further development will provide clinicians and scientists great insight into the vascular workings of the brain. Functional studies of blood flow within the microvascular system of the brain will be greatly aided with this technology.

Apparently, because this report includes the use of some proprietary software and methods, there is no supplementary material describing the algorithms or mathematics used to analyze the data. The textual descriptions of the technical aspects of this outstanding achievement are terse, though mostly clear.

There probably should be a discussion about the relevance of using SonoVue in the US.
The word data should be treated as plural.
Materials and Methods(M and M) is defined on page 5, but used earlier in the manuscript. It should be moved earlier in the text.
Page 5; It is not clear what “two more degrees of ramification observable” means.
Page 5; “change or direction” should be “change of direction”
Page 5; not sure what is meant by “to λ/12 or resolution/24 at this depth”
Page 7; ACPD should be defined or have a reference.
Page 7: (Moya Moya-like disease) should have a reference.
Fig 4; Frame j is not labeled.
Page 8: “easiness of use” should probably be “ease of use”
Page 8; it is mentioned that ultrasound has real-time capabilities, but the times for computation of the various images in this report are not mentioned. This statement needs to be modified relative to those considerations.
Page 9; it would be important for the authors to describe the state of approval for SonoVue in the US for brain imaging.
Page 9; What length of time was used for calculation of the TI in the skull? 1 minute 15 seconds is a long exposure when not moving the probe.
Page 9; it is not clear what the sequence of data acquisition is. It is repeated every 2 seconds but how does

that relate to the 45 second acquisition, for example?
Page 12; the sentence beginning with “Finally, animation was” has many jargon words that need to be defined.

Reviewer #2 (Report for the authors (Required)):

The paper by Demene et al presented the ULM technique as applied transcranially in certain human pathologies. This is achieved at variable resolutions using contrast agents injected intravenously. The paper is well presented and several complex anatomies are presented as a list of different cases. ULM is a technique that was presented and published before with impressive results as shown here. The same group has shown brain flow in humans without contrast. Therefore, it is not clear what is the novelty of this paper, except to show ULM’s application in a few complex human anatomy cases, i.e., the authors need to clearly specify the novelty of the methodology presented and what is the problem that was solved and how was it solved. The geometry and frequency of the transducer were presented but it is not clear of why that specific geometry and frequency were selected and how the technique was optimized. Similarly, the bolus of the contrast agent seems to also be arbitrarily chosen
without a clear justification. The diverging wave bean sequence was indicated as a previous development in cardiac imaging but the depth and the transcranial application clearly make the brain a different target than the heart. The confirmation with CT is a strong aspect of the paper but it is only done in large vessels while in smaller vessels there is no validation. It is also difficult in most figures to discern the similarity of the anatomy between ULM and CT/MRI.

1. Abstract- ‘Ultrasound’ is too general of a word and it is not clear why a capital letter is used here. Ultrasound imaging typically does not use contrast as ULM does. Therefore, contrast-enhanced ultrasound (CEUS) may need to be specified. Also, the bolus injection is larger than the approved dose for the heart and microbubbles are not necessarily approved for clinical brain imaging, even at lower doses. So, it is important not to give a false impression of conventional ultrasound providing such imaging.
2. Abstract- there are no quantitative or novel findings specified and therefore reads like a review paper on brain ULM.
3. Abstract- the flow vortex in an aneurysm is not ‘unprecedented’ That’s known
4. P. 2, para 2 - Replace ‘broke’ with ‘solved’
5. P. 2, para 2 – The same group has published on functional ultrasound (fUS) in the brain of humans for imaging flow without microbubbles. How does human ULM compare to fUS in humans? Since the group has experience in both modalities, a fair comparison could be made. There is obviously a very big advantage in achieving this without contrast.
6. P. 5, para 1- Replace ‘Microbubbles’ with ‘Microbubble’
7. Fig. 3: The curves in (e) and (f) are very difficult to interpret. What do these bins indicate? Why are they different in systole vs. diastole?
8. Fig. 1C: It appears that the skull bone is hand drawn. The aberration correction needs to be clearly shown with before and after maps for a specific known skull anatomy.
9. P.12, para 1: please provide details on the particle animation solver.

Reviewer #3 (Report for the authors (Required)):

This is a manuscript that demonstrates dramatic improvements in transcranial ultrasound imaging and analysis using a combination of methods, several of which were pioneered by this group. These include ultrafast ultrasound imaging, on the orders of hundreds to thousands of imaging frames per second, ultrahigh resolution vessel detection using ultrasound gas bubble contrast agents injected such that low vascular concentrations permit imaging solitary bubbles allowing subwave length resolutions, on the order of 10s of micron diameters, singular value decomposition motion and clutter cancellation, phase aberration correction through the skull, and motion correction. The results are quite extraordinary depicting very small vascular structures and dynamics of flow not visible on standard brain imaging modalities such as CT or MRI. Although the all of the methods employed are not completely new, this group has demonstrated several of these methods before, the union of these methods
is new and has produced images of exceptional quality and detail. The consequences of such methods could be huge, with better definition of blood vessels and evaluations of flow dynamics, potential for such methods are almost hard to predict. Functional imaging with MRI has already had major impacts in clinical diagnosis and research. The methods described here could be used in conjunction with MRI or stand alone with better definition of local vascular anatomy and flow. It is very possible that this improve could lead to new levels of

functional imaging and diagnosis of brain diseases.
Major technical questions:
1) The biggest problem with this method is the limited access. Although the investigators do an excellent job of correcting for skull aberration of the ultrasound signals, this method still uses the temporal sutures as sites through which sound can enter the brain. These are the typical sites used in standard transcranial Doppler studies. Because of this, the entire brain cannot be imaged. Thus the method has a major limitation relative to MRI and CT. Do the investigators have any ideas of how to overcome this major problem? Given this problem, it is highly likely that the ultrasound methods described herein will not be used for primary diagnosis or evaluations, and will only be used in conjunction with CT and/or MRI after an initial evaluation detects regions of interest that can be assess using ultrasound.
2) Transcranial Doppler studies have failure rates of about 15%. These are studies using focused ultrasound beams produced by small aperture ultrasound probes to detect spectral Doppler signals from basal arteries. These probes need very small access windows relative to the imaging these investigators will perform using compounding of multiple diverging beams. Besides loosing energy on passage through the suture, the probe's aperture could be severely limited preventing compounding. I would guess that the number of failed examinations will be much higher than the 15% figure. I know the impressive results demonstrated here are preliminary, but can the investigators predict how many of their studies will in fact fail due to the skull transmission? The investigators acknowledge this problem, but if you can't get sound into the brain, deaberrating reflected signals will not be of much help.

3) Superresolution requires that intravascular scatterers be single contrast agent bubbles. Further, their aberration correction methods also depend on reflections from single bubbles. The investigators inject small boluses of contrast to insure that their signals are coming from individual bubbles. How do they guarantee that they are looking at individual bubbles when they generate their images? What do they do to compensate for decreased resolution if they think their echoes are being produced by groups of bubbles?

4) Multiple injections of small boluses of contrast agent are required for this method. Although definitely possible, this will require new modifications to injection protocols.

5) Please describe the vesselness filter in more detail.
Minor technical criticisms:
1) i and j are not visible in frame b of figure 3. I and j should be e and f.
2) Being able to see a flow vortex in a small MCA aneurysm is a beautiful demonstration of the power of the method. However, the clinical value of this information is not clear at this time. Predicting a potential thrombus in an MCA aneurysm based on the presence of a vortex is a bit of a stretch.
3) "j" label is missing in Figure 4.
4) At the end of paragraph 2, page 2, "…is traditionally handheld performed" should be "…is traditionally handheld and performed"
5) aneurism should be aneurysm on page before references.

Tue 08/12/2020
Decision on Article NBME-20-1300A

Dear Dr. Tanter,

Thank you for your revised manuscript, "Deep Transcranial Adaptive Ultrasound Localization Microscopy of the Human Brain Vascularization". Having consulted with the original reviewers (whose comments you will find at the end of this message), I am pleased to say that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

Also, please address the remaining minor points from the reviewers.

