

Longitudinal Awake Imaging of Mouse Deep Brain Microvasculature with Super-resolution Ultrasound Localization Microscopy

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This study presents **important** methodologies for repeated brain ultrasound localization microscopy (ULM) in awake mice and a set of results indicating that wakefulness reduces vascularity and blood flow velocity. The data supporting these findings are **solid**. This study is relevant for scientists investigating vascular physiology in the brain.

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Abstract

Ultrasound localization microscopy (ULM) is an emerging imaging modality that resolves microvasculature in deep tissues with high spatial resolution. However, existing preclinical ULM applications are largely constrained to anesthetized animals, introducing confounding vascular effects such as vasodilation and altered hemodynamics. As such, ULM quantifications (e.g., vessel diameter, density, and flow velocity) may be confounded by the use of anesthesia, undermining the usefulness of ULM in practice. Here we introduce a method to address this limitation and achieve ULM imaging in awake mouse brain. Pupillary monitoring was used to support the presence of the awake state during ULM imaging. Vasodilation induced by isoflurane was observed by ULM. Upon recovery to the awake state, reductions in vessel density and flow velocity were observed across different brain regions. In the cortex, the effects induced by isoflurane are more pronounced on venous flow than on arterial flow. In addition, serial *in vivo* imaging of the same animal brain at weekly intervals demonstrated the highly robust longitudinal imaging capability of the proposed technique. The consistency was further verified through quantitative analysis on individual vessels, cortical regions of arteries and veins, and subcortical regions. This study demonstrates

longitudinal ULM imaging in the awake mouse brain, which is crucial for many ULM brain applications that require awake and behaving animals.

Introduction

Sensitive imaging of correlates of activity in the awake brain is fundamental for advancing our understanding of neural function and neurological diseases. In the field of neuroscience, there is a growing interest in utilizing imaging techniques to study the rodent brain, which serves as a valuable model for investigating brain function^{1,2}. Brain imaging modalities for rodents encompass a wide range of techniques, including but not limited to functional magnetic resonance imaging (fMRI)^{3,4}, positron emission tomography (PET)^{5,6}, one- and two-photon imaging^{7,8}, photoacoustic imaging^{9,10}, and more recently ultrasound localization microscopy (ULM)¹¹. ULM is uniquely capable of imaging microvasculature situated in deep tissue (e.g., at a depth of several centimeters). ULM can also be combined with the principles of functional ultrasound (fUS)^{12–15} to image whole-brain neural activity at a microscopic scale¹⁶. The structural and functional imaging capabilities of ULM have opened new doors for numerous basic research and clinical applications that involve cerebral microvasculature^{17–19}.

At present, a key limitation associated with existing ULM brain imaging studies is the use of anesthesia, which induces profound alterations to cerebral blood flow (CBF), including changes in vessel size (e.g., diameter) and flow velocity²⁰. As such, CBF measurements under anesthesia do not reflect the blood flow under the normal physiological state of the brain. In addition, anesthetics also have a significant attenuating effect on neural responses to sensory inputs, thereby impacting the neurovascular coupling process^{21–26}. Therefore, in neuroscience research, brain imaging in the awake state is often preferred over imaging under anesthesia (e.g., fluorescence imaging²⁷, photoacoustic imaging^{28,29}, fMRI⁴, PET⁶, and fUS^{30–33}). As ULM is gaining traction in many preclinical brain imaging applications, enabling ULM for awake animals has become crucial to eliminate the confounding vascular effects of anesthetics and obtain accurate structural and functional cerebrovascular measurements.

Another challenge associated with preclinical ULM brain imaging is to conduct long-term, longitudinal studies, which are essential for tracking disease progression or therapeutic impacts for many neurological disease applications^{34–36}. The key technical challenge for longitudinal ULM brain imaging is to find the same imaging plane and reconstruct consistent ULM images across different imaging sessions. Misalignment of imaging planes or tissue movement will undermine ULM imaging quality and result in inconsistent cerebrovascular quantifications. Although intact skull imaging has been shown to be feasible for short-term studies (e.g., over a few days)¹⁸, long-term monitoring can be challenging because changes in skull properties over time (e.g., thickness and composition) could negatively impact ULM imaging quality. Currently there is a strong need for methodological developments to enable longitudinal brain imaging with ULM.

In this study, we developed a method for awake and longitudinal ULM brain imaging in a mouse model to eliminate the confounding vascular effects of anesthesia and enable monitoring of cerebral vasculature over a three-week period in the same animal. We constructed a head-fixed awake imaging platform and established a ULM image reconstruction metric to allow comparisons of ULM images acquired at different states of wakefulness (e.g., awake vs. anesthesia). Our method allowed detailed comparisons of local and global variations of the cerebral vasculature and blood flow under awake and anesthesia conditions. Detailed quantitative analysis of vessel diameter and blood flow velocity was performed. We also demonstrated robust longitudinal ULM brain imaging on same animals with high repeatability over multiple weeks.

Results

ULM brain imaging can be performed in head-fixed awake mice

To achieve consistent ULM brain imaging while allowing limited movement in awake animals, a head-fixed imaging platform with a chronic cranial window was used in this study. Pre-surgery handling and post-surgery habituation were employed to alleviate animal stress and facilitate awake imaging³³. To further minimize tissue motion, a 3D-printed body tube was adopted³³ for animal immobilization (**Fig. 1a**). A headpost was implanted during cranial window surgery to fix the animal's head to the body tube. In this procedure, the skull was removed, and the brain tissue was protected using a polymethyl pentene (PMP) membrane³⁷, which was further protected by a layer of silicone rubber (**Fig. 1b**). Animals were imaged after one week of surgical recovery and head-fixation habituation. Prior to each imaging session, the silicone rubber was gently removed with forceps, and the transparent PMP membrane allowed detailed examination of the brain surface to ensure absence of tissue damage (**Fig. 1c**).

Contrast enhancing microbubbles (MBs) were administered intravenously via tail vein in this study (details provided in Methods). Isoflurane anesthesia was terminated right after microbubble (MB) injection, marking the beginning of the data acquisition (i.e., $T = 0$ s). Mice were allowed to gradually regain consciousness. Throughout the awakening process, an infrared camera was used to monitor the pupil to provide a reference for the state of arousal³⁸ (**Fig. 1d**). **Fig. 1e** shows the change in pupillary area over time, which reveals a gradual enlargement of the pupil after the cessation of anesthesia. Due to differences in tail vein injection timing and anesthesia depth, the time required for each mouse to fully awaken varied. Although it was not feasible to get pupil size stabilized just after 500 seconds for each animal, ULM reconstruction only used the data that acquired after the animal reached full pupillary dilation, to ensure that ULM accurately captures the cerebrovascular characteristics in the awake state. Ultrafast plane wave imaging data were collected for ULM reconstruction (details provided in Methods) and a representative awake ULM brain image is presented in **Fig. 1f**.

Microbubble count serves as a quantitative metric for awake ULM image reconstruction

Due to the stochastic nature of MB localization and fluctuations of MB concentration in the blood stream, it is challenging to compare ULM images acquired at different states of wakefulness with different cerebral blood flow conditions. Therefore, to obtain complete, fully saturated ULM images under different physiological conditions and MB concentrations, we used MB count as a measure of vessel saturation to determine the completeness of ULM reconstructions. To ensure high quality ULM imaging, only MBs that were successfully tracked by the uTrack algorithm (details provided in Methods) were considered as effective MB signals that were utilized for ULM image quantification.

To facilitate equitable comparison of brain perfusion at different states, a practical saturation point enabling stable quantification of most vessels needs to be established. Our observations indicated that when the cumulative MB count reached 5 million, ULM images achieved a relatively stable state. Accordingly, in this study, the saturation point was defined as a cumulative MB count of 5 million. There are also possible alternatives for ULM image normalization. For example, different ULM images can be normalized to have the same saturation rate. However, the proposed method of using the same number of cumulative MB count for normalization enables the analysis of blood flow distribution across different brain regions from a probabilistic perspective. The following analysis substantiates this criterion.

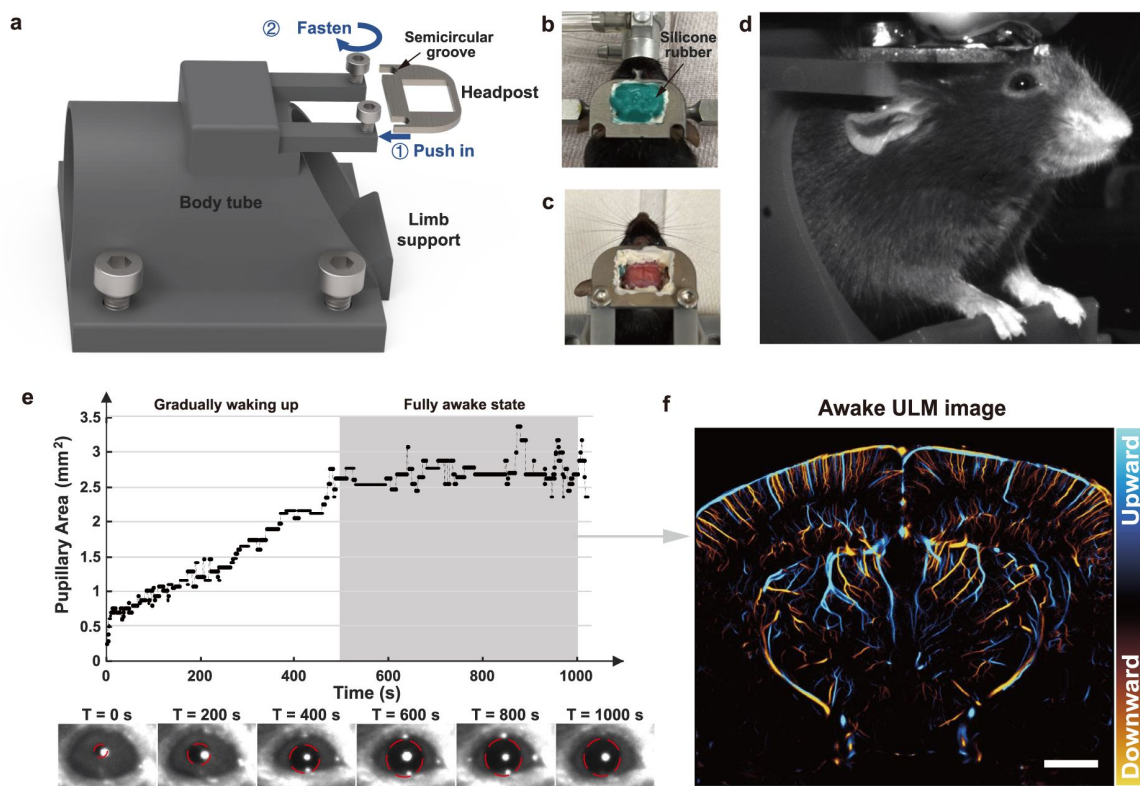


Fig. 1

Experimental setup for awake mice ULM brain imaging.

a, 3D model of the body tube enabling rapid fixation of the headpost onto the tube. **b**, Top view photograph of the mouse after cranial window surgery, with the headpost protected by silicone rubber. **c**, Photograph taken after the post-surgery recovery, with the removal of the silicone rubber protection. **d**, Image captured by the camera positioned in front of the mouse during the imaging session. **e**, Change of the pupillary area over time, after microbubble injection ($T = 0$ s), along with magnified portion of the pupil photo in **d** at six time points ($T = 0$, 200, 400, 600, 800, and 1000 second). Pupil is outlined with red dashed circle. **f**, Awake ULM image obtained from data collected during a fully awake state, as indicated by the gray shaded area in **e**. (scale bar: 1 mm)

Fig. 2a compares ULM directional vessel density maps and flow speed maps generated with 1, 3, 5, and 6 million MBs, using the same animal as shown in **Fig. 1**. To quantitatively confirm saturation, multiple vessel segments were selected for further analysis. **Fig. 2b** presents the measured vessel diameter for a specific segment at various MB counts. After binarizing the ULM map, the vessel diameter was measured by calculating the distance from the vessel centerline to the edge. Each point along the centerline of the segment provided a diameter measurement, enabling calculation of the mean and standard error. At low MB counts, vessels appeared incompletely filled, leading to inaccurate estimation of vessel diameter due to incomplete profiles. For example, at 1–2 million MBs, the binarized ULM map displayed a width of only one or two pixels along the segment. As a result, the measurements always yielded the same diameter values (two pixels, ~10µm) with a consistently low standard error of the mean across the entire segment. With increased MB counts, the measured vessel diameter gradually rose, ultimately reaching saturation. The plots in **Fig. 2b** show that vessel diameter stabilized at 5 million MB count. Additionally, **Fig. 2c** illustrates the changes in flow velocity measured at different cumulative MB counts. The violin plots display the distribution of flow speed estimates for all valid centerline pixels within the selected segment. At low MB counts (1–3 million), flow velocity estimates fluctuated, but they stabilized as the MB count increased (4–6 million MBs). At 5 million MBs, flow velocity estimates were nearly identical to those at 6 million MBs, corroborating previous findings that vessel velocity measurements stabilize as MB count grows³⁹. To assess the generalizability of the 5 million MB saturation condition, vessel segments from three different mice across various brain regions were examined. The results, shown in **Supplementary Fig. 2**, confirm that this saturation criterion applies broadly. Although the 5 million MB threshold may not ensure absolute saturation for all vessels, it is generally effective for vessels larger than 15 µm. This MB count threshold was therefore adopted as a practical criterion.