In the meantime, we will assess the main text in more detail, in particular the title and abstract, for clarity, accessibility and readability. Please expect an update to this message with additional points to address. However, you don't need to wait for the update to act on the instructions in the attached document and to submit the final files.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly
agree to release their name, we will include the names in the peer-review supplementary file. More information on transparent peer review is available.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Rosy

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Dr Rosy Favicchio
Senior Editor, *Nature Biomedical Engineering*

P.S. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol-sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

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Reviewer #1 (Report for the authors (Required)):

Summary of Improvements
The authors have more clearly described how the use of microbubbles allows super-resolution imaging and phase aberration correction through the skull.
They have improved the description of probe and frequency choices for the study.
A more complete description of the international vagaries of microbubble use is included.
Although the description of the use of hemodynamics for predicting embolus incidence is farfetched, it is not inappropriate.

A more complete description of the computational cost of the method is now included.
The addition of a section describing considerations of decreased center frequency and increased aperture and their probable impact on this method being able to image with higher SNR the entire brain will provide the ultrasound community with great incentive to pursue these methods.
The addition of a description of the vesselness filter was especially interesting to this reviewer.
The inclusion of data and codes will provide an important value add to this significant contribution to the ultrasound literature.
Any remaining major technical criticisms or questions.
none
Any remaining minor technical criticisms or questions.
none
Any missing or unclear details about statistics, protocols or materials (please check the reporting summary provided, and note that the form is a dynamic PDF file that when not flattened can only be properly viewed via Acrobat Reader).
none
Any missing citations to relevant literature.
none
Any optional suggestions for improvement.
See some specific edits listed below
Any stylistic issues or recommendations.
Very little grammatical editing is needed.

Line 69 objects should be object
Line 329 Come should be came
Line 328 do should be does.
Line 602 The authors should state that they “thank…” not that they “want to thank…”

Reviewer #2 (Report for the authors (Required)):

The authors revised the manuscript substantially to address most of the concerns raised. The rigor is enhanced and several of the methodologies have been clarified. There are two points that remain:

1) Since novelty remains unclear in the introduction, the following text in the responses to reviews regarding novelty should be incorporated in the introduction of the revised manuscript:
"Our group showed brain flow imaging in humans without contrast (Demené et al., 2014; Demene et al., 2017; Imbault et al.,
2017), the so called ultrafast Doppler Ultrasound, but we never did that both transcranially and with superresolution: it was either in neonates through the fontanel or during brain surgery (a result reproduced by (Soloukey et al., 2020)) using an opened skull flap. Moreover, imaging without contrast agent does not allow so far to beat the diffraction limit (and would result in the case of our paper to images with a resolution of the order of 1 mm to a few mm, 2 orders of magnitude above
what is shown). Here, what we did is to show that not only ULM is doable in human adults transcranially, but also that at this low ultrasound frequency, with an expected low SNR and in the presence of motion we could achieve resolutions of the order of 25 µm far beyond the typical 1 mm resolution of fUS imaging, a resolution never reached to our best knowledge by any other modality."

2) There are several typos and colloquialisms throughout the paper, these should be fixed prior to final submission. An example is shown below:
'We choses a bolus dose that: -gave enough dilution for the microbubbles for individual identification, and enough concentration for an acquisition in a short time compared to what is described in the ULM literature'
Please replace 'choses' with 'chose' and 'gave enough' with 'provided sufficient'

Reviewer #3 (Report for the authors (Required)):

Deep Transcranial Adaptive Ultrasound
Localization Microscopy of the Human Brain
Vascularization

This is a revision of the initial submission of this manuscript. The work remains an impressive presentation of high resolution imaging of the brain. The work will have major impact on brain imaging. The breadth of the innovation in this work is very high, and the authors have done an impressive job in developing this work. They have also very adequately responded to the reviewer critiques of the initial submission. I have no major issues with this revision. There are some grammatical, structural, and spelling errors that need to be corrected in order to make the work easier to read.

Abstract:
There are several words that are capitalized incorrectly. These include Computed Tomography Angiography (line 19), Magnetic Resonance Angiography (line 19), Ultrafast (line 27), and Ultrasound (line 34).

Line 65 – Please spell out PALM and STORM.
Line 90 – transducer should be transducer's
Line 92 – transducer should be transducers
Figure 1 caption. SVD should be spelled out as singular value decomposition when the abbreviation is first encountered in the text.
Figure 3 caption: There are no i and j sections in this figure. Are these referring to figure 4? I would suggest changing dissymmetric to asymmetric.
Line 215: Color should be lower case, color.
Line 217: calculation should be calculations
Line 221: calculation should be calculations
Line 224: display should be displays
Line 225: display should be displays
Line 226: stenosis should be stenoses
Line 226: "This aspect is" should be "These aspects are"
Line 227: display should be displays
Line 326: pass should be passes
Line 328: do should be does
Line 329: come should be comes
Line 343: The statement "to summarise" is hard to understand. Perhaps it should be written as: To summarise: for 1s, the ultrasound is on and both pulsing diverging waves at 4900Hz and acquiring data, for 1s, the ultrasound is off and saving the RF data that were just acquired to disk …….
Line 367: trough should be through
Line 395: belong should be belongs
Line 447: "for the diastole" should be "for diastole"
Line 455: "for the diastole" should be "for diastole"
Line 473: cerebral angiography should be cerebral angiogram
Line 478: "MR angiography of the circle of willis" should be "MR angiogram of the Circle of Willis"

Finally,
The statement in the review of the initial submission that approximately 15% of transcranial Doppler ultrasound scans of the brain fail is supported for example by the following reference:
Lee C-H, Jeon S-H, Wang S-J, Shin B-S, Kang HG. Factors associated with temporal window failure in transcranial Doppler sonography. Neurological Sciences 2020; https://doi.org/10.1007/s10072-020-04459-6

Rebuttal 1

Dear Editor,

We thank you for giving us the opportunity to answer the comments of the reviewers. We followed your two main advices:

- First, we added comments in the methodological section and better clarified the degree of technical advances of the work. In addition to the extended methodological explanations, we added data sample and code sample (matlab) regarding the most important steps of the processing routine: Beamforming corresponding to image formation, Aberration correction demo code detailing the important steps of aberration correction, data Filtering with the SVD filtering and display codes for visualization of the microbubbles, super-localisation data with display code. We believe now that every aspect of the technique is covered and explained with a much wider level of details.
- Second, we discussed into further details the limitations of the work in regards to its clinical applicability.

We also added more data and information about the choice of the ultrasound transducer and of the ultrasound frequency range used in the study (suppl fig 1).

We would also like to thank the reviewers for their thoughtful comments that helped us to further improve the manuscript quality.

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Reviewer #1 (Report for the authors (Required)):

The authors have achieved microscopic brain angiography by combining ultrafast ultrasound localization microscopy (ULM) of intravenously injected microbubbles both with adaptive corrections of ultrasonic wave aberrations induced during transcranial propagation and micrometric brain motion corrections using speckle tracking. Performance of this highly innovative achievement is illustrated by clinical examples in human brain.

This is a world class advancement in the field of brain imaging. Not only is it done with non-ionizing and available technology, but with better spatial and temporal resolution than conventional and much more expensive methods. This new technology will have wide application throughout the world. Further development will provide clinicians and scientists great insight into the vascular workings of the brain. Functional studies of blood flow within the microvascular system of the brain will be greatly aided with this technology.

We want to warmly thank the reviewer for his/her kind comment and enthusiasm for this work. This is very encouraging for us.

Apparently, because this report includes the use of some proprietary software and methods, there is no supplementary material describing the algorithms or mathematics used to analyze the data. The textual descriptions of the technical aspects of this outstanding achievement are terse, though mostly clear.

We agree that algorithms and methods were described succinctly, we mentioned every aspect of it while trying to keep short the length of the method section. To assess the concern of the reviewer and of the future reader, we improved strongly the methodological section by adding several codes and data samples covering all the steps of the imaging process:
First, we added data sample and code sample (matlab) regarding the most important steps of the processing routine, per se:

- Beamforming (image formation): we supply raw RF data and the matlab based beamforming routine.
- Aberration correction: we supply a demo code detailing the important steps of aberration correction. Output is an aberration correction profile for different patches of the image that can be fed into the previous bullet point.
- Filtering: we supply the beamformed data (output of the 2 previous bullet points) and the SVD filtering and display code for visualization of the microbubbles.
- We provide super-resolution data based on the data of the previous bullet point, along with display code along with the raw data.

Second, we added supplementary details in the method section. We believe now that every aspect of the technique is covered and explained with a much wider level of details.

There probably should be a discussion about the relevance of using SonoVue in the US.

This is an interesting comment. Sonovue® has been approved in more than 40 countries, is widely used in Europe, and has been approved in the US under the name LUMASON for echocardiographic and liver imaging applications both in adults and children, underlining its innocuousness. Although we performed our clinical translation with Sonovue®, the concept of Ultrasound Localization Microscopy can be applied with any other approved contrast agent (Optison, Levovist, Definity) which could be substituted to Sonovue®, as their acoustical properties are very similar. We added a comment in the discussion about the possibility to substitute other contrast agents to Sonovue®.

"Ultrasound contrast agent innocuousness achieves a consensus among radiologists. Sonovue® has been approved in more than 40 countries, is widely used in Europe for various imaging applications. It is routinely used for transcranial ultrasound Doppler imaging in a lot of European countries such as Germany, Switzerland, Portugal or France. It has been approved in the US under the name LUMASON for echocardiographic and liver imaging applications both in adults and children, underlining its innocuousness. Nevertheless, the concept of ULM should be applicable to any other approved contrast agents (Optison, Levovist, Definity)."

The word data should be treated as plural.

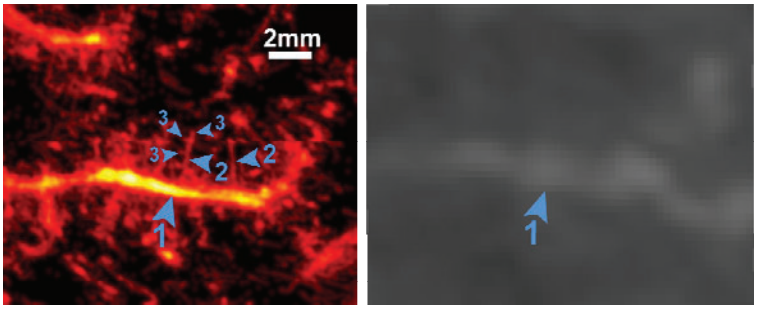
This has been checked and corrected.

Materials and Methods (M and M) is defined on page 5, but used earlier in the manuscript. It should be moved earlier in the text.

Methods section has to be placed after the discussion according to the Nature BME guidelines. Therefore we remove the reference to M&M in the main text and added relevant information.

Page 5; It is not clear what "two more degrees of ramification observable" means.

This was indeed unclear. We propose to change the text as "with at least two more orders of vessel branching delineated » and to add a supporting supplemental figure as such:



On this figure, we can see that vessel 1 is visible both on t-ULM and CT, but that branching order 2 and 3 can only be seen on t-ULM.

Page 5; "change or direction" should be "change of direction"
Agreed.

Page 5; not sure what is meant by "to $\lambda/12$ or resolution/24 at this depth"
Resolution in ultrasound imaging is depth-dependent, and in a different way for the axial and the lateral resolution. This is illustrated by supplemental figure 1a, where we see that at a 20 mm depth resolution is (0.75 mm axial, 1.02 mm lateral), at a 58 mm depth resolution is (0.83 mm axial, 2.68 mm lateral) and at a 88 mm depth resolution is (0.89 mm axial, 4.17 mm lateral). Loss of axial resolution with depth is low, and can be attributed to higher attenuation of the high frequency content, resulting is a slightly longer ultrasound pulse at depth. Lateral resolution is given by the aperture (the f-number in optics) of the ultrasound probe, and is commonly accepted as being given by $\lambda F/d$, where λ is the wavelength, F the focal distance (i.e. the depth), and d the width of the probe (the aperture).
We changed the formulation around line 96 to improve the understanding of the depth dependency of the resolution.
We changed the sentence at line 176 to "corresponding to $\lambda/12$ (equivalent to conventional axial resolution/12 and conventional lateral resolution /24 at this 38 mm depth, with a numerical aperture of 2, see suppl. Fig. 1). »
We completed the legend of supplemental figure 1 with the above information for the reader's sake.

Page 7; ACPD should be defined or have a reference.
Sorry for this acronym. This was a mistake (French acronym), and the sentence has been replaced with "In a 63-year-old patient (Supplemental Figure 6) presenting with chronic cerebral hypoperfusion, 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. »

Page 7: (Moya Moya-like disease) should have a reference.
Reference has been added. Scott, R.M., Smith, E.R., 2009. Moyamoya Disease and Moyamoya Syndrome. New England Journal of Medicine 360, 1226–1237.

Fig 4; Frame j is not labeled.
Yes. There was actually a display problem with this figure, with a Word® clipping box that partially cropped the image, making the letter j disappear. This is now corrected.

Page 8: "easiness of use" should probably be "ease of use"
This has been corrected.

Page 8; it is mentioned that ultrasound has real-time capabilities, but the times for computation of the various images in this report are not mentioned. This statement needs to be modified relative to those considerations.
The statement has been rephrased into "Its low cost, ease of use, sensitivity and quantification capabilities joined with almost two orders of magnitude improvement in terms of resolution ».
Real time capabilities are discussed in details further in the text, balanced with the current processing time. In particular we added near line 215:
"With our current ultrasound machine, raw data are saved to disk for off-line processing due to limited computation capabilities and fine tuning of image reconstruction parameters. Currently, calculation are performed on a regular computer (Intel® Xeon® CPU E5-2630 @2.40 GHz, NVidia GeForce GTX Titan X) without optimization (mostly Matlab® code), and typical post processing time for 1s of acquisition is: beamforming + aberration correction (8 s + 45 s + 8 s), filtering (0.5 s),

localization (45 s), bubble tracking (1 min). The two last operations are poorly optimized matlab based calculation that can be heavily accelerated. In particular localization is heavily parallelizable as localization is image independent, and bubble tracking can be done in parallel on image sub-patches, which would drastically reduce the calculation time as it is non-linear with the number of bubbles considered. »

Page 9; it would be important for the authors to describe the state of approval for SonoVue in the US for brain imaging.

We added in the discussion a comment on the worldwide approval of Sonovue (line 226).
Sonovue® has been approved in more than 40 countries, is widely used in Europe for various imaging applications. It is routinely used for transcranial color-coded ultrasound imaging in a lot of European countries such as Germany, Switzerland, Portugal or France… It has been approved in the US under the name LUMASON for echocardiographic and liver imaging applications both in adults and children, underlining its innocuousness. Nevertheless, the concept of ULM should be applicable to any other approved contrast agents (Optison, Levovist, Definity).” As transcranial color-coded ultrasound is only performed in a very few specialized medical centers in the US, SonoVue/LUMASON is not registered for this application.

Page 9; What length of time was used for calculation of the TI in the skull? 1 minute 15 seconds is a long exposure when not moving the probe.

This is an interesting remark as safety was our first concern. Actually a thermal index is defined by regulation agencies (in the US the Food and Drug Administration (FDA) recommendations, in Europe the International Electrotechnical Commission (IEC) standards) as the ratio of the current acoustic power output from the transducer to the power required to cause a maximum tissue temperature rise of 1°C (Martin, 2010). As it results from a modelling of the heat transfer in a diffusive steady-state, it does not use an exposure time in its calculation. More precisely: “the TI is defined as $TI = W_p/W_{deg}$, where W_p is the relevant (attenuated) acoustic power at the depth of interest, and W_{deg} is the estimated power necessary to raise the tissue equilibrium temperature by 1°C according to a chosen specific tissue model. Therefore, a TI value of 2 would correspond to a 2°C increase in equilibrium temperature […]The model for soft tissue assumes a reasonable worst case […] likewise, the model for bone assumes that most of the acoustic power is absorbed in a thin disk at the surface of the bone (i.e., approximately infinite absorption coefficient).” Adapted from (Bigelow et al., 2011). Therefore a TI of 1 indicates that maximum temperature rise for **any examination duration** will be 1°C.
More specifically to our case: the most important index is the cranial TI (TIC). It is evaluated the same way for a scanned or non-scanned ultrasound beam, using measurements of the acoustic power on a whole cross section of the ultrasound beam close to the transducer, and modelling an absorption by the bone at this distance. In our case the TI largely overestimate the temperature rise as it is designed for continuous ultrasound imaging, while in our case ultrasound is on only half of the duration of the examination (1s of acquisition, 1 s of pause).
We also gave the TI bone (TIB) and the TI soft tissue (TIS) as indicative value, but they are even more overestimated for 2 reasons: 1/the same 50% duty cycle argument 2/ the pressure in depth is actually way lower than measured in water due to the skull bone absorption.
We completed the methods with a summary of the above information (around line 307).