To further verify that the proposed MB bolus injection method can help to achieve ULM image saturation shortly after mice awaken from anesthesia, an analysis on the change in MB concentration over time was conducted once pupil size had stabilized (T = 500s). **Fig. 2d** shows a clear trend of decreasing MB concentration in the blood stream and increasing cumulative MB count with time. **Fig. 2e** demonstrates a flattened vessel saturation curve and a rapidly reducing vessel filling rate, which is typical for ULM reconstruction³⁹. The pixel filling rate dropped below 5% of the initial rate after 300 seconds of ULM data accumulation (T = 800s), indicating ULM image saturation. **Fig. 2f** further examines the relationship between the pixel filling rate and the cumulative MB count, which is independent of data acquisition time. The pixel filling rate at 5 million cumulative MBs is always below 5% of the initial rate for each experiment, ensuring image saturation. In summary, all the quantitative measurements indicate that ULM images obtained using the proposed metric (i.e., 5 million MBs) were complete and can be used to consistently measure cerebral vascular properties such as vessel diameter and blood flow velocity. All subsequent ULM images in this study were produced using this criterion.

ULM reveals increase in blood flow induced by isoflurane anesthesia

Fig. 3 presents a comparison of ULM directional vessel density maps and flow velocity maps in awake and anesthetized states for three different coronal planes from three animals. Four regions of interest (ROIs) were selected within each imaging plane to provide detailed comparisons. When comparing vessel density maps, ULM images that were acquired in the awake state demonstrate a global reduction of vascularity, which refers to percentage of pixels that occupied by blood vessels. The reduction is also clearly observed in magnified local regions especially for regions 5, which encompasses the pretectal region. In addition to reduced vascularity, ULM also reveals decreased vessel diameter which reflects vasoconstriction after the animals woke up (e.g., white arrows in regions 1, 3, and 6, corresponding to the thalamus, cortex and midbrain/cortex overlap region).

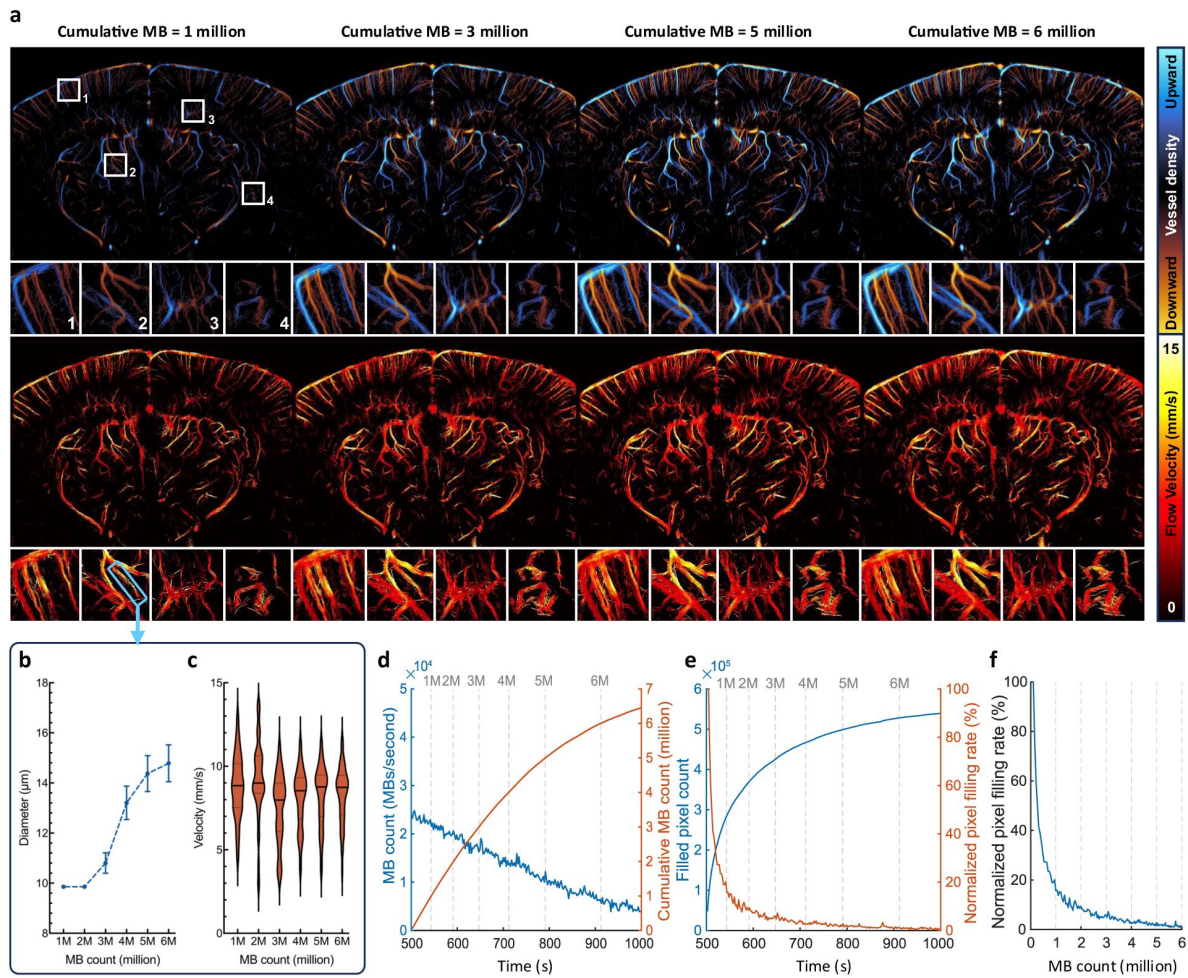


Fig. 2

Data processing standards for awake mice ULM imaging.

a, ULM directional vessel density maps and flow velocity maps at cumulative MB count of 1, 3, 5, and 6 million. **b**, Vessel diameter measurements along the selected segment at varying cumulative MB counts. Each point along the vessel centerline provides a measurement, with the mean and standard error of the mean (SEM) plotted to show average diameter and variability. **c**, Flow velocity measurements along the selected segment at varying cumulative MB counts. Each non-zero centerline pixel provides a velocity value, with the distribution across positions shown as a violin plot. **d**, Time courses of MB count in each second (blue curve) and the cumulative MB count (orange curve) starting from $T = 500\text{s}$. The vertical gray dashed lines indicate the time points when the cumulated MB count reaches 1, 2, 3, 4, 5, and 6 million. **e**, Time courses of saturation level of the ULM image (blue curve) and the filling rate of pixels (orange curve). The filling rate is calculated by taking the derivative of the filled pixel count, and then normalized to the initial filling rate at the beginning of ULM reconstruction ($T = 500\text{s}$). **f**, Relationship between pixel filling rate and cumulative MB count, eliminating the time axis by plotting the orange curves from **d** and **e** together. This figure presents a case study based on the same mouse shown in **Fig 1**. The x-axis for **d-f** begins at 500 seconds because, at this point, the mouse's pupil size stabilized, indicating it had recovered to an awake state. Consequently, ULM images were accumulated starting from this time. It is important to note that not every mouse requires 500 seconds to fully awaken; the time to reach a stable awake state varies across individual mice.

Focusing on ULM flow speed maps, a global reduction in flow speed can be clearly observed when transitioning from anesthetized to awake. Regional maps further revealed the significant flow speed reduction for most of the vessels throughout the brain (e.g., blue arrows).

To quantitatively compare the results from **Fig. 3**, we performed various analyses in **Fig. 4**. Since previous studies have shown that isoflurane induces different effects on arteries and veins^{20,40,41}, we first selected a cortical artery and vein for comparison. In **Fig. 4a**, a cortical region near Region 3 from **Fig. 3** was selected, and one artery and one vein in this region were analyzed. **Fig. 4b** compares the vessel diameters of these segments in anesthetized and awake states. For the selected artery, no significant change in diameter was observed, with both anesthetized and awake states showing an average diameter of 22 μm . In contrast, the selected vein displayed a notable response to isoflurane, with its diameter decreasing significantly from 54 μm under isoflurane anesthesia to 41 μm in the awake state. Flow velocity analysis of the same vessels further suggests that isoflurane may have a greater impact on venous blood flow velocity: arterial flow velocity increased slightly from 7.33 mm/s under anesthesia to 8.04 mm/s in the awake state, whereas venous flow velocity decreased from 10.48 mm/s to 8.11 mm/s, with both the magnitude and statistical significance of the decrease greater in the vein.

While single-vessel analysis provides valuable insights, its generalizability is limited. To validate the broader applicability of our findings, we conducted ROI-based analyses of cortical arteries and veins, using vascularity and mean velocity as primary metrics. The relationship between arteriovenous classification and blood flow direction is more straightforward to infer on cortex, with downward flow representing cortical arteries and upward flow representing cortical veins¹⁶. **Fig. 4d** shows, for Mouse 1, the comparison of cortical upward (venous) and downward (arterial) flow density maps under different states. **Supplementary Fig. 3** also provides a detailed comparison of upward and downward flow for all three mice in both anesthetized and awake conditions. **Fig. 4e** demonstrates that transitioning from isoflurane anesthesia to the awake state led to a reduction in vascularity for both arteries and veins within the selected cortical ROI across all three mice. However, the decrease in venous vascularity (averaging 55% across mice) was greater than that of arterial vascularity (averaging 35%). **Fig. 4f** further illustrates the change in mean velocity within the selected ROI, where both arteries and veins showed a decreasing trend; however, venous flow velocity reduced by an average of 38%, compared to a 19% reduction in arterial flow velocity across the cortical ROI. These results underscore the different responses of arteries and veins to isoflurane anesthesia, which is also indicated by previous studies^{20,40,41}.

To visualize flow velocity changes more clearly, we highlighted the flow velocity comparison for the same region in **Fig. 4g** and provided a violin plot of velocity estimates within the ROI in **Fig. 4h**. The violin plots reveal a consistently greater reduction in venous flow velocity from anesthesia to the awake state across all three mice.

For deeper brain regions, flow analyses were conducted without separating arteries and veins, as this physiological separation is not established for ULM in these regions. **Fig. 4i** displays the selected ROIs for each mouse, while **Fig. 4j** and **4k** present the trends in vascularity and flow velocity changes across different brain regions. Results indicate that vascularity and flow velocity decreased across the brain as mice transitioned from anesthetized to awake states, with subcortical ROIs showing more pronounced changes than cortical ROIs. For example, in the thalamus and midbrain regions, average vascularity decreased by 37% and average flow velocity by 17%, whereas cortical ROIs exhibited smaller decreases of 4% in vascularity and 11% in flow velocity. This work is consistent with a previous fMRI study, where it was also observed that isoflurane-induced cerebral hyperemia was not most pronounced in the cerebral cortex compared with other deeper brain regions⁴².

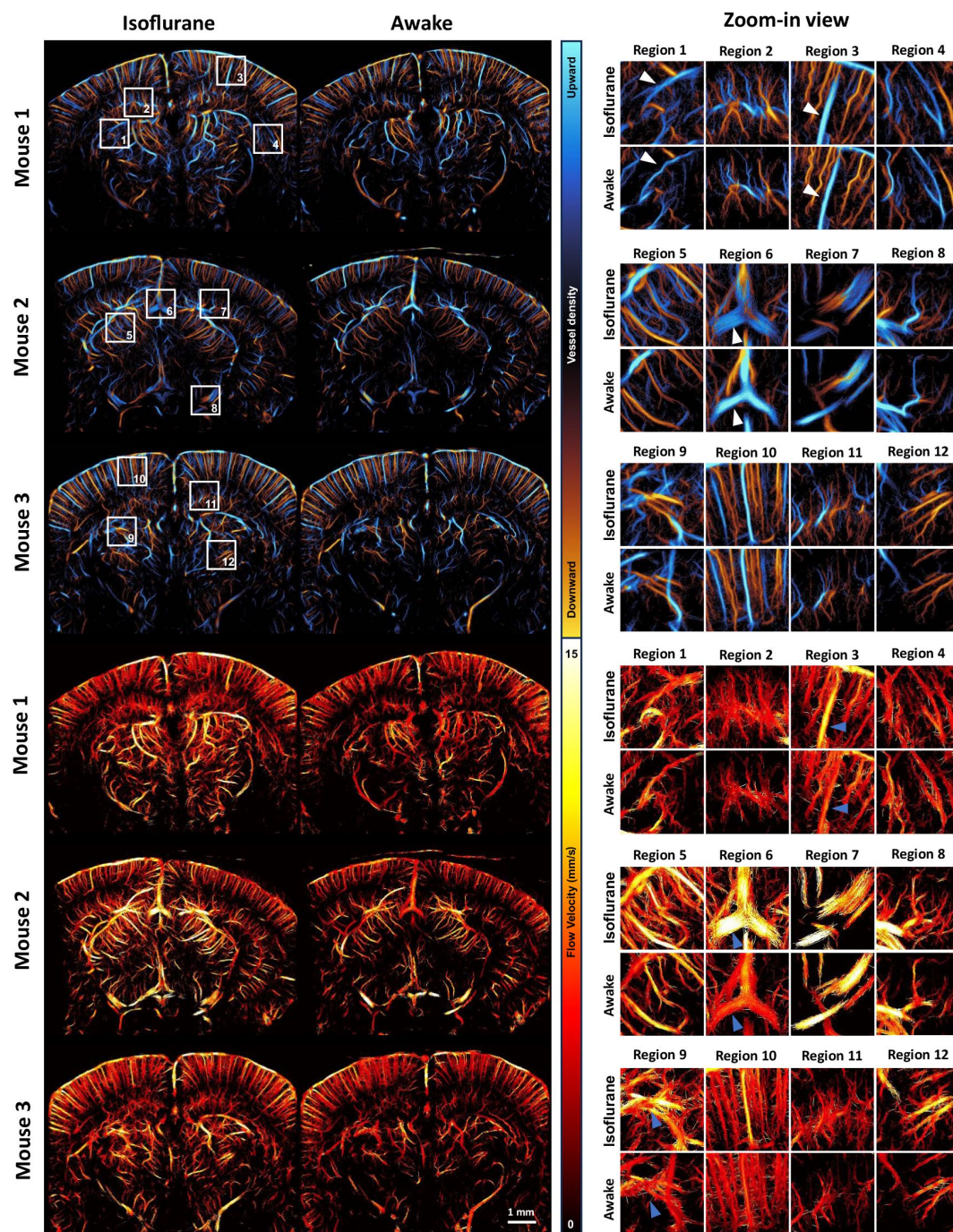


Fig. 3

Comparison of ULM images in isoflurane anesthetized and awake states.