Page 9; it is not clear what the sequence of data acquisition is. It is repeated every 2 seconds but how does that relate to the 45 second acquisition, for example?

Sorry for this lack of clarity. We rephrased around line 342 because it should be very simple: « 1s the ultrasound is on pulsing diverging wave at 4900 Hz and acquiring data, 1s the ultrasound is off and saving to disk the RF data that were just acquired, and this process loops during a desired duration (45 s for patient of figure 1, 2 min 15 s for patient of figure 2 and 3, 24 s for patients of figure 4)."

Page 12; the sentence beginning with "Finally, animation was" has many jargon words that need to be defined.

We apologize for this technical jargon. We modified the sentence to clarify.

"Finally, animation was rendered using the "3D renderer Mantra" library of Houdini software after setting-up an additional dome light effect."

Reviewer #2 (Report for the authors (Required)):

The paper by Demene et al presented the ULM technique as applied transcranially in certain human pathologies. This is achieved at variable resolutions using contrast agents injected intravenously. The paper is well presented and several complex anatomies are presented as a list of different cases. ULM is a technique that was presented and published before with impressive results as shown here.

We warmly thank the reviewer for his/her positive comment.

The same group has shown brain flow in humans without contrast. Therefore, it is not clear what is the novelty of this paper, except to show ULM's application in a few complex human anatomy cases, i.e., the authors need to clearly specify the novelty of the methodology presented and what is the problem that was solved and how was it solved.

We apologize for this aspect being not clear enough in the paper, as there is a huge step to take compared to prior articles for showing such results. First of all, it is true that our group showed brain flow imaging in humans without contrast (Demené et al., 2014; Demene et al., 2017; Imbault et al., 2017), the so called **ultrafast Doppler Ultrasound**, but we never did that both **transcranially** and with **superresolution**: it was either in neonates through the fontanel or during brain surgery (a result reproduced by (Soloukey et al., 2020)) using an opened skull flap. Moreover, imaging without contrast agent does not allow so far to beat the diffraction limit (and would result in the case of our paper to images with a resolution of the order of 1 mm to a few mm, 2 orders of magnitude above what is shown).
Here, what we did is to show that not only **ULM** is doable in human adults transcranially, but also that at this low ultrasound frequency, with an expected low SNR and in the presence of motion we could achieve resolutions of the order of 25 µm far beyond the typical 1 mm resolution of fUS imaging, a resolution never reached to our best knowledge by any other modality.

We listed all these challenges in the paragraph *"However, 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. Imaging through the skull via the acoustic temporal bone window imposes the use of a phased array small acoustic aperture (19.2 mm) and a low imaging frequency (2 MHz) that both strongly limit the axial resolution to 0.82 mm ± 0.07 mm, and the lateral resolution between 1 and to 5 mm, depending on the imaging depth (Supplemental Figure 1). It also limits the sensitivity due to low Rayleigh scattering of red blood cells at these frequencies10. Furthermore, the skull bone is responsible for large attenuation of the recorded ultrasonic signal through two mechanisms11: irreversible absorption in the bone and*

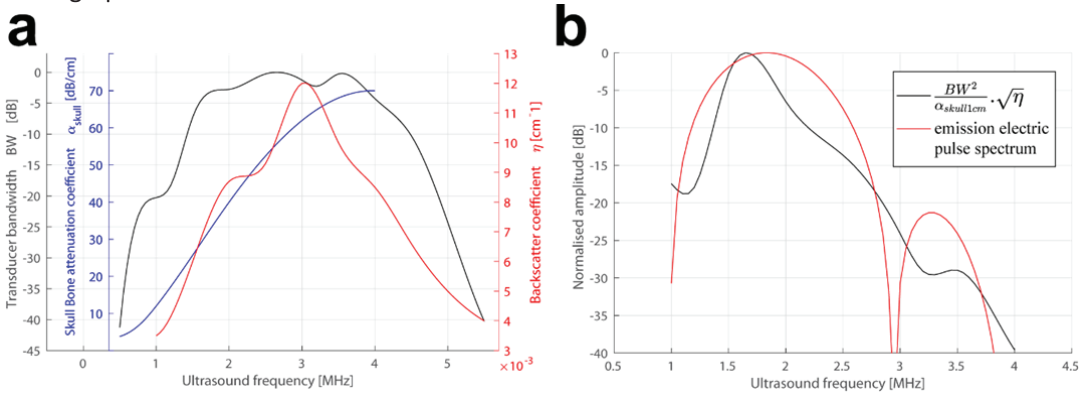
theoretically reversible diffraction effects due to the speed of sound mismatch between the brain tissue (~1500m.s-1) and bone (~3000m.s-1) that distorts the acoustic wave front (Figure 1c), a process called wave aberration12,13. Finally, the influence of motion artefacts escalates in the micrometric resolution range, and is of first importance as ultrasound is traditionally handheld performed and as the brain moves inside the skull cavity." at line 73, and the beginning of the paper is about how to overcome each of this difficulties.

We want to emphasize here that despite the presentation of the ULM concept in 2011 (Couture et al., 2011) and a proof of concept for the rat brain published in Nature in 2015 (Errico et al., 2015), no group has ever shown so far ULM imaging in the human brain, even though papers have been published about ULM in other organs such as the kidney or liver, in animals (Foiret et al., 2017; Song et al., 2018).
That being said, in the text:
- Line 73 we added the sentence "However, even though the ULM concept was introduced almost 10 years ago (Couture et al., 2011), no translation of ULM imaging to the human brain has been shown so far. Indeed the application of ULM to microvascular brain imaging in humans faces major challenges » to emphasize on the novelty of the research.
- We emphasized the list of challenges by making it a paragraph of its own.
- We followed the advice of reviewer 2 by structuring more the text into 2 paragraphs: what are the problems (line 73 to 91) and how were they solved (from line 88). Therefore, we removed from the above list of challenges the mention of the probe characteristics and frequency because it belonged more to the solutions than to the problems. We gave more information about skull bone attenuation. We added details in the following paragraph about the solutions and their justifications (line 92 to 108)
- We completed the methods in order to give more details about the solving of those challenges.

The geometry and frequency of the transducer were presented but it is not clear of why that specific geometry and frequency were selected and how the technique was optimized.

Agreed, this information was missing and we apologize for that, as this optimization was an important process.
Identification of those 2 specific challenges is now done through the sentence:
"Imaging through the skull trans-temporally imposes the use of a small acoustic aperture because the acoustic temporal bone window rarely exceeds 20 mm in diameter, and the use of a low imaging frequency because the ultrasound attenuation coefficient in the skull is enormous, and highly increasing with frequency: it scales in $f^{2.1}$ around 1MHz, 2.1 being the highest frequency exponent of all biological tissues (Bamber, 2005) » (line 76 to 81).
Moreover we added comments on the choice of the transducer array, and on the choice of emission pulse to drive it:
"First, we used a phased array transducer of 19.2 mm acoustic aperture, driven at a 2 MHz central frequency: this choice corresponds to a trade-off (suppl fig 1a-b) between emitting as much as possible within the transducer available bandwidth (it is 1 to 5 MHz and central frequency is 3MHz, most available low frequency imaging transducer are not designed to efficiently emit ultrasound below 2 MHz), keeping the skull bone attenuation coefficient as low as possible10,14 (10 dB/cm at 1 MHz, 40 dB/cm at 2 MHz, 60 dB/cm at 3 MHz) (suppl fig 1a), targeting as much as possible an efficient backscatter coefficient for the contrast agent15 (η = 3.5 10-3 cm-1 at 1 MHz, η = 8.9 10-3 cm-1 at 2 MHz. η = 1.2 10-2 cm-1 at 3 MHz), and keeping the highest possible resolution (at 2MHz, axial resolution to 0.82 mm ± 0.07 mm and lateral resolution between 1 and 5 mm, depending on the imaging depth) (suppl fig 1c-e).» (line 88 to 97).
To support this paragraph, we added 2 graphs in supplemental figure 1 (see below): supplemental figure 1 a shows the evolution of those parameters with ultrasound frequency, supplemental figure 1 b shows in black the curve combining those parameters (BW is squared as the signal pass through the

transducer at emission and at reception, α_{skull} is actually calculated for a total distance of skull propagation of 1 cm (0.5 cm thickness back and forth, this length actually do not influence the position of the peak), the backscatter coefficient η is taken as a square root as it come from a power measurement and we are considering amplitude of the received signal) and overlays in red the spectrum of the 2 MHz-2 cycle-squared electric pulse send from the ultrasound scanner to the ultrasound transducer: it fits nicely above the black curve, we chose a slightly higher frequency in order to keep as much resolution as possible. We also completed the methods with description of these graphs.



Suppl. Fig. 1: Choice of the emission frequency and effect on the diffraction-limited resolution of the ultrasonic probe used in the study. **a.** Choice of the emission frequency is influenced by the available transducer bandwidth (black), the skull bone attenuation (blue) and the power of the backscattering of the Sonovue® microbubbles (red). **b.** Combining these parameters gives a relatively narrow window between 1.5 and 2 MHz for t-ULM (black). The frequency spectrum (red) of the electric pulse used to drive the ultrasound transducer targets this peak.

Similarly, the bolus of the contrast agent seems to also be arbitrarily chosen without a clear justification.

Optimization of the bolus size is actually difficult and would require large cohorts and a lot of practice for t-ULM with several sonographers. Indeed the optimal dose will probably depend on individual factors, such as weight, heart rate, but also temporal bone thickness, etc. Due to the limited size of our cohort in such a prospective and proof-of-concept study, and also to the lack of immediate real time feedback for the t-ULM imaging, we chose a bolus size that: -gave enough dilution for the microbubbles for individual identification, while in the same time being concentrated enough for an acquisition in a short time compared to what is described in the ULM literature – would allow for multiple injections if we wanted to do multiple acquisitions after repositioning of the probe – would not in total be higher than the maximum recommended (by the swiss "Compendium des medicaments") dose of 2.4 mL. The Swiss guideline regarding the safe use of Sonovue can be found here :
https://compendium.ch/fr/product/115837-sonovue-subst-seche-c-solv/mpro.
This has been added in the text near line 104.

The diverging wave bean sequence was indicated as a previous development in cardiac imaging but the depth and the transcranial application clearly make the brain a different target than the heart.

Agreed. We modified the sentence in order to emphasize that we adapted the diverging wave sequence to transcranial human brain imaging.

"…a technique that has previously been proposed for ultrafast cardiac imaging[16] and been adapted here to human brain imaging"

The confirmation with CT is a strong aspect of the paper but it is only done in large vessels while in smaller vessels there is no validation.

This is a very good point, there was actually no possibility for us to do such a validation on the smallest visible vessels. To reach such a level of detail, it would require either very complex post-mortem injection techniques, which is out of the question in our case for clinical imaging, or maybe a very high field (>10T) MRI, which is not available in Switzerland.

It is also difficult in most figures to discern the similarity of the anatomy between ULM and CT/MRI.
We are quite surprised by this comment as the similarity between vascular structures seemed obvious to all the authors. However, we have added a few more arrows in the figures to point to identifiable landmarks.

1. Abstract- 'Ultrasound' is too general of a word and it is not clear why a capital letter is used here. Ultrasound imaging typically does not use contrast as ULM does. Therefore, contrast-enhanced ultrasound (CEUS) may need to be specified.

This is a good point. We used a capital letter for Ultrasound just as we did for Computed Tomography Angiography or Magnetic Resonance Angiography, in order to name the imaging modality and not just the physical phenomenon.
We have the feeling that the expression "contrast-enhanced ultrasound" used in the abstract would categorize our findings in an improper way, as what we present here is really different from what is commonly accepted in the clinics under the name "contrast-enhanced ultrasound". Even if some elements are the same (the presence of microbubbles, the use of an ultrasound probe), most of the technique (use of ultrafast imaging, diverging waves instead of typical focused beams, dynamic aberration correction, SVD filtering, microbubble centers super-localization, sub-wavelength motion correction, bubble density and speed image reconstruction) is different from the clinical practice of "contrast-enhanced ultrasound". ULM is based on a localization process whereas Contrast Enhanced Ultrasound is an imaging process. Therefore, we proposed to use "microbubbles aided Ultrasound" in the abstract, and contrast-enhanced ultrasound when relevant in the text (line 55, 60).

Also, the bolus injection is larger than the approved dose for the heart and microbubbles are not necessarily approved for clinical brain imaging, even at lower doses. So, it is important not to give a false impression of conventional ultrasound providing such imaging.

We agree that it is important to highlight the need of microbubbles and not give the impression that conventional ultrasound can provide such imaging. Regarding the type and concentration of contrast agents used, we humbly believe that we should not write the article with a country-centered consideration, and leave the reader appreciate to applicability regarding his available regulation. In particular, in Europe the acceptable use for contrast brain imaging is 2 mL (in Switzerland 2 mL for cardiac imaging, 2.4 mL for vascular Doppler imaging), and can be doubled to a second dose after benefit/risk evaluation by the medical doctor if one dose did not lead to significant diagnosis. In the paper we only mention 0.2 mL boluses.
https://compendium.ch/fr/product/115837-sonovue-subst-seche-c-solv/mpro.
Precisions near line 104.

2. Abstract- there are no quantitative or novel findings specified and therefore reads like a review paper on brain ULM.

We tried to strictly follow the nature guidelines (available here https://www.nature.com/documents/nature-summary-paragraph.pdf) to construct our abstract. According to those guidelines, there should be "Two or three sentences explaining what the main result reveals in direct comparison to what was thought to be the case previously, or how the main result adds to previous knowledge", and we tried to do so in the part "Microscopic brain angiography is attained by combining Ultrafast ultrasound Localization Microscopy (ULM) of intravenously injected microbubbles both with adaptive corrections of ultrasonic wave aberrations induced during transcranial propagation and micrometric brain motion corrections using speckle tracking. Its performance is illustrated by clinical examples. Complex and pathological vascular networks of tangled arteries were mapped and functionally characterized by high resolution (down to 25µm) quantification of flow dynamics. Unprecedented functional information was observed, such as the localization and dynamics of blood vortices in a deep-seated aneurysm. »

3. Abstract- the flow vortex in an aneurysm is not 'unprecedented' That's known.

The reviewer is right. The sentence was not clearly written. The terminology "unprecedented" was not applicable to the visualization of flow vortex which was recently performed in 4D flow MRI imaging (1mm resolution, 30 minutes acquisition) but rather to the resolution obtained (20 µm) and acquisition time (some tens of seconds). We modified the sentence by:

"~~Unprecedented~~ Functional information was observed at unprecedented microscopic level, such as the localization and dynamics of blood vortices in a small deep-seated aneurysm."

4. P. 2, para 2 - Replace 'broke' with 'solved'
Agreed.

5. P. 2, para 2 – The same group has published on functional ultrasound (fUS) in the brain of humans for imaging flow without microbubbles. How does human ULM compare to fUS in humans? Since the group has experience in both modalities, a fair comparison could be made. There is obviously a very big advantage in achieving this without contrast.