The ULM images of three different mice are shown. The upper panel shows the comparison of directional vessel density maps, while the lower panel shows the comparison of flow velocity. Four regions of interest (ROIs) are selected within each coronal plane (indicated by white dashed boxes in the whole-plane view of the vessel density map) for zoom-in comparison.

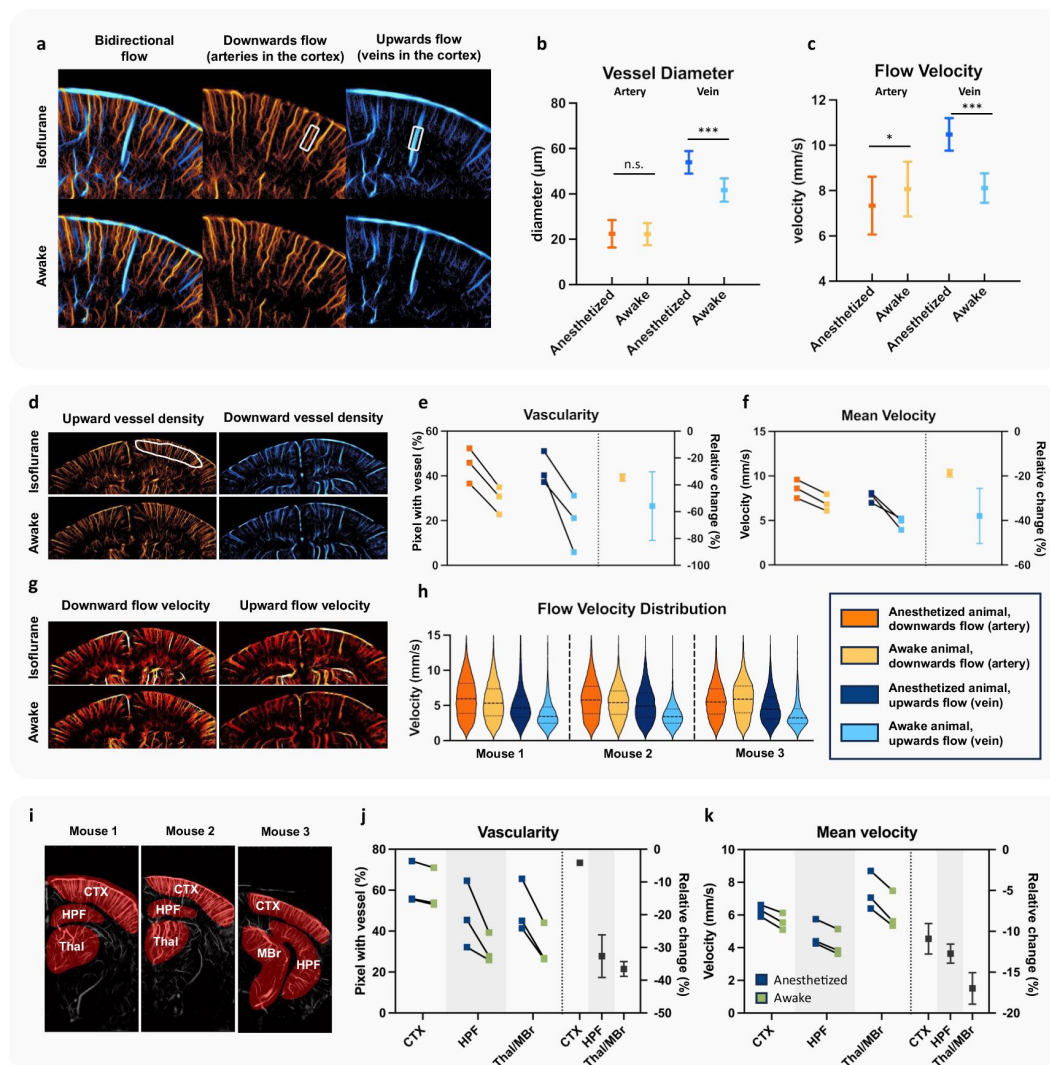


Fig. 4

Quantitative comparison of the ULM images in isoflurane anesthetized and awake states.

a, Example cortical region near Region 3 in Mouse 1 (from **Fig. 3**), with vessel density maps displayed separately by flow direction. In this cortical region, upward flow corresponds to venous blood, while downward flow corresponds to arterial blood. One artery and one vein segment were selected within the boxed areas for analysis of flow velocity and diameter. **b,c**, Comparisons of vessel diameter (**b**) and flow velocity (**c**) for the selected arterial and venous segments. Statistical analysis was conducted using t-test at each measurement point along the segments. **d**, Whole cortical region in Mouse 1, showing separate blood flow density maps for upward (venous) and downward (arterial) flow, with a defined ROI (white circled region) used for quantitative analysis. **e,f**, Quantitative assessment of vascularity (**e**) and mean flow velocity (**f**) within the cortical ROI for Mouse 1–3, presented as individual values in each state (left y-axis) and the relative reduction from the anesthetized baseline to the awake state (right y-axis). **g**, Flow velocity map for the same cortical region shown in **d**, using Mouse 1 as an example. **h**, Flow velocity distributions within the cortical ROI for Mouse 1–3, comparing anesthetized and awake states. **i**, Regional analysis across different brain areas using bidirectional ULM maps, with selected ROIs from the cortex (CTX), hippocampal formation (HPF), thalamus (Thal), and midbrain (MBr) for each mouse. **j,k**, Quantitative analysis of vascularity (**j**) and mean blood flow velocity (**k**) for the ROIs in each brain region in Mouse 1–3. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

In summary, statistical analysis revealed a decrease in individual vessel diameter, vascularity, and blood flow velocity (particularly in venules) after awakening. These findings align with existing research, indicating higher blood perfusion during isoflurane anesthesia^{20,40,41,43,44}.

Awake ULM imaging demonstrates high consistency in longitudinal imaging across different weeks

Longitudinal awake ULM brain imaging was feasible using the surgical and imaging techniques presented in this study. **Fig. 5** presents the results of awake brain imaging performed on the same brain region over three consecutive weeks. Two ROIs at different depths were selected for each mouse to compare micro vessel reconstruction across different time points. **Fig. 5** demonstrates a high level of consistency in both directional vessel density maps and flow speed maps obtained from the three imaging sessions, although some minor discrepancies were observed. The inconsistency could potentially be attributed to physiological variation and/or slight misalignment.

To quantitatively evaluate the consistency of ULM imaging in longitudinal studies, we conducted a similar analysis to that shown in **Fig. 4**. First, a case study was performed on individual vessels by selecting an artery and a vein in the cortex of Mouse 4 (**Fig. 6a**) and measuring vessel diameter (**Fig. 6b**) and flow velocity (**Fig. 6c**) along the selected segments. The results show small variation in measurements across weeks. For vessel diameter, both arterial and venous segments demonstrated statistical equivalence across the three weekly measurements. Statistical testing of equivalence was conducted using the two one-sided test (TOST) procedure, which evaluates the null hypothesis that the difference between the two weeks is larger than three times the standard deviation of one week. Large p-values from the TOST analyses indicate rejection of the null hypothesis, supporting that the measurements are equivalent. For flow velocity, apart from a slight decrease in the venous flow during the second week (4.49 mm/s, compared to 6.03 mm/s in the first week and 6.00 mm/s in the third week), no other significant differences were observed. Overall, quantitative measurements maintained high consistency, though slight variations were expected due to differences in probe positioning and physiological variations among the mice.

To further generalize these findings and examine longitudinal variation in ROI-based analysis, we used Mouse 4 as an example to show the consistency of blood flow density across different flow directions in the cortex (**Fig. 6d**) and extended the quantitative analysis to all three mice (**Fig. 6e**). In **Supplementary Fig. 4**, we present a comparison of upward and downward flow images for all three mice over the three-week longitudinal study period. In the comparative study shown in **Fig. 4**, clear differences were seen between arterial and venous responses under various conditions, while in the longitudinal study, both arteries and veins exhibited similar levels of variation. For instance, in Mouse 6, both arterial and venous vascularity showed a notable decrease from around 60% in the first week to around 40% in the second week, which is a relatively large variation compared with other measurements. This quantitative analysis aims to inform readers of the expected range of variation of this technique. **Fig. 6f** displays the flow velocity map for the same region as in **Fig. 6d**, with flow velocity distributions inside the selected ROI for all three mice shown in **Fig. 6g**. While arterial and venous flow velocity distributions exhibit clear distinctions, their variations over the three weeks remained acceptable. Even in the previously mentioned case of Mouse 6 during Week 1 and Week 2, arterial mean flow velocity only shifted from 6.31 mm/s in the first week to 7.00 mm/s in the second week.

We also conducted ROI analyses without distinguishing between arteries and veins for subcortical regions (**Fig. 6h–k**), using the same ROIs as those shown in **Fig. 5**. Although subcortical regions show larger difference to anesthesia-induced changes, they maintained relatively stable values in the longitudinal study. In terms of vascularity (**Fig. 6i**), Mouse 5 exhibited the greatest variation among the three mice, with vascularity increasing from 55% in the first week to 67% in

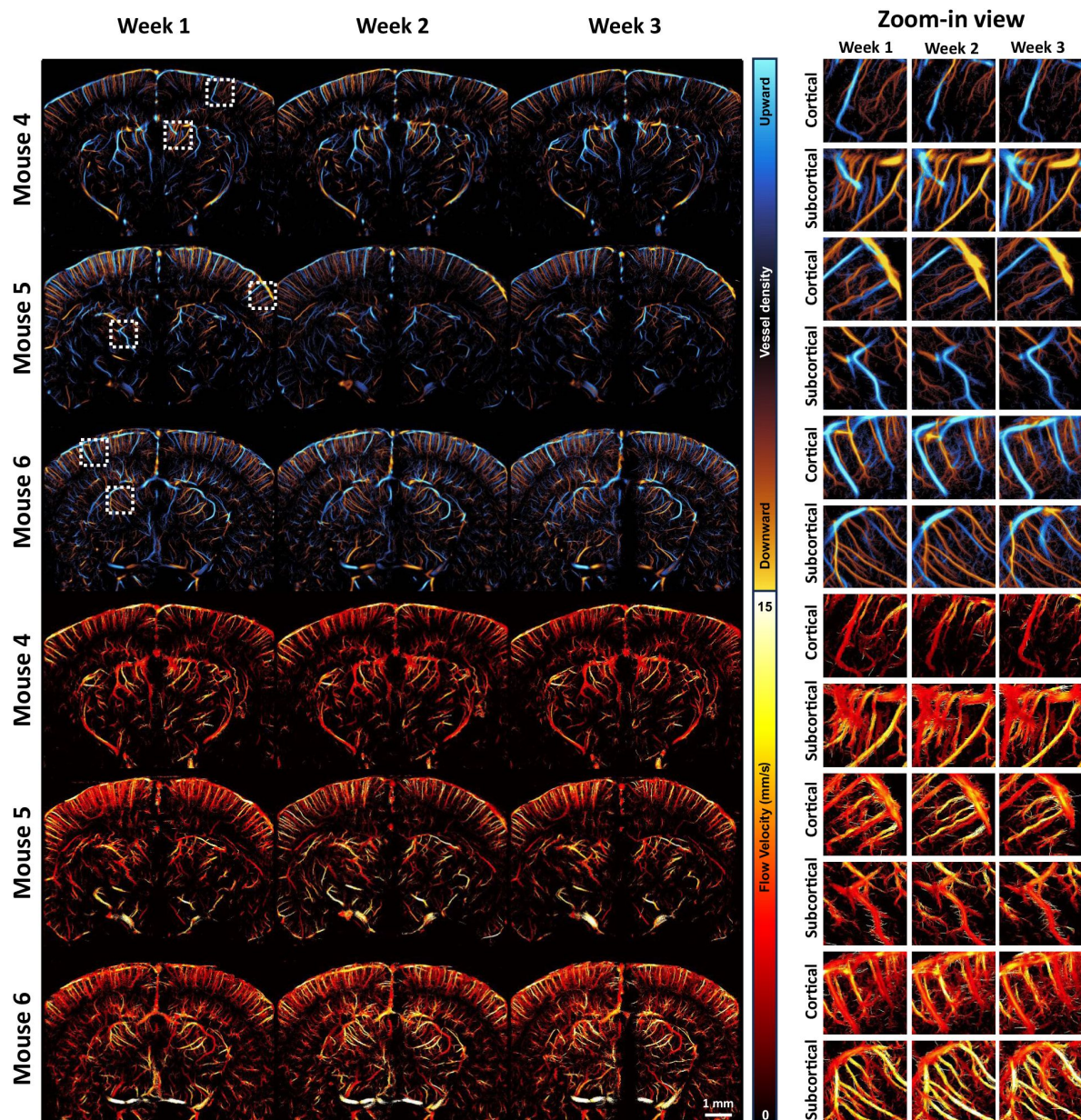


Fig. 5

Longitudinal awake ULM imaging results on the same coronal plane for three consecutive weeks.