This is a very good point and it emphasizes that we did not clearly explained in the main text of the paper the core concept of ULM and its fundamental difference with ultrafast Doppler only (and by extension with fUS). We apologize for this, as we took for granted that the paper by Errico et al in the trepanned rat was known. We added this paragraph at line 65 to briefly summarize the concept;
"ULM relies on the same kind of concept that has been the basis for the development of PALM[3] and STORM[4] 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 center 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 device5. In the case of ultrasound imaging, this isolated objects consists in diluted ultrasound contrast agents, and the method gains from imaging at the fastest rate possible[6]."
Therefore it is difficult to draw a fair comparison between ULM and fUS, as they are very different and pursue different goals. ULM is a super-resolution imaging technique using contrast agent for morphological (including flow estimation) vascular imaging. fUS is a brain functional imaging technique based on the repeated use of Ultrafast Power Doppler without contrast agent in order to assess local cerebral blood volume changes that can be linked to underlying neuronal activity.
Also, just to clarify: fUS imaging has not been done so far trans-cranially in human. There are fUS papers published in neonates through the fontanel, and papers reporting use of fUS in adults per-operatively with the skull flap removed.
Super-localisation or super-resolution ultrasound imaging without contrast agent is a coveted grail, but so far no research group has demonstrated such a method.

6. P. 5, para 1- Replace ‘Microbubbles’ with ‘Microbubble’
Agreed

7. Fig. 3: The curves in (e) and (f) are very difficult to interpret. What do these bins indicate? Why are they different in systole vs. diastole?
We measure individual events (positions of bubbles) and extract information (bubble speed at certain positions). Therefore to extract a speed profile, we have to locally average or bin the data on a certain spatial extent a various position of the vessel cross section. We chose to bin in order show the data distribution.
Therefore, for example on figure 3f for example, the first whisker plot is drawn based on the velocity of bubbles that crossed the blue line of figure 3c between the edge of the vessel and 62.5 µm from the edge of the vessel. The second whisker plot is based on the binning of the speed of the bubbles that crossed this blue line between 62.5 and 125 µm from the edge, and so on.
As we know when a bubble is occurring in time relative to the systole and diastole, we can also subdivide this binning between events occurring in systole (on 0.3 s around the systolic peak, that is evaluated independently for the bubble positions and speed, see methods) and in diastole (0.6 s around the diastolic minimal speed, same thing), as was detailed in the methods.

8. Fig. 1C: It appears that the skull bone is hand drawn. The aberration correction needs to be clearly shown with before and after maps for a specific known skull anatomy.

We apologize as Fig 1C was just meant to be a schematics. The complete figure on aberration correction is actually suppl fig 2, that showed computed aberration laws and the before and after maps that reviewer 2 was looking for. We clarified this in the text by calling several time subpanels of suppl fig 2 within the text.

9. P.12, para 1: please provide details on the particle animation solver.
The sentence was unclear, we clarified and simplified as:
“Then, particle position, size, color and life duration are 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 10^4 to 10^5 particles.s^{-1} with a lifetime of 3 seconds.[…]”

Reviewer #3 (Report for the authors (Required)):

This is a manuscript that demonstrates dramatic improvements in transcranial ultrasound imaging and analysis using a combination of methods, several of which were pioneered by this group. These include ultrafast ultrasound imaging, on the orders of hundreds to thousands of imaging frames per second, ultrahigh resolution vessel detection using ultrasound gas bubble contrast agents injected such that low vascular concentrations permit imaging solitary bubbles allowing subwavelength resolutions, on the order of 10s of micron diameters, singular value decomposition motion and clutter cancellation, phase aberration correction through the skull, and motion correction. The results are quite extraordinary depicting very small vascular structures and dynamics of flow not visible on standard brain imaging modalities such as CT or MRI. Although the all of the methods employed are not completely new, this group has demonstrated several of these methods before, the union of these methods is new and has produced images of exceptional quality and detail. The consequences of such methods could be huge, with better definition of blood vessels and evaluations of flow dynamics, potential for such methods are almost hard to predict. Functional imaging with MRI has already had major impacts in clinical diagnosis and research. The methods described here could be used in conjunction with MRI or stand alone with better definition of local vascular anatomy

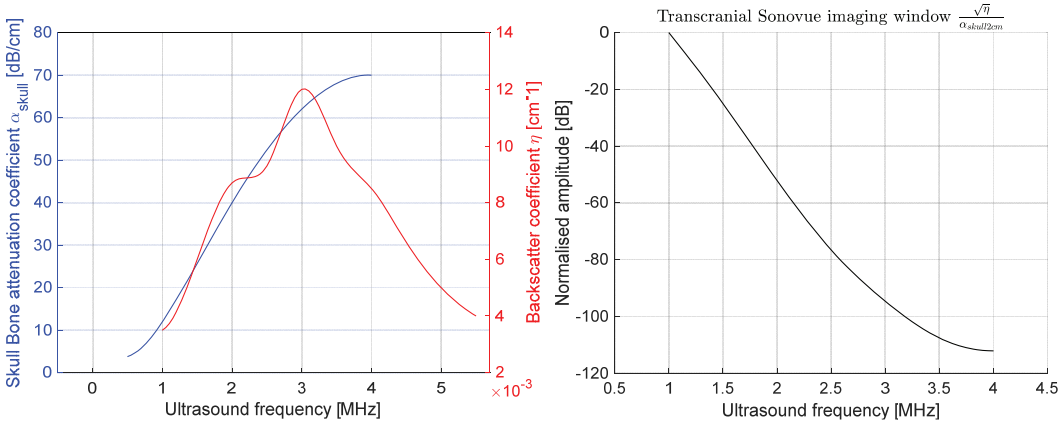
and flow. It is very possible that this improve could lead to new levels of functional imaging and diagnosis of brain diseases.

We are very grateful to the reviewer for this very enthusiastic comment, and we feel much rewarded that he grasped so acutely what this whole work means to our group.

Major technical questions:

1) The biggest problem with this method is the limited access. Although the investigators do an excellent job of correcting for skull aberration of the ultrasound signals, this method still uses the temporal sutures as sites through which sound can enter the brain. These are the typical sites used in standard transcranial Doppler studies. Because of this, the entire brain cannot be imaged. Thus the method has a major limitation relative to MRI and CT. Do the investigators have any ideas of how to overcome this major problem? Given this problem, it is highly likely that the ultrasound methods described herein will not be used for primary diagnosis or evaluations, and will only be used in conjunction with CT and/or MRI after an initial evaluation detects regions of interest that can be assess using ultrasound.

The reviewer highlights the crux for t-ULM. There are two ways to answer this question:
- The first one is given by the reviewer, and t-ULM will be used complementary to regular MRI or CT, for exploration of a particular brain area with the increased resolution and functional information. This solution would be readily available, as it requires no further technical development than what is proposed in this paper, and a lot of brain areas are actually accessible like this. Our images have better quality near 60-70 mm depth, because it corresponds to the elevation focus of our probe, and are degraded at large depth due to the loss of this elevation focalization (as long with attenuation of course, but we can see in figure 2d that we do not lose that much of sensitivity around 110mm, but we lose precision in the localization of the bubbles). Therefore maybe with a slightly lower central frequency and deeper elevation focus we could envision whole hemisphere imaging (see figure 1b and 2a for the field of view extent) from the contralateral temporal window, and therefore whole brain imaging using both temporal windows and perhaps also the occipital window.
- The second way needs an explanation of our strategy for doing this paper. We wanted to use hardware (programmable ultrasound scanner and ultrasonic probe) already available in order to do a fast proof of concept as close to the clinics as possible. This hardware (due to the transducer bandwidth but also to the electronic board bandwidth) could not efficiently reach central emission frequencies below 1.7 MHz. Also we did not know which kind of precision to expect and were somehow comforted to be able to reach a 25µm precision with this setting, at 2 MHz and with a small aperture <20mm. **Therefore we are now confident that it is likely possible to lower the frequency down 1MHz and below for t-ULM**, and we can now envision different ultrasound systems and different imaging probes. By taking into account only the skull bone attenuation coefficient and the backscatter coefficient of Sonovue (graph below, left), we can see that when we want to cross a large skull thickness there is a huge advantage at using a central frequency of 1MHz or below (graph below, right, simulating the relative receive signal amplitude when using Sonovue and propagating back and forth through 1cm of skull bone, there is a typical 50 dB difference between working at 2 MHz and 1 MHz).



At 0.5 MHz, the attenuation coefficient is 8 times lower than at 2 MHz, therefore 1cm of skull bone to cross at 2MHz (which correspond ~ to a temporal bone window back and forth) is the same than 8 cm of skull bone to cross at 0.5 MHz, largely enough to envision imaging through any site on the skull. There will of course be a loss of resolution, but that might be compensated by larger apertures and thus larger antenna gains, as the temporal window will not be a constraint anymore. Also, working on contrast agents with a peak of backscatter coefficient at lower frequency will help.
We added a shortened version of these considerations within the text (line 143-148) and in the discussion (line 239-254) and added a reference to the foundation work of F.J. Fry and J.E. Barger on the acoustic properties of the human skull bone.

2) Transcranial Doppler studies have failure rates of about 15%. These are studies using focused ultrasound beams produced by small aperture ultrasound probes to detect spectral Doppler signals from basal arteries. These probes need very small access windows relative to the imaging these investigators will perform using compounding of multiple diverging beams. Besides loosing energy on passage through the suture, the probe's aperture could be severely limited preventing compounding. I would guess that the number of failed examinations will be much higher than the 15% figure. I know the impressive results demonstrated here are preliminary, but can the investigators predict how many of their studies will in fact fail due to the skull transmission? The investigators acknowledge this problem, but if you can't get sound into the brain, deaberrating reflected signals will not be of much help.

That last sentence is of prime importance, and explains the care we took in optimizing the ultrasound frequency used for this study (supplemental fig 1), we agree very much with the reviewer. It would have been very beneficial to add a reference for the failure rate of 15% for transcranial Doppler study, in order for us to understand what the cause of failure is really: problem of transmission through the skull, or problem of positioning for a given anatomy, as this kind of basal artery monitoring system cannot perform imaging and as positioning of the probe is blindly done by an experienced technician based on the measured spectra. Also, does this kind of study enable the use of contrast agents? If not, it is very difficult to infer a failure rate for t-ULM from this 15% in transcranial Doppler monitoring.
From our experience in a swiss university medical center neurosonology laboratory: most of the patients requiring an examination performed with transcranial color-coded Doppler imaging (Color Doppler and Pulse Wave Doppler), if they have sufficient acoustic transcranial bone windows, can be imaged without contrast agents. If this is not the case, injection of the echocontrast agent Sonovue® is performed and enables to enhance the signal enough to detect the arterial proximal segment of the circle of willis. This constitutes the main indication of Sonovue® for vascular brain imaging.
We added a comment on that aspect in the discussion line 230-236.

3) Superresolution requires that intravascular scatterers be single contrast agent bubbles. Further, their aberration correction methods also depend on reflections from single bubbles. The investigators inject small boluses of contrast to insure that their signals are coming from individual bubbles. How do they guarantee that they are looking at individual bubbles when they generate their images? What do they do to compensate for decreased resolution if they think their echoes are being produced by groups of bubbles?

First of all most of the time the bubbles are indeed individual bubbles. According to (Schneider, 1999), there is 1 to 5.10^8 bubbles in 1 mL of injectable Sonovue. Injecting 0.2mL therefore corresponds to a maximum of 10^8 bubble sin the blood stream, whose total volume can be estimated to 5L in an adult. We end up with a maximum of 20 bubbles/mm^3, but that is right after the injection, and bubble filtering occurs rapidly in the lungs, therefore we rapidly obtain an average concentration below the unity per resolution cell. However randomness of the bubble distribution makes that having a group of bubble is very likely to occur, especially in the big vessels. We compensate for that by comparing the neighborhood of local maxima with a stereotyped bubble response, and discarding those who look more like speckle than isolated bubbles. This was indicated in the methods as "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 λ and lateral (angular) dimension of $\arctan(\frac{\lambda}{d})$, and local maxima with weak correlation (< 0.6) were discarded (local spatial speckle fluctuation can generate local maxima but are not like a point spread function). » Moreover, it occurs in the biggest vessels, where precision is not the most important.

4) Multiple injections of small boluses of contrast agent are required for this method. Although definitely possible, this will require new modifications to injection protocols.

We found small boluses convenient for 2 reasons:
- For now the acquisition is so demanding in terms of computer resources that our machine is really at the edge of its capacity, and need pauses between sessions of 1 min 30 s of acquisition. Therefore a small bolus of 0.2 mL is enough to obtain a convenient number of bubbles per image during this period of time. Continuous infusion of microbubbles would be unnecessary in that context, and would not follow the ALARA principles.
- Although we did not have enough data to rigorously study this phenomenon, it might be possible that injecting a small bolus before the acquisition gave access to a diversity of bubble concentration during the whole imaging session of 1 min 30, and therefore helping to delineate different population of vessels: high concentration of bubbles would be efficient to relatively rapidly delineate the small vessels where bubbles are still isolate but give speckle in the biggest vessels preventing the super-localisation to occur, and low concentration of bubbles at the end of the bolus would help to have information on the bigger vessels.

That being said, with higher computer power and the possibility of doing continuous t-ULM, even real-time ULM, it might seem reasonable to use microbubble infusion, for example using the dedicated Vueject pump (by Bracco). We need further study to optimize the dose in such a context.
Bolus injection is part of the clinical routine in Switzerland.

5) Please describe the vesselness filter in more detail.
We added details and references from line 393 to 404.

Minor technical criticisms:

1) i and j are not visible in frame b of figure 3. I and j should be e and f.
This has been corrected

2) Being able to see a flow vortex in a small MCA aneurysm is a beautiful demonstration of the power of the method. However, the clinical value of this information is not clear at this time. Predicting a potential thrombus in an MCA aneurysm based on the presence of a vortex is a bit of a stretch.
We totally agree and we felt that the very hypothetical nature of the clinical value of this kind of functional information was clearly stated in this sentence, but this is maybe due to our non-native English speaker limitations. We used the formulation of reviewer 3 and rephrased more explicitly into "The potential for such imaging is hard to predict, but it could be possible that the degree of clotting or the risk of rupture of a given aneurysm might be influenced by the local fluid mechanics and blood flow speeds reached in the aneurysm. Numerous studies try to link local flow mechanics, wall shear stress and health of the vessel wall(Febina et al., 2018; Goudot et al., 2019; Paszkowiak and Dardik, 2016). Therefore such unique functional information could be of clinical relevance for making the decision regarding surgical resection or coiling», and added references for clarity.

3) "j" label is missing in Figure 4.
There was actually a display problem with this figure, with a Word® clipping box that partially cropped the image, making the letter j disappear. This is now corrected.

4) At the end of paragraph 2, page 2, "…is traditionally handheld performed" should be "…is traditionally handheld and performed"
Agreed. We change the sentence to "Finally, the influence of motion artefacts escalates in the micrometric resolution range and is of first importance, as ultrasound is traditionally handheld and as the brain moves inside the skull cavity. »

5) aneurism should be aneurysm on page before references.
Agreed and corrected.

Dear Editor,

We deeply thank you for accepting the publication of our manuscript in Nature Biomedical Engineering. We took into account all final reviewer comments in our revised manuscript. You will find below a list of answers to all remaining editorial and reviewer comments.

Reviewer #1 (Report for the authors (Required)):

Very little grammatical editing is needed.

Line 69 objects should be object
Corrected

Line 329 Come should be came
corrected

Line 328 do should be does.
corrected

Line 602 The authors should state that they “thank…” not that they “want to thank…”
corrected

Reviewer #2 (Report for the authors (Required)):

The authors revised the manuscript substantially to address most of the concerns raised. The rigor is enhanced and several of the methodologies have been clarified. There are two points that remain:

1) Since novelty remains unclear in the introduction, the following text in the responses to reviews regarding novelty should be incorporated in the introduction of the revised manuscript:
"Our group showed brain flow imaging in humans without contrast (Demené et al., 2014; Demene et al., 2017; Imbault et al.,
2017), the so called ultrafast Doppler Ultrasound, but we never did that both transcranially and with superresolution: it was either in neonates through the fontanel or during brain surgery (a result reproduced by (Soloukey et al., 2020)) using an opened skull flap. Moreover, imaging without contrast agent does not allow so far to beat the diffraction limit

(and would result in the case of our paper to images with a resolution of the order of 1 mm to a few mm, 2 orders of magnitude above
what is shown). Here, what we did is to show that not only ULM is doable in human adults transcranially, but also that at this low ultrasound frequency, with an expected low SNR and in the presence of motion we could achieve resolutions of the order of 25 µm far beyond the typical 1 mm resolution of fUS imaging, a resolution never reached to our best knowledge by any other modality."