The ULM images of three different mice are shown. The upper panel shows the comparison of directional vessel density maps, while the lower panel shows the comparison of flow velocity. Two regions of interest (a cortical ROI and a sub cortical ROI) are selected within each coronal plane (indicated by white dashed boxes in the whole-plane view of the vessel density map) for zoom-in comparison.

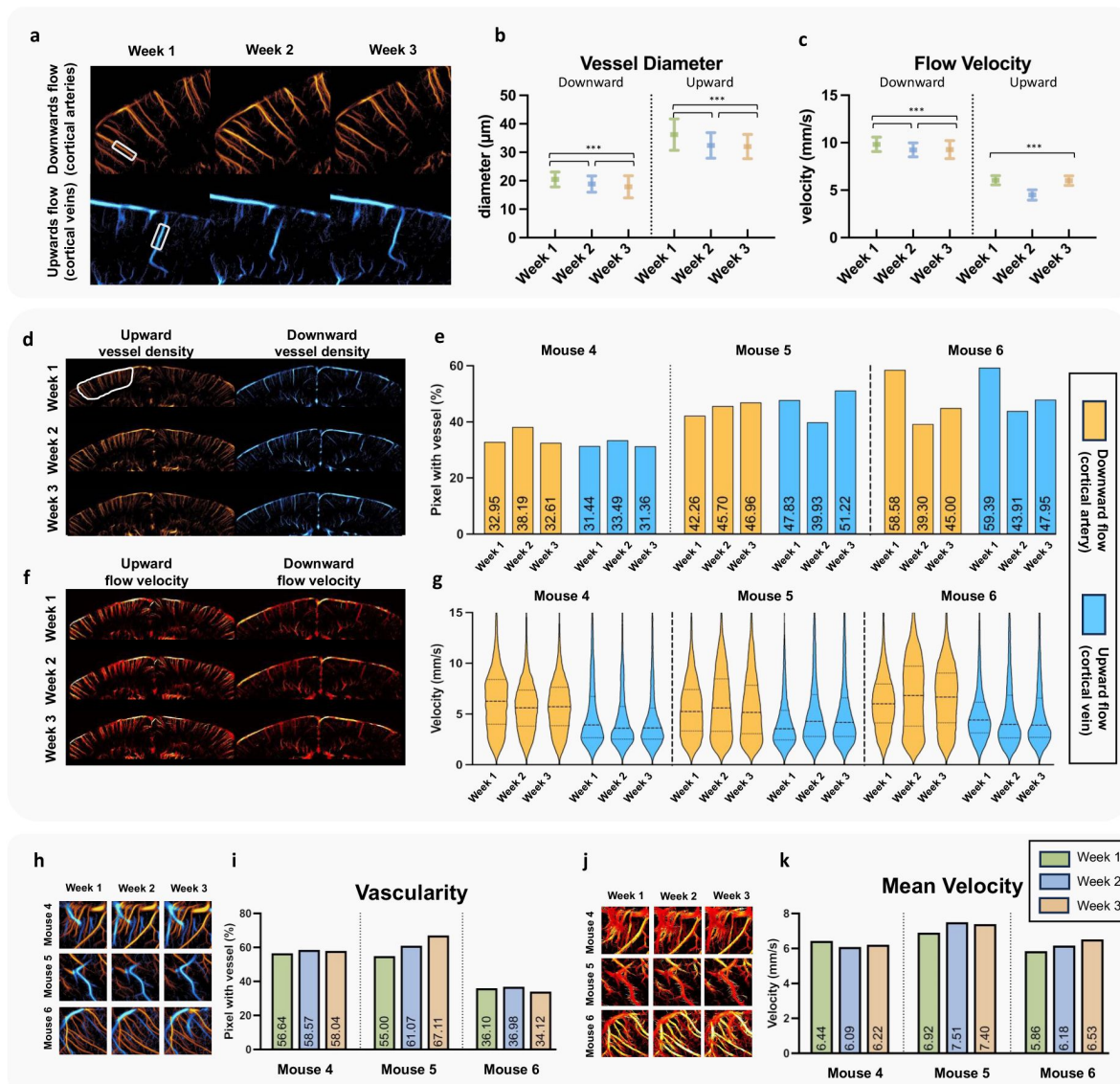


Fig. 6

Analysis of ULM images acquired in the three consecutive weeks.

a, Two example cortical regions in Mouse 4 (from Fig. 5), with one for artery selection and the other for vein. The vessel segments were selected within the boxed areas for analysis of flow velocity and diameter. **b, c**, Comparisons of vessel diameter (**b**) and flow velocity (**c**) for the selected arterial and venous segments. Statistical analysis was conducted using the two one-sided test (TOST) procedure, which evaluates the null hypothesis that the difference between the two weeks is larger than three times the standard deviation of one week. **d**, Whole cortical region in Mouse 4, showing separate blood flow density maps for upward (venous) and downward (arterial) flow, with a defined ROI (white circled region) used for quantitative analysis. **e**, Bar plot for the vascularity comparison of the cortical artery and vein among Mouse 4-6. **f**, Flow velocity map for the same cortical region shown in **d**, using Mouse 4 as an example. **g**, Flow velocity distributions within the cortical ROI for Mouse 4-6, comparing arterial and venous flow across three weeks. **h**, Bidirectional ULM vessel density map of subcortical ROIs for the three different mice. **i**, Bar plot for the vascularity comparison of the subcortical ROIs in **h**. **j**, ULM flow velocity map of the same subcortical ROIs for the three different mice. **k**, Bar plot for the mean velocity comparison of the subcortical ROIs. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

the third week. Compared to the maximum 40% reduction seen from anesthesia to awake states in **Fig. 4j**, vascularity measurement in subcortical regions remained consistent. This observation also applied to mean velocity measurements.

Discussion

In this study, we introduced a method for performing ULM brain imaging in awake mice under a longitudinal study setting. Our method enabled high-resolution imaging of deep cerebral microvasculature with the animal under the awake state. We translated the awake imaging techniques previously described in fUS³³ to our study to enable awake ULM, and established a quantitative metric for ULM image reconstruction. Based on the setup above, we studied CBF changes induced by anesthesia, which aligned well with literature. Isoflurane has been shown to increase vascular diameter and CBF in mice, as validated by multiple imaging modalities including optical coherence tomography⁴⁰, photoacoustic microscopy⁴¹, two-photon microscopy⁴³, and laser speckle imaging^{20,44}. These effects have also been validated in larger animal models such as rats⁴², dogs⁴⁵, and marmosets⁴⁶. In human, vasodilation and increased CBF caused by volatile anesthetics such as isoflurane have also been reported²².

Statistical analysis from **Fig. 4** shows that certain vessels exhibit a larger diameter under isoflurane anesthesia, and the vascularity, calculated as the percentage of vascular area within selected brain region ROIs, is also higher in the anesthetized state. These findings suggest a vasodilation effect induced by isoflurane, consistent with existing research^{20,40,41,43,44}. It is worth noting that although our data indicate a global elevation of CBF under isoflurane anesthesia, individual vessels exhibit large discrepancies in behavior. For example, the vessel at the left lower corner from Mouse 3 in the entorhinal cortex (**Fig. 3**) shows almost no blood flow during anesthesia but then exhibits high vessel pixel density after awakening. The wide range of vessel behaviors were also previously reported in literature^{20,46}. Our results indicate that awake ULM imaging has ample spatial resolution and imaging depth of penetration to resolve individual vessel variations down to micron-sized vessels deep into the brain. This is a unique capability that is not available from other biomedical imaging modalities.

Increased blood flow velocity induced by isoflurane has also been reported by other studies^{40,41}. However, previous research presented different speculations on the predominant factor contributing to the increase in CBF induced by anesthesia, specifically whether the increase is attributed to vasodilation or increase in blood flow velocity²⁰. One study found significant changes in both blood flow and vessel diameter but minor changes in flow velocity, suggesting that the increase in blood flow was largely driven by vasodilation⁴⁰. Conversely, another study drew the opposite conclusion²⁰. Benefitting from the large field of view (FOV) of ULM and its capability to directly quantify microvascular blood flow velocity, we can make a more comprehensive inference regarding the relationship among the changes in vessel diameter, flow speed, and flow volume from anesthetized to awake states. For arteries, the change in blood flow velocity is not significant, indicating that the alteration in blood flow may be primarily due to vasodilation instead of velocity change. Isoflurane causes vasodilation by acting on the ion channels (e.g., potassium channel) of smooth muscle⁴⁵, which is more abundantly found in arteries than in veins. In the case of veins, which do not actively dilate or constrict, their vessel diameter and blood flow variations are more likely controlled by passive mechanisms. **Fig. 5** reveals significant differences in flow velocity of veins between anesthesia and awake state, suggesting that the changes in flow velocity may have a greater impact on venous blood flow volume compared with arterial volume.

The differences in cerebral vasculature between anesthetized and awake states observed using ULM are also in agreement with other studies^{20,40,41,44}. However, previous studies mostly used optical imaging techniques, which have limited penetration depth and can only observe

surface pial vessels in the cortex. Some other studies using fMRI can detect deeper CBF changes in the whole brain, but they do not provide insights about small vessel blood flow variations due to insufficient spatial resolution⁴². As a bridging imaging modality between MRI and optical techniques, awake ULM enables observations of detailed microvascular variations induced by anesthesia across the whole depth of the brain, which provides complementary information to existing biomedical imaging modalities.

Although isoflurane is widely used in ultrasound imaging because it provides long-lasting and stable anesthetic effects, it is important to note that the vasodilation observed with isoflurane is not representative of all anesthetics. Some anesthesia protocols, such as ketamine combined with medetomidine, do not produce significant vasodilation and are therefore preferred in experiments where vascular stability is essential, such as functional ultrasound imaging⁴⁷. Therefore, in future studies, it would be valuable to design more rigorous control experiments with larger sample sizes to systematically compare the effects of isoflurane anesthesia, awake states, and other anesthetics that do not induce vasodilation on cerebral blood flow.

Our proposed method enabled repeatable longitudinal brain imaging over a three-week period, addressing a key limitation of conventional ULM imaging and offering potential for various preclinical applications. However, there are still some limitations in this study.

One of the limitations is the lack of objective measures to assess the effectiveness of head-fix habituation in reducing anxiety. This may introduce variability in stress levels among mice. Recent studies suggest that tracking physiological parameters such as heart rate, respiratory rate, and corticosterone levels during habituation can confirm that mice reach a low stress state prior to imaging⁴⁸. This approach would be highly beneficial for future awake imaging studies. Furthermore, alternative head-fixation setups, such as air-floated balls or treadmills, which allow the free movement of limbs, have been shown to reduce anxiety and facilitate natural behaviors during imaging³⁰. Adopting these approaches in future studies could enhance the reliability of awake imaging data by minimizing stress-related confounds.

Another limitation of this study is the potential residual vasodilatory effect of isoflurane anesthesia on awake imaging sessions. The awake imaging sessions were conducted shortly after the mice had emerged from isoflurane anesthesia, required for the MB bolus injections. The lasting vasodilatory effects of isoflurane may have influenced vascular responses, potentially contributing to an underestimation of differences in vascular dynamics between anesthetized and awake state. Future applications of awake ULM in functional imaging using an indwelling jugular vein catheter presents a promising alternative to enable more accurate functional imaging in awake animals, addressing current limitations associated with anesthesia-induced vascular effects.

In our longitudinal study, consistent imaging results were obtained over a three-week period, demonstrating the feasibility of awake ULM imaging for this duration. However, for certain research applications, a monitoring period of several months would be valuable. Extending the duration of longitudinal awake ULM imaging to enable such long-term studies is a potential direction for future development.

Tissue motion is also a critical concern of ULM imaging. Benefiting from the robust head-fixing protocols and successful habituation, we did not observe significant motion artifacts in the ULM images. Nevertheless, we still employed the correlation method to register the ULM images⁴⁹ in order to remove subtle tissue movement and improve the ULM image quality. This method preserves the maximum fidelity for the MB tracks, but may not be adequate for non-rigid motion. While rigid motion correction is often effective in anesthetized animals, awake animal imaging presents greater challenges due to the more prominent non-rigid motion, particularly in deeper brain regions. This is evidenced in **Supplementary Fig. 1** (Mouse 7), where cortical vessels

remain relatively stable, but regions around the colliculi and mesencephalon exhibit more noticeable motion artifacts, indicating that displacement is more pronounced in deeper areas. To address these deeper, non-rigid motions, recent studies suggest estimating non-rigid transformations from unfiltered tissue signals before applying corrections to ULM vascular images^{16,50}. Such advanced motion correction strategies may be more effective for awake ULM imaging, which experiences higher motion variability. The development of more robust and effective motion correction techniques will be crucial to reduce motion artifacts in future awake ULM applications. Meanwhile, with 2D imaging, we cannot correct for out-of-plane motion, which necessitates 3D imaging. In the future, 3D motion correction techniques that account for complex tissue motions and are computationally efficient need to be developed for awake and longitudinal ULM imaging.

Based on the maximum linking distance and gap closing parameters outlined in the Methods section, blood flow with velocities below 50 mm/s can be detected. However, the use of a directional filter to estimate flow direction may introduce aliasing. MBs moving at higher velocities may be subject to incorrect flow direction estimation due to aliasing effects. Given that the compounded frame rate is 1000 Hz, with an ultrasound center frequency of 20 MHz and a sound speed of 1540 m/s, the relationship between Doppler frequency and the axial blood flow velocity¹² indicates that aliasing will not occur for axial flow velocities below 19.25 mm/s. In all flow velocity maps presented in this study, the range is limited to a maximum of 15 mm/s, remaining below the critical threshold for aliasing. Additionally, all vessels analyzed in the violin plots for arteriovenous flow comparisons fall within this range. While cortical arterioles and venules generally exhibit moderate flow speeds, aliasing remains a factor to consider when combining directional filtering with velocity analysis.

Advances in ULM imaging methods can benefit longitudinal awake imaging. For instance, dynamic ULM can differentiate between arteries and veins by leveraging pulsatility features⁵¹. 3D ULM, with volumetric imaging array^{52,53}, enables the reconstruction of whole-brain vascular network, providing a more comprehensive understanding of vessel branching patterns. Meanwhile, 3D ULM also helps to mitigate the challenge of aligning the identical coronal plane for longitudinal imaging, a process that requires precise manual alignment in 2D ULM to ensure consistency. Additionally, this alignment issue can also be alleviated in 2D imaging using backscattering amplitude method, which may assist in estimating out-of-plane positioning during longitudinal imaging⁵⁴.

Longitudinal brain imaging in the awake state offers a promising tool for neuroscience research as it not only avoids the confounding effects of anesthesia on cerebral vasculature, but also enables observations of intrinsic dynamics of the vasculature within the same subject, minimizing potential sources of bias associated with inter-subject variability. In the future, this technique is expected to be further integrated with disease models to study the changes in cerebral vasculature during the development of diseases. Also, this technique can be further combined with the latest functional ULM (fULM) studies¹⁶ to allow awake fULM imaging. Our study laid the foundation for these studies with awake fULM, which is expected to improve the sensitivity of conventional fULM techniques because hemodynamic responses are much stronger in the awake state than in anesthesia^{24–26}.

Methods

Animal preparation

Six healthy female C57 mice (8–12 weeks) were used for this study. All experimental procedures were conducted in accordance with the guidelines set by the University of Illinois Institutional Animal Care and Use Committee (IACUC Protocol number #22165). The animals were housed in an

environment with a 12-hour light/dark cycle and had free access to food and water. Prior to the cranial window surgery, the mice were kept in group housing, and they were individually housed post-surgery.

Pre-surgery handling

A week before performing the cranial window surgery, the animals underwent tunnel handling, a procedure shown to significantly reduce the levels of anxiety in mice^{55,56}. Specifically, a commercially available polycarbonate mouse transfer tube (TRANS-TUBE 130X50MM, Braintree Scientific, Inc.) was used for the initial three days. The mice were encouraged to enter the tube from their cage, after which the tube was lifted, allowing the mouse to remain inside for 30 seconds. The mouse was then returned to its cage for free movement for one minute, before a second identical tunnel handling was carried out. This procedure was done twice daily. Approximately three days into this routine, the mice were accustomed to the tunnel handling method. The animals were then picked up using the commercial tunnel, and then a 3D-printed body tube was attached to the other end of the tunnel. The mice were allowed to enter the body tube voluntarily. Subsequently, the body tube was utilized for handling the mice in the following 5 days as a replacement for the commercial tunnel.

Cranial window surgery

The entire procedure closely followed a previously published protocol³³. To minimize brain swelling, the animal received an intraperitoneal injection of Dexamethasone (0.5 mg/kg body weight). The mouse was anesthetized by inhalation of isoflurane (3% for induction and 1%-1.5% for maintenance). After confirming that the mouse was anesthetized, the head of the animal was fixed in the stereotaxic frame. Afterward, the scalp of the mouse was incised, and the temporalis muscle was dissected from the skull using forceps. Tissue adhesive (3M, cat. no. 70200742529) was applied to secure the retracted muscles and incision edges, ensuring proper closure of the skin. Subsequently, a headpost was fixed to the skull using tissue adhesive and dental cement (Sun Medical Co., Ltd, cat. no. Super-Bond C&B). The skull was then carefully thinned using a high-speed rotary micromotor (Freedom, cat. no. K.1070) to create a cranial window with a width of approximately 8 mm laterally and which extending from bregma to lambda in the anterior-posterior direction. The skull was continuously thinned along this outline until it became loose, allowing for separation of the bone from the dura mater using forceps. After removing the skull, a polymethyl pentene plastic sheet (Goodfellow, cat. no. ME311051) was placed over the cranial window and fixed to the bone using tissue adhesive and dental cement. To provide protection, the window was covered with biocompatible silicone rubber (Smooth-on, cat. no. Body Double-Fast Set). Once the silicone rubber was secure, the anesthesia was discontinued, and the mouse was allowed to regain consciousness.

Post-surgical care and head-fix habituation

Following the surgery, subcutaneous administration of Carprofen (5-10 mg/kg body weight) was provided to the animals for immediate post-operative pain management. The animals were allowed to recover from anesthesia in their individual cages, with a heating pad provided to maintain optimal body temperature during the recovery period. Observations were made every 15 minutes until the animals reached sternal recumbency. To aid in their recovery, dry food soaked in water was provided, along with the use of recovery gel (ClearH2O, cat. no. 72-06-5022) to facilitate chewing and hydration. In the case that any signs of pain were observed, additional subcutaneous doses of 5-10 mg/kg Carprofen were administered every 12 to 24 hours to alleviate surgical discomfort for 3 days. Post-operative monitoring of the animals was performed daily for a duration of 14 days following the surgery. Once the animals had fully recovered from the surgical procedure, the animals were habituated daily to head fixation after walking through the body tube. The duration of the head-fixation periods was gradually increased over time, starting at

approximately 10 minutes on day 1 and extending to up to 1 hour after 3 days³³. If any signs of discomfort, such as excessive movement or vocalization, were observed during the head-fixation sessions then the sessions would be immediately discontinued.

The use of 3D-printed body tube

The body tube was secured to the table using four screws on the outer side, while two small screws on the top were used to clamp the headpost. Two semicircular grooves that fit the size of the small screws were made on the headpost (**Fig. 1a**). During the process of head-fixing the animal, the experimenter guided the animal to enter the body tube from the rear. Once the animal protruded its head from the front end, the experimenter manually grasped the headpost, gently restrained the head of the animal, and subsequently aligned the semicircular grooves with the screws to immobilize the animal.

Experimental procedure of imaging sessions

On the day of imaging, the animal was guided to walk into the body tube, and the headpost was firmly secured to the tube. The protective silicone rubber and headpost were cleaned using 70% ethanol, followed by rinsing with sterile saline. Then, forceps were inserted between the silicone rubber and the cement to detach the silicone rubber from the headpost. Due to the challenges of conducting continuous infusion in awake animals, contrast microbubbles were administered via tail vein bolus injection in this study. Animals were anesthetized with isoflurane (3% for induction and 1% for maintenance) before tail vein catheterization to alleviate pain and stress. Ultrasound coupling gel was applied, and the transducer was positioned to find the imaging plane. When the imaging plane was identified, the relative position of the probe and the headpost was recorded, serving as a reference point for subsequent longitudinal studies. B-mode and power Doppler images were also saved as references to facilitate the identification of the same imaging plane in the following weeks.

Once the imaging plane was confirmed, the isoflurane anesthesia was terminated, and 0.1 ml of DEFINITY® (Lantheus, North Billerica, MA) microbubble was administered via the tail vein catheter. The completion of the injection was considered as the starting point, denoted as $T = 0$. From $T = 0$ onwards, continuous ultrasound data was saved. After completion of the imaging session, the cranial window was filled with the same biocompatible silicone rubber described above, allowing approximately 10 minutes for solidification. The headpost was then removed from the body tube, and the animal was returned to its home cage. Imaging sessions were conducted once per week over a three-week period.

For the experiment of comparing differences between anesthesia and awake state (**Fig. 3-5**), once the mice were fixed to the body tube, they were continuously anesthetized with 2% isoflurane for more than 15 minutes. During this process, the tail vein catheterization was completed, and the imaging plane was confirmed. Following the bolus injection of 0.1 ml MB, ULM data under anesthesia was acquired for 1000 seconds. The tail vein catheter was remained in place. After the ULM acquisition under anesthesia, it was essential to wait until no bubbles were circulating within the blood stream before terminating anesthesia. Once the animal approached full arousal, another bolus injection of 0.1 ml MB was administered. The catheter was then removed, and ULM acquisition started (marked as $T = 0$). Other procedures were the same as those described in the previous two paragraphs. For the ULM data collected under anesthesia, since there was no specific time point at which mice achieved full pupillary dilatation, we consistently employed the RF dataset starting from $T = 500$ s until the cumulative MB count reached 5 million for ULM reconstruction to make the MB concentration similar as in the awake cases.

Pupillary recording and measurement

Monitoring the pupillary area of rodents is commonly used to determine their level of arousal⁵⁷. The pupils constrict under isoflurane anesthesia and enlarge upon awakening^{38,58}. In this study, video recording of pupillary area performed using a camera (ace acA800-510um, Basler Inc., Exton, PA) placed in front of the mouse, with timestamps synchronized to the ultrasound acquisition. The ULM imaging session was carried out in a dark room. An infrared (IR) flashlight (EVOLVA Future Technology T20) was positioned above the camera. Since mice are functionally blind to IR wavelengths^{59,60}, the use of IR illumination can minimize the effect of light exposure on pupil changes so that the pupillary area is mostly influenced by anesthesia. The videos recorded by the camera were analyzed using ImageJ software, and the contour of the pupil was manually circled. The equivalent area of a single pixel was calibrated, which allowed for the quantification of the pupillary area according to the number of pixels occupied by the pupil.

Ultrasound imaging sequence

A Vantage 256 system (Verasonics Inc., Kirkland, WA) was used for this experiment. The ultrasound system was connected to a linear-array transducer (L35-16vX, Verasonics Inc.), which was fixed onto a 3D-printed holder attached to a translation motor (VT-80, Physik Instrumente, Auburn, MA). The motor allowed precise control of transducer movement in the elevational direction, enabling adjustment of the imaged coronal plane. Ultrasound was transmitted at a center frequency of 20 MHz. A 9-angle compounded plane wave technique was used (angles: -8°, -6°, -4°, -2°, 0°, 2°, 4°, 6°, and 8°), with a post-compounded frame rate of 1000 Hz. Interleaved sampling is employed to capture high-frequency echoes more effectively. With the system's sampling rate limited to 62.5 MHz, the upper limit of the center frequency of the transducer passband is 15.625 MHz. To mitigate aliasing, two transmissions are sent per angle, staggered in time. This approach effectively doubles the sampling rate, ensuring more accurate image reconstruction. The ensemble size of each dataset was 800 frames, and the acquired radiofrequency (RF) data was saved for offline reconstruction. Beamforming was conducted using the Verasonics built-in program to reconstruct the in-phase and quadrature (IQ) data. The IQ data had a pixel size of half the wavelength in the axial direction and one wavelength in the lateral direction.