This is now done in the introduction to emphasize on the novelty of the work, and we inserted this text (in two parts). The abstract was also modified accordingly.

2) There are several typos and colloquialisms throughout the paper, these should be fixed prior to final submission. An example is shown below:
'We choses a bolus dose that: -gave enough dilution for the microbubbles for individual identification, and enough concentration for an acquisition in a short time compared to what is described in the ULM literature'
Please replace 'choses' with 'chose' and 'gave enough' with 'provided sufficient'

We corrected these typos and some others throughout the paper.

Reviewer #3 (Report for the authors (Required)):

Deep Transcranial Adaptive Ultrasound
Localization Microscopy of the Human Brain
Vascularization

This is a revision of the initial submission of this manuscript. The work remains an impressive presentation of high resolution imaging of the brain. The work will have major impact on brain imaging. The breadth of the innovation in this work is very high, and the authors have done an impressive job in developing this work. They have also very adequately responded to the reviewer critiques of the initial submission. I have no major issues with this revision. There are some grammatical, structural, and spelling errors that need to be corrected in order to make the work easier to read.

Abstract:
There are several words that are capitalized incorrectly. These include Computed Tomography Angiography (line 19), Magnetic Resonance Angiography (line 19), Ultrafast (line 27), and Ultrasound (line 34).
Corrected

Line 65 – Please spell out PALM and STORM.
corrected

Line 90 – transducer should be transducer’s
corrected

Line 92 – transducer should be transducers
corrected

Figure 1 caption. SVD should be spelled out as singular value decomposition when the abbreviation is first encountered in the text.

corrected

Figure 3 caption: There are no i and j sections in this figure. Are these referring to figure 4?
Agreed, there was a mistake as figure 2 and 3 were gathered in the same figure at first. We were referring to figure 3 c and d. This is now corrected.

I would suggest changing dissymmetric to asymmetric.
corrected

Line 215: Color should be lower case, color
corrected.

Line 217: calculation should be calculations
corrected

Line 221: calculation should be calculations
corrected

Line 224: display should be displays
corrected

Line 225: display should be displays
corrected

Line 226: stenosis should be stenosis
corrected

Line 226: “This aspect is” should be “These aspects are”
corrected

Line 227: display should be displays
corrected

Line 326: pass should be passes
corrected

Line 328: do should be does
corrected

Line 329: come should be comes
corrected

Line 343: The statement “to summarise” is hard to understand. Perhaps it should be written as: To summarise: for 1s, the ultrasound is on and both pulsing diverging waves at 4900Hz and acquiring data, for 1s, the ultrasound is off and saving the RF data that were just acquired to disk …….
Thank you very much, this is corrected.

Line 367: trough should be through
corrected

Line 395: belong should be belongs

corrected

Line 447: “for the diastole” should be “for diastole”
corrected

Line 455: “for the diastole” should be “for diastole”
Corrected

Line 473: cerebral angiography should be cerebral angiogram
corrected

Line 478: “MR angiography of the circle of willis” should be “MR angiogram of the Circle of Willis”
corrected

Finally,
The statement in the review of the initial submission that approximately 15% of transcranial Doppler ultrasound scans of the brain fail is supported for example by the following reference:
Lee C-H, Jeon S-H, Wang S-J, Shin B-S, Kang HG. Factors associated with temporal window failure in transcranial Doppler sonography. Neurological Sciences 2020; https://doi.org/10.1007/s10072-020-04459-6
Thank you for this pertinent reference. It has been added in the discussion.

Editor comments:

☒ Please make sure that all acronyms are spelled out at first use. Note that we have now edited the abstract and therefore acronyms that were spelled out in your abstract will need placing in the main sections, as appropriate.
Corrected

☒ To adhere to house style, please avoid any use of ‘breakthrough’, 'paradigm shift', 'innovative', 'striking', ‘major advance’, ‘unprecedented’, and of similar wording denoting the importance of the results described in the manuscript; it is up to readers to judge the advance. Also, please remove any instances of 'for the first time', 'novel' and ‘new’, unless strictly needed. ‘Platform’ (an often misused term) should be substituted by ‘technology’, ‘method’, ‘approach’ or similar wording.
Corrected

☒ Please clarify how many patients have been tested to date and clarify the patients in Fig. 1, 2 and 4 are different individuals
Corrected

☒ As noted in the editorial 'Show the dots in plots', we request that, for all bar plots (in the main figures and in the SI), measurements are shown as individual data points (in addition to measures of central tendency and error bars), unless sample numbers are large.
As asked, we added all sample numbers in the figure captions. Regarding the ‘show the dots in plot’ policy, we did not show the dots as the number of samples is very large (between some hundreds to thousands of bubble positions).

☒ All *p*-values (unless smaller than 0.001) should be provided as precise figures (rather than as $p <$ number). Statistical tests, whether they are one-sided or two-sided, and whether adjustments were made for multiple comparisons, should be specified in each relevant figure caption.
Corrected in all figure captions

☒ For all graphs with error bars, please define in the figure caption the centre values, measure of dispersion, and sample numbers (please do not provide sample ranges). Please note that this information is missing in the legends of figures: 3e, 3f; supplementary figure: 5. 3.Please indicate what '*, **, ***' represents; if this represents p values, please indicate the statistical test used and where appropriate, specify whether it was one-sided or two-sided and whether adjustments were made for multiple comparisons and the exact p value in the legend of Supplementary figure: 5, 3f.
Corrected

☒ Representative images: please indicate in the caption how many images were taken. We strongly suggest that all the images taken (or a subset of them if there are too many) be included in the SI.
Corrected in the figure captions

☒ Measurement replicates: for all relevant figures, please state in the caption whether replicates are biological or technical. Reporting statistics on technical replicates is discouraged (in such cases, a clear justification should be provided). Please note that this information is missing in the legends of figures: 2e, 2b, 2d; 4a, 4g; Supplementary figures: 2a-2f, 2i; 3a-3f; 4 (left panel).
Corrected in the figure captions

☒ Box plots: the minima, maxima, centre, and percentiles need to be defined in the figure captions. Please note that the box plots need to be defined in terms of minima, maxima, centre, bounds of box and whiskers and percentile in the legend of supplementary figure: 5
Corrected

☒ For clinical images, please clarify what the intensity units (i.e. the heat-colourmap in the ULM images and the greysclae in the MDCT and the MIP-TOF angiograms) refer to. Please ensure that the colour bar is displayed, with intensities reported in numerical values. Please avoid the use of arbitrary units or min/max.
Corrected

☒ Please clearly state what the black arrows are (ie the flow vector field needs to be explained in more detail, also in the manuscript), and please replace the word "arrow" with "arrowhead" when describing the green, blue and red tips in the images, so as to avoid confusion.
Corrected

☒ Please ensure that all micrographs include a scale bar and this scale bar is defined on the panels or in the figure legends, this is missing for Supplementary figure: 2i.
Corrected

☒ Please note that the handheld device graphic displayed in Fig 1a-c, Fig. 2a, SI Fig1c-d, SI Fig 2g and SI Fig.6-7 may be erroneously mistaken for the SuperMicroConvex 12-3

transducer (Supersonic Imagine), rather than the Single Crystal Phased Array XP5-1, stated in the Methods section. Please confirm the product used and modify the graphic to include the somewhat more angular features (and proportions) of the XP5-1.
We added a clear statement in the figure caption that the probe is a Phased Array XP5-1 (in addition to the Methods section). Although this phased array probe XP5-1 has less rounded shapes than the 12-3 transducer from Supersonic Imagine, it has round shapes. The important part of the graphic is the front face of the probe which is here flat (Phased array) making it distinct from the convex front face of a microconvex probe.

Finally, we also modified the reporting summary in agreement with all required changes. We also provide in this last version the information about the data and codes available along with the manuscript. All codes and data examples were uploaded on the public Zenodo repository website and the information is provided in the Data Availibility section and Code Availibility section. We also proposed cover and aesthetic image suggestions as issue cover for the journal. We hope it could perhaps convince the editorial board to highlight it.

We thank you again for your trust and interest in our research work. Many thanks for your detailed edition and modification of the abstract of the manuscript.