ULM image processing

To ensure consistent localization of bubbles at different depths, a noise equalization method was employed to adjust signal intensity⁶¹. High pass filtering with a cutoff frequency of 30Hz was applied to the IQ data to enhance sensitivity for MB extraction. MBs moving toward and away from the transducer were separated into two distinct datasets (upward flow and downward flow) based on the positive and negative Doppler shifts⁶². Subsequently, singular value decomposition (SVD) filtering was applied to the IQ data to further eliminate clutter signals from tissue and extract MB signals^{63,64}. The singular value cutoff was adaptively determined to achieve objective and stable filtering results⁶⁵. The filtered MB data was then spline interpolated to a resolution with a lateral and axial pixel size of one-tenth of the wavelength (4.928 μm). Each interpolated frame was subjected to 2D normalized cross-correlation with the point spread function of the imaging system, which was empirically determined. Regional maxima in the cross-correlation results indicated the center position of MBs⁴⁹. Thresholding of image intensity was applied to reject low-intensity values and prevent noise from being erroneously identified as bubbles. The coordinates of the microbubble center points obtained from each frame were tracked using the uTrack algorithm^{66,67}. To ensure the detection of reliable MB tracks, only tracks with a minimum length of 10 consecutive frames were considered valid. The choice of 10 consecutive frames (10 ms) was based on established practice¹¹ but can be adjusted as needed⁶⁸. For the uTrack algorithm, two additional key parameters were specified: the maximum linking distance and the gap-filling distance, both set to 10 pixels (~50 microns). This

configuration means that only bubble centroids within 10 pixels of each other across consecutive frames are considered part of the same bubble trajectory. Additionally, when the start and end points of two tracks fall within this threshold, the gap-filling parameter merges them into a single, continuous track. It is important to select these parameters carefully, as overly large values could lead to an overestimation of flow velocity. By setting the maximum linking distance to 10 pixels, we effectively limited the measurable velocity to 50 mm/s, under the assumption that no bubble would exceed a 50-micron displacement within the 1 ms interval between frames. Considering the velocity distribution across the mouse brain⁶⁹, this 50 mm/s limit ensures that most of the blood flow is captured accurately. After determining bubble tracks with the specified parameters for uTrack algorithm, accumulating the MB tracks resulted in the flow intensity map. The final values were square-root transformed to compress the dynamic range of the image for display. Furthermore, the distance traveled by MB between adjacent frames was calculated during the tracking process to determine blood flow velocity, which was then assigned to each individual bubble track. The average velocity computed from multiple tracks was used to generate a comprehensive flow speed map.

Quantitative analysis of ULM images

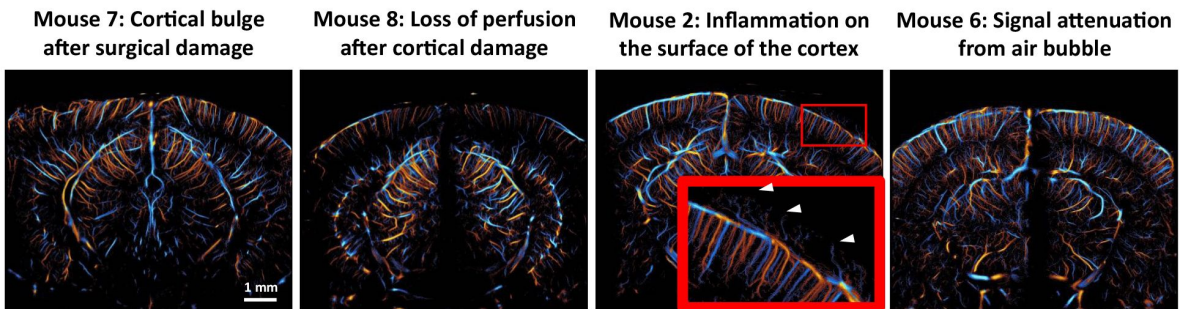
For single-vessel analysis, the vessel density map (either bidirectional or unidirectional) was binarized, and the center line of the vessel was extracted using MATLAB “bwskel” function. The radius of each vessel was defined as the distance from the central line to the nearest vessel edge on the binarized map, with diameter calculated by doubling this radius. Given a pixel size of approximately 4.928 μm , the minimum detectable vessel diameter using this approach is 9.856 μm , explaining why the smallest measured radius in unsaturated conditions, as shown in **Fig. 2b**, always falls into this value. To analyze a specific vessel, we manually selected a vessel segment. Each point on the central line provided a diameter measurement. For flow velocity, each non-zero pixel within the chosen segment was used to estimate effective blood flow velocity in the vessel.

For ROI-based analysis, ROIs were manually defined based on a brain atlas to ensure consistency across both anesthetized and awake states, as well as across weekly imaging sessions, enhancing comparability of results. In ROI-based analysis, we focused on two primary parameters: vascularity and mean velocity. Vascularity was defined as the proportion of the pixel count occupied by blood vessels within each ROI, obtained by binarizing the ULM vessel density maps and calculating the percentage of the pixels with MB signal. Mean velocity was calculated by averaging all non-zero pixel of velocity estimates within the ROI. The velocity distribution within each ROI was also visualized using violin plots, as shown in **Fig. 2c**, **4c** and **6c**, to illustrate the range and density of flow velocity estimates across different acquisition.

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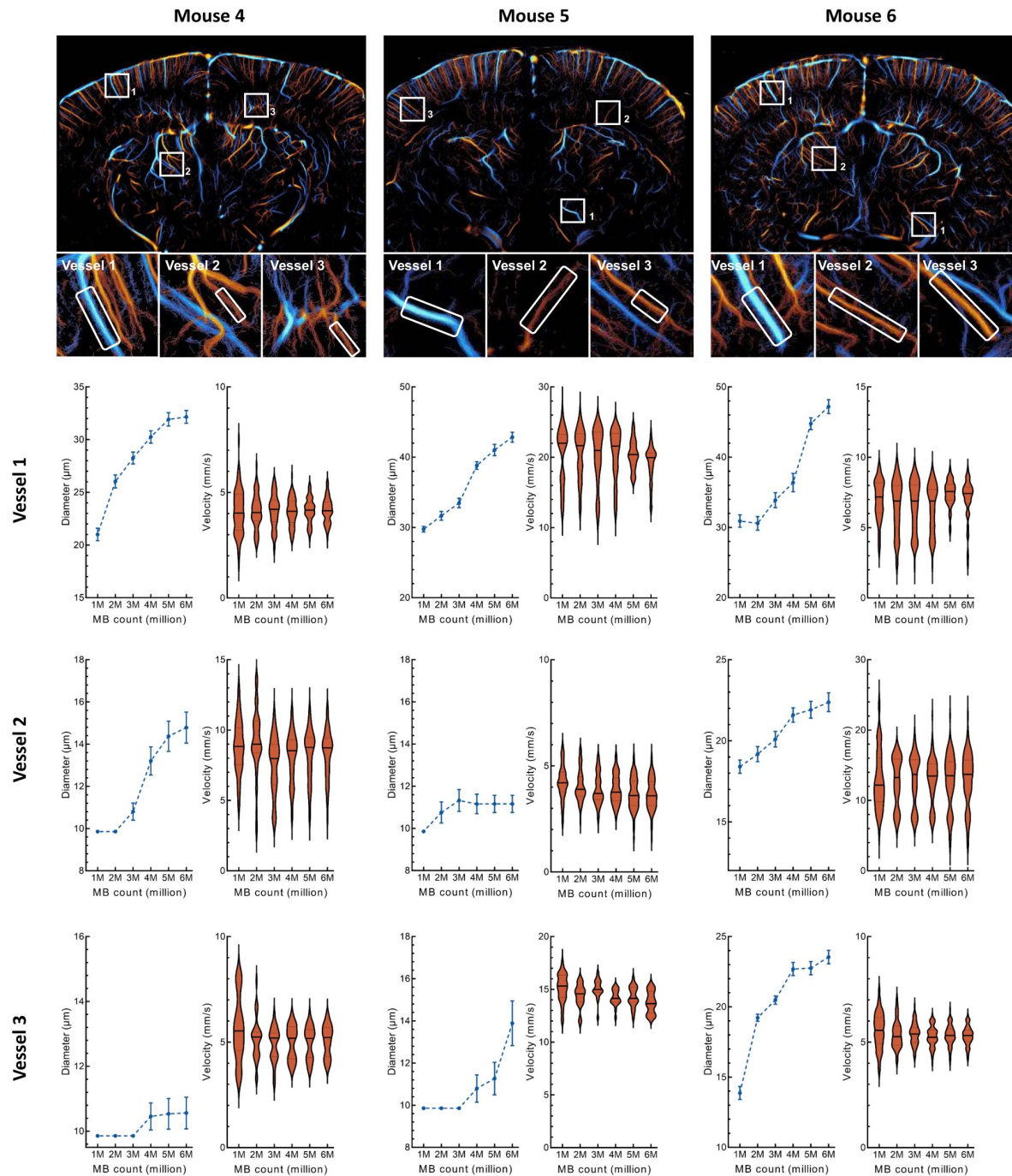
Animal ID	Use of the animal	Corresponding figures
Mouse1	• Comparison between anesthetized and awake imaging	Figure 3 Figure 4
Mouse2	• Comparison between anesthetized and awake imaging • Showcase of surgical defect or problematic ULM image	Figure 3 Figure 4 Supplementary figure 1
Mouse3	• Comparison between anesthetized and awake imaging	Figure 3 Figure 4
Mouse4	• Longitudinal imaging across 3 weeks • Example of demonstrating system schematic and image saturation criteria	Figure 1 Figure 2 Figure 5 Figure 6 Supplementary figure 2
Mouse5	• Longitudinal imaging across 3 weeks • Example of demonstrating image saturation criteria	Figure 5 Figure 6
Mouse6	• Longitudinal imaging across 3 weeks • Example of demonstrating image saturation criteria • Showcase of surgical defect or problematic ULM image	Figure 5 Figure 6 Supplementary figure 1 Supplementary figure 2
Mouse7	• Showcase of surgical defect or problematic ULM image	Supplementary figure 1
Mouse8	• Showcase of surgical defect or problematic ULM image	Supplementary figure 1



Supplementary Fig. 1

Overview of all experimental mice and examples of poor imaging outcomes.

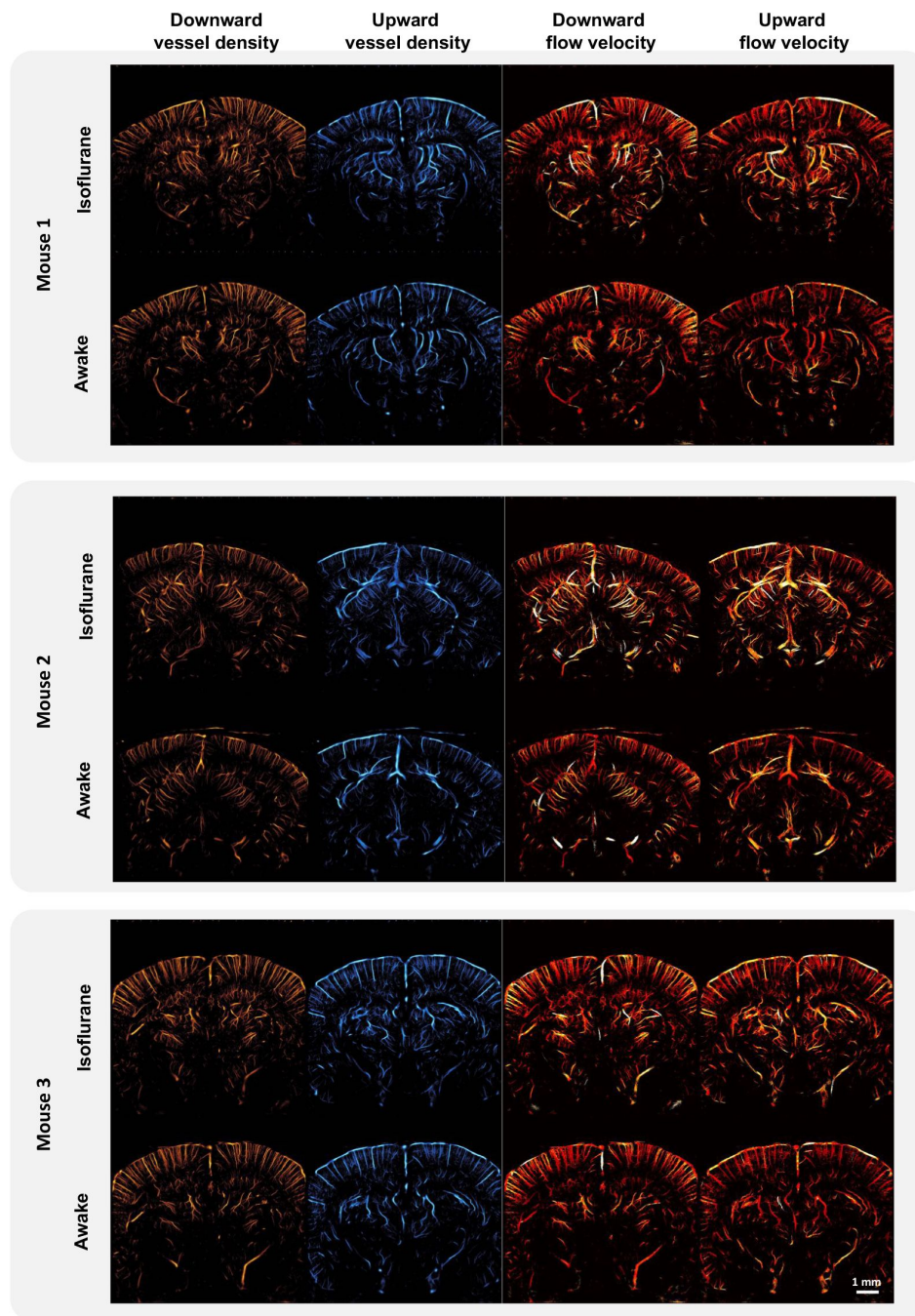
The table details the usage and locations where each experimental mouse is referenced in this study. Not all mice yielded optimal imaging results. Mice with poor imaging outcomes are included as examples of failed cases.



Supplementary Fig. 2

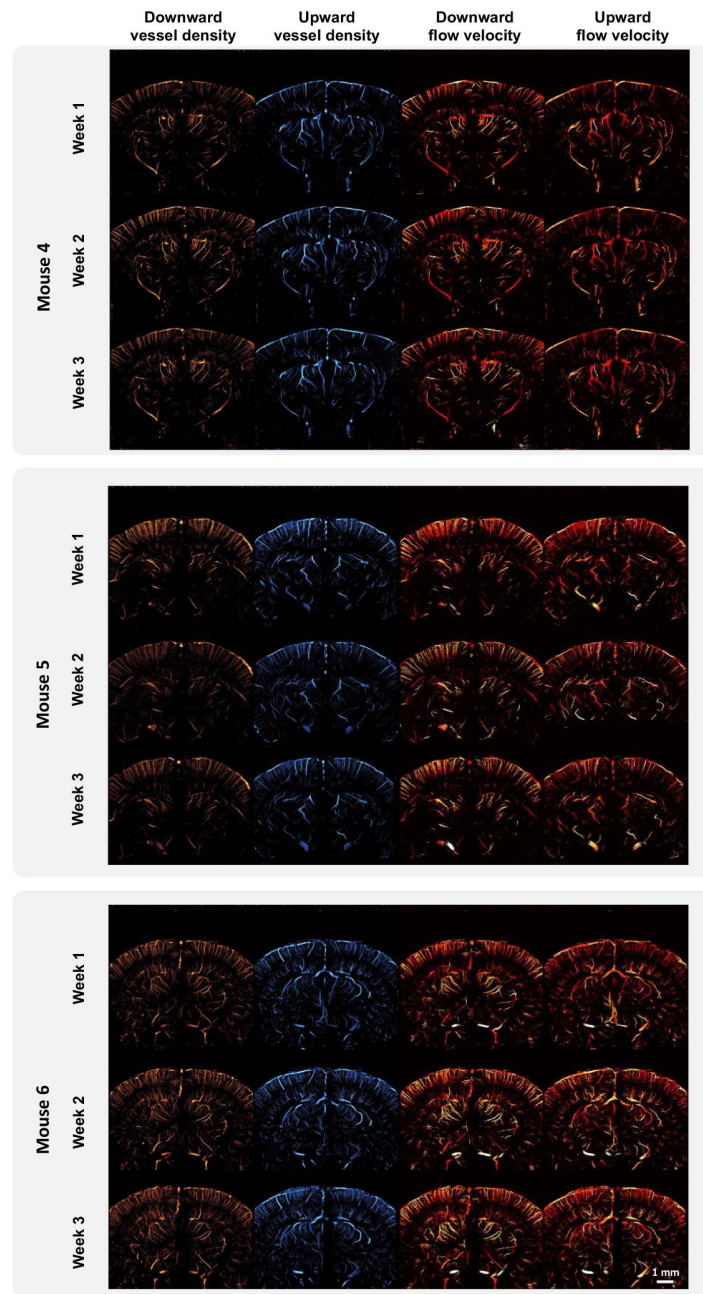
Perfusion under the 5 million saturation standard for various small vessels.

To ensure the findings in [Figure 2b](#) are not specific to a single vessel, vascular segments were selected from three different brain regions in three different mice. Comparisons of vessel diameter and blood flow velocity



Supplementary Fig 3

Comparison of ULM images in isoflurane anesthetized and awake states (Mouse 1 to Mouse 3), with distinctions made between upward and downward flow.



Supplementary Fig 4

Longitudinal comparison of Mouse 4 to Mouse 6 with distinctions made between upward and downward flow.

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Reviewer #1 (Public review):

Summary:

Wang and Colleagues present a study aimed at demonstrating the feasibility of repeated ultrasound localization microscopy (ULM) recording sessions on mice chronically implanted with a cranial window transparent to US. They provided quantitative information on their protocol, such as the required number of Contrast enhancing microbubbles (MBs) to get a clear image of the vasculature of a brain coronal section. Also, they quantified the co-registration quality over time-distant sessions and the vasodilator effect of isoflurane.

Strengths:

Strengths: the study showed a remarkable performance in recording precisely the same brain coronal section over repeated imaging sessions. In addition, it sheds light on the vasodilator effect of isoflurane (an anesthetic whose effects are not fully understood) on the different brain vasculature compartments, although, as the Authors stated, some insights in this aspect have already been published with other imaging techniques. The experimental setting and protocol are very well described.

Wang and co-authors submitted a revised version of their study, which shows improvements in the clarity of the data description.

However, the flaws and limitations of this study are substantially unchanged.

The main issues are:

- Statistics are still inadequate. The TOST test proposed in this revised version is not

equivalent to an ANOVA. Indeed, multivariate analyses should be the most appropriate, given that some quantifications were probably made on multiple vessels from different mice. The 3 reviewers mentioned the flaws in statistics as the primary concern.

- No new data has been added, such as testing other anesthetics.

- The Authors still insist on using the term Vascularity which they define as: 'proportion of the pixel count occupied by blood vessels within each ROI, obtained by binarizing the ULM vessel density maps and calculating the percentage of the pixels with MB signal.'. Why not use apparent cerebral blood volume or just CBV? Introducing an unnecessary and redundant term is not scientifically acceptable. In this revised version, vascularity is also used to indicate a higher vascular density (Line 275), which does not make sense: blood vessels do not generate from the isoflurane to the awake condition in a few minutes. Rev2 also raised this point.

- The long-term recordings mentioned by the Authors refer to the 3-week time frame analyzed in this study. However, within each acquisition, the time available from imaging is only a few minutes (< 10', referring to most of the plots showing time courses) after the animals' arousal from isoflurane and before bubbles disappear. This limitation should be acknowledged.

- The more precise description of the number of mice and blood vessels analyzed in Figure 6 makes it apparent the limited number of independent samples used to support the findings of this work. A limitation that should be acknowledged. The newly provided information added as Supplementary Figure 1 should be moved to the main text, eventually in the figure legends. The limited data in support of the findings was also highlighted by Rev2 and, indirectly, by Rev3.

<https://doi.org/10.7554/eLife.95168.2.sa2>

Reviewer #2 (Public review):

Summary:

The authors present a very interesting collection of methods and results using brain ultrasound localization microscopy (ULM) in awake mice. They emphasize the effect of the level of anesthesia on the quantifiable elements assessable with this technique (i.e. vessel diameter, flow speed, in veins and arteries, area perfused, in capillaries) and demonstrate the possibility of achieving longitudinal cerebrovascular assessment in one animal during several weeks with their protocol.

The authors made a good rewriting of the article based on the reviewers' comments. One of the message of the first version of the manuscript was that variability in measurements (vessel diameter, flow velocity, vascularity) were much more pronounced under changes of anesthesia than when considering longitudinal imaging across several weeks. This message is now not quite mitigated, as longitudinal imaging seems to show a certain variability close to the order of magnitude observed under anesthesia. In that sense, the review process was useful in avoiding hasty conclusion and calls for further caution in ULM awake longitudinal imaging, in particular regarding precision of positioning and cancellation of tissue motion.

Strengths:

Even if the methods elements considered separately are not new (brain ULM in rodents, setup for longitudinal awake imaging similar to those used in fUS imaging, quantification of vessel diameters/bubble flow/vessel area), when masterfully combined as it is done in this paper, they answer two questions that have been long-running in the community: what is the impact of anesthesia on the parameters measured by ULM (and indirectly in fUS and other techniques)? Is it possible to achieve ULM in awake rodents for longitudinal imaging? The manuscript is well constructed, well written, and graphics are appealing.

The manuscript has been much strengthened by the round of review, with more animals for the longitudinal imaging study.

Weaknesses:

Some weaknesses remain, not hindering the quality of the work, that the authors might want to answer or explain.

- When considering fig 4e and fig 4j together: it seems that in fig 4e the vascularity reduction in the cortical ROI is around 30% for downward flow, and around 55% for upward flow; but when grouping both cortical flows in fig 4j, the reduction is much smaller (~5%), even at the individual level (only mouse 1 is used in fig 4e). Can you comment on that?

- When considering fig4e, fig 4j, fig6e and fig6i altogether, it seems that vascularity can be highly variable, whether it be under anesthesia or vascular imaging, with changes between 5 to 40%. Is this vascularity quantification worth it (namely, reliable for example to quantify changes in a pathological model requiring longitudinal imaging)?

<https://doi.org/10.7554/eLife.95168.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer 1 (Public Review):

• *While the title is fair with respect to the data shown, in the summary and the rest of the paper, the comparison between anesthetized and awake conditions is systematically stated, while more caution should be used.*

First, isoflurane is one of the (many) anesthetics commonly used in pre-clinical research, and its effect on the brain vasculature cannot be generalized to all the anesthetics. Indeed, other anesthesia approaches do not produce evident vasodilation; see ketamine + medetomidine mixtures. Second, the imaged awake state is head-fixed and body-constrained in mice. A condition that can generate substantial stress in the animals. In this study, there is no evaluation of the stress level of the mice. In addition, the awake imaging sessions were performed a few minutes after the mouse woke up from isoflurane induction, which is necessary to inject the MB bolus. It is known that the vasodilator effects of isoflurane last a long time after its withdrawal. This aspect would have influenced the results, eventually underestimating the difference with respect to the awake state.

These limitations should be clearly described in the Discussion.

Looking at Figure 2e, it takes more than 5' to reach the 5 Millions MB count useful for good imaging. However, the MB count per pixel drops to a few % at that time. This information tells me that (i) repeated measurements are feasible but with limited brain coverage since a single 'wake up' is needed to acquire a single brain section and (ii) this approach cannot fit the requirements of functional ULM that requires to merge the responses to multiple stimuli to get a complete functional image. Of course, a chronic i.v. catheter would fix the issue, but this configuration is not trivial to test in the experimental setup proposed by the authors, hindering the extension of the approach to fULM.

Thank you for highlighting these limitations, as they address aspects that were not fully considered during the experimental design and manuscript writing. In response, we have added the following paragraphs to the discussion section, addressing these limitations of our study:

(Line 310) “Although isoflurane is widely used in ultrasound imaging because it provides long-lasting and stable anesthetic effects, it is important to note that the vasodilation observed with isoflurane is not representative of all anesthetics. Some anesthesia protocols, such as ketamine combined with medetomidine, do not produce significant vasodilation and are therefore preferred in experiments where vascular stability is essential, such as functional ultrasound imaging(47). Therefore, in future studies, it would be valuable to design more rigorous control experiments with larger sample sizes to systematically compare the effects of isoflurane anesthesia, awake states, and other anesthetics that do not induce vasodilation on cerebral blood flow.

Our proposed method enabled repeatable longitudinal brain imaging over a three-week period, addressing a key limitation of conventional ULM imaging and offering potential for various preclinical applications. However, there are still some limitations in this study.

One of the limitations is the lack of objective measures to assess the effectiveness of head-fix habituation in reducing anxiety. This may introduce variability in stress levels among mice. Recent studies suggest that tracking physiological parameters such as heart rate, respiratory rate, and corticosterone levels during habituation can confirm that mice reach a low stress state prior to imaging(48). This approach would be highly beneficial for future awake imaging studies. Furthermore, alternative head-fixation setups, such as air-floated balls or treadmills, which allow the free movement of limbs, have been shown to reduce anxiety and facilitate natural behaviors during imaging(30). Adopting these approaches in future studies could enhance the reliability of awake imaging data by minimizing stress-related confounds.

Another limitation of this study is the potential residual vasodilatory effect of isoflurane anesthesia on awake imaging sessions. The awake imaging sessions were conducted shortly after the mice had emerged from isoflurane anesthesia, required for the MB bolus injections. The lasting vasodilatory effects of isoflurane may have influenced vascular responses, potentially contributing to an underestimation of differences in vascular dynamics between anesthetized and awake state. Future applications of awake ULM in functional imaging using an indwelling jugular vein catheter presents a promising alternative to enable more accurate functional imaging in awake animals, addressing current limitations associated with anesthesia-induced vascular effects.”

• *Statistics are often poor or not properly described.*

The legend and the text referring to Figure 2 do not report any indication of the number of animals analyzed. I assume it is only one, which makes the findings strongly dependent on the imaging quality of THAT mouse in THAT experiment. Three mice have been displayed in Figure 3, as reported in the text, but it is not clear whether it is a mouse for each shown brain section. Figure 5 reports quantitative data on blood vessels in awake VS isoflurane states but: no indication about the number of tested mice is provided, nor the number of measured blood vessels per type and if statistics have been done on mice or with a multivariate method.

Also, a T-test is inappropriate when the goal is to compare different brain regions and blood vessel types.

Similar issues partially apply to Figure 6, too.

Thank you for bringing this to our attention.

We acknowledge that the statistical analyses were not clearly explained in the original version. In the revised manuscript, we have ensured that the statistical methods are clearly described.

(Fig.4 caption) “b,c, Comparisons of vessel diameter (b) and flow velocity (c) for the selected arterial and venous segments. Statistical analysis was conducted using t-test at each measurement point along the segments.”

(Fig.6 caption) “b,c, Comparisons of vessel diameter (b) and flow velocity (c) for the selected arterial and venous segments. Statistical analysis was conducted using the two one-sided test (TOST) procedure, which evaluates the null hypothesis that the difference between the two weeks is larger than three times the standard deviation of one week.”

Additionally, we corrected an error in the previous comparison of the violin plots on flow velocities, where a t-test was incorrectly applied; this has now been removed.

We acknowledge that the original version did not clearly indicate the numbers of animals in the statistical analysis. In the revised manuscript, we have added Supplementary Figure 1 to specify the mice used, and we have labeled each mouse accordingly in the figures or captions. In the revised Figures 4 and 6, we have ensured that each quantitative analysis figure or its caption clearly indicate the specific mice.

For original Figures 1 and 2, these are presented as case studies to illustrate the methodology. Since the anesthesia time required for tail vein injection for each animal varies slightly, it is challenging to have the consistent time taken for each mouse to recover from anesthesia across all mice. For instance, in Figure 1, the mouse took nearly 500 seconds to recover from anesthesia, but this duration is not consistent across all animals, which is a limitation of the bolus injection technique. We have noted this point in the discussion (discussion on the limitation of bolus injection), and we have also clarified in the results section and figure captions that these figures represent a case study of a single mouse rather than a standardized recovery time for all animals.

We further clarified this point in the end of the Figure 2 caption:

(Fig.2 caption) “This figure presents a case study based on the same mouse shown in Fig 1. The x-axis for d-f begins at 500 seconds because, at this point, the mouse’s pupil size stabilized, indicating it had recovered to an awake state. Consequently, ULM images were accumulated starting from this time. It is important to note that not every mouse requires 500 seconds to fully awaken; the time to reach a stable awake state varies across individual mice.” We added the following statement before introducing Figure 1e:

(Line 93) “Due to differences in tail vein injection timing and anesthesia depth, the time required for each mouse to fully awaken varied. Although it was not feasible to get pupil size stabilized just after 500 seconds for each animal, ULM reconstruction only used the data that acquired after the animal reached full pupillary dilation, to ensure that ULM accurately captures the cerebrovascular characteristics in the awake state.”

We added the following statement before introducing Figure 2d:

(Line 139) “To further verify that the proposed MB bolus injection method can help to achieve ULM image saturation shortly after mice awaken from anesthesia, an analysis on the change in MB concentration over time was conducted once pupil size had stabilized (T = 500s).”

For Figures 3, 4, and 5 (in the revised version, Figures 4 and 5 have been combined into a single Figure 4), the data represents results from three individual mice, with each coronal plane corresponding to a different mouse. In the revised version, we have added labels to indicate the specific mouse in each image to improve clarity. We also recognize that some analyses in the original submission (original Figure 5) may have lacked sufficient statistical power due to the small sample size. Therefore, in the revised version, we have focused only

on findings that were consistently observed across the three mice to ensure robust conclusions.

Reviewer 1 (Recommendations For the Authors):

- *If the study's main goal is to compare awake vs anesthetized ULM, the authors should test at least another anesthetic with no evident vasodilator effect.*

Thank you for this valuable suggestion. We would like to clarify that the primary aim of our study is not to comprehensively compare the effects of anesthesia versus the awake state, as a rigorous comparison would indeed require a more controlled experimental design, including additional anesthetics, a larger cohort of mice, and broader controls to ensure sufficient statistical power. We also add the following statement in the Discussion to clarify this point:

(Line 314) “Therefore, in future studies, it would be valuable to design more rigorous control experiments with larger sample sizes to systematically compare the effects of isoflurane anesthesia, awake states, and other anesthetics that do not induce vasodilation on cerebral blood flow.”

We acknowledge that the initial organization of Figures 3–5 placed excessive emphasis on comparisons between the awake and anesthetized states, but without yielding consistently significant findings. Meanwhile, our longitudinal observations in original Figure 6 were underrepresented, despite their potential importance.

In the revised version, we shifted our focus toward the main goal of awake longitudinal imaging. By consolidating the previous Figures 4 and 5 into the new Figure 4, we emphasize conclusions that are both more consistent and broadly applicable, avoiding areas that may lack sufficient rigor or consensus. Additionally, we expanded the quantitative analysis related to longitudinal imaging, highlighting its role as the ultimate objective of this study. The awake vs. anesthetized ULM comparison was intended to demonstrate the value of awake imaging and introduce the importance of awake longitudinal imaging. In the revised text, we have reframed this comparison to emphasize the specific response to isoflurane rather than a general response to anesthesia. For example, in Figures 3 and 4, we have replaced the original term “Anesthetized” with “Isoflurane”. We have also added a discussion noting that isoflurane may induces more vasodilation than other anesthetic agents.

(Line 310) “Although isoflurane is widely used in ultrasound imaging because it provides long-lasting and stable anesthetic effects, it is important to note that the vasodilation observed with isoflurane is not representative of all anesthetics. Some anesthesia protocols, such as ketamine combined with medetomidine, do not produce significant vasodilation and are therefore preferred in experiments where vascular stability is essential, such as functional ultrasound imaging(47).”

- *The claims made about the proposed experimental protocol to be suitable for the “long-term” (line 255) are not supported by the data and should be modified according to the presented evidence.*

Thank you for your valuable feedback. We agree that our current three-week experimental results do not yet fulfill the requirements for extended longitudinal imaging that may span several months. We have revised the relevant text accordingly. For instance, the phrase “Our proposed method enabled long-term, repeatable longitudinal brain imaging” has been modified to “Our proposed method enabled repeatable longitudinal brain imaging over a threeweek period.” (Similar changes also in Line 67, Line 318, and Line 337) Additionally, we have added the following paragraph in the discussion section to indicate that extending the monitoring period to several months is a meaningful direction for future exploration:

(Line 337) “In our longitudinal study, consistent imaging results were obtained over a three-week period, demonstrating the feasibility of awake ULM imaging for this duration. However, for certain research applications, a monitoring period of several months would be valuable. Extending the duration of longitudinal awake ULM imaging to enable such long-term studies is a potential direction for future development.”

Recommendations for improving the writing and presentation:

- *Reporting the number of mice and blood vessels and statistics for each quantitative figure.*

Thank you for highlighting this issue. We acknowledge that the quantitative figures in the previous version lacked clarity in specifying the number of mice, vessels, and associated statistics. In the revised version, we have ensured that each quantitative figure or its caption clearly indicate the specific mice, vessels, and statistical methods used. To further minimize any potential confusion, we have also added Supplementary Figure 1 to clearly label and reference each individual mouse included in the study.

Minor corrections to the text and figures.

- *Line 22: “vascularity reduction from anesthesia” is not clear, nor it is a codified property of brain vasculature. Explain or rephrase.*

Thank you for your comment. We apologize for any confusion caused by the phrase “vascularity reduction from anesthesia” in the abstract. We agree that this phrasing was unclear without context. To improve clarity, we have revised this statement in the abstract to make it more straightforward and easier to understand.

(Line 24) “Vasodilation induced by isoflurane was observed by ULM. Upon recovery to the awake state, reductions in vessel density and flow velocity were observed across different brain regions.”

Additionally, we have added a section in the Methods titled Quantitative Analysis of ULM Images to provide a clear definition of vascularity. This section outlines how vascularity is quantified in our study, ensuring that our terminology is well-defined.

The following sentence shows the definition of vascularity:

(Line 547) “Vascularity was defined as the proportion of the pixel count occupied by blood vessels within each ROI, obtained by binarizing the ULM vessel density maps and calculating the percentage of the pixels with MB signal.”

We have also added an instant definition when it was firstly used in Results part:

(Line 161) “When comparing vessel density maps, ULM images that are acquired in the awake state demonstrate a global reduction of vascularity, which refers to percentage of pixels that occupied by blood vessels.”

- *Line 76: putting the mice in a tube is also intended “To further reduce animal anxiety and minimize tissue motion” I agree with tissue motion, not with animal anxiety, which, indeed, I expect to be higher than if it could, for example, run on a ball or a treadmill.*

Thank you for pointing this out. We acknowledge the limitations of our setup regarding reducing animal anxiety. We have replaced the original phrase “to further reduce animal anxiety and minimize tissue motion” with “to further minimize tissue motion.” (Line 78)

Additionally, we have added the following paragraph in Discussion section to address the limitations of our setup in reducing anxiety.

(Line 321) “One of the limitations is the lack of objective measures to assess the effectiveness of head-fix habituation in reducing anxiety. This may introduce variability in stress levels among mice. Recent studies suggest that tracking physiological parameters such as heart rate, respiratory rate, and corticosterone levels during habituation can confirm that mice reach a low stress state prior to imaging(48). This approach would be highly beneficial for future awake imaging studies. Furthermore, alternative head-fixation setups, such as air-floated balls or treadmills, which allow the free movement of limbs, have been shown to reduce anxiety and facilitate natural behaviors during imaging(30). Adopting these approaches in future studies could enhance the reliability of awake imaging data by minimizing stress-related confounds.”

• *Line 79: PMP has been used by Sieu et al., Nat Methods, 2015; it should be acknowledged.*

Thank you for highlighting this. We have now included the reference to Sieu et al. Nat Methods, 2015 to appropriately acknowledge their use of PMP. (Line 81)

• *Figure: is there a reason why the plots start at 500 sec? What happened before that time?*

Thank you for your question regarding the starting time in the plots. Figures 1 and 2 are case studies using a single mouse to demonstrate the feasibility of our method. The “zero” timepoint was defined as the moment when anesthesia was stopped, and the microbubble injection began. However, the mouse does not fully recover immediately after anesthesia is stopped. As shown in Figure 1e, there is a period of approximately 500 seconds during which the pupil gradually dilates, indicating recovery. Only after this period does the mouse reach a relatively stable physiological state suitable for ULM imaging, which is why the plots in Figure 2 begin at T = 500 seconds.

We recognize that this was not sufficiently explained in the main text and figure captions. In the revised manuscript, we have clarified this timing rationale in both the results section and the figure captions. We added the following sentence to the result section to introduce Fig.2d:

(Line 139) “To further verify that the proposed MB bolus injection method can help to achieve ULM image saturation shortly after mice awaken from anesthesia, an analysis on the change in MB concentration over time was conducted once pupil size had stabilized (T = 500s).”

We also added the following statement to note that this recover time varies across individual mice:

(Line 154, Fig.2 caption) “This figure presents a case study based on the same mouse shown in Fig 1. The x-axis for d-f begins at 500 seconds because, at this point, the mouse’s pupil size stabilized, indicating it had recovered to an awake state. Consequently, ULM images were accumulated starting from this time. It is important to note that not every mouse requires 500 seconds to fully awaken; the time to reach a stable awake state varies across individual mice.”

Reviewer 2 (Public Review):

• *The only major comment (calling for further work) I would like to make is the relative weakness of the manuscript regarding longitudinal imaging (mostly Figure 6), compared to the exhaustive review of the effect of isoflurane on the vasculature (3 rats, 3 imaging planes, quantification on a large number of vessels, in 9 different brain regions). The 6 cortical vessels evaluated in Figure 6 feel really disappointing. As longitudinal imaging is*

supposed to be the salient element of this manuscript (first word appearing in the title), it should be as good and trustworthy as the first part of the paper. Figure 6c. is of major importance, and should be supported by a more extensive vessel analysis, including various brain areas, and validated on several animals to validate the robustness of longitudinal positioning with several instances of the surgical procedure. Figure 6d estimates the reliability of flow measurements on 3 vessels only. Therefore I recommend showing something similar to what is done in Figures 4 and 5: 3 animals, and more extensive quantification in different brain regions.

We thank the reviewer for pointing out this issue. We acknowledge that the first version of the manuscript lacked in-depth quantitative analysis in the section on the longitudinal study, which should have been a focal point. It also did not provide a sufficient number of animals to demonstrate the reproducibility of the technique. In this revised version, we have included results from more animals and conducted a more comprehensive quantitative analysis, with the corresponding text updated accordingly. Specifically, we combined the previous Figures 4 and 5 into the current Figure 4 (corresponding revised text from Line 169 to Line 207). The revised Figures 5 and 6

compare the results of the longitudinal study, presenting data from three mice (corresponding revised text from

Line 224 to Line 258). Detailed information about the mice used has been added to Supplementary Figure 1, and Supplementary Figure 4 further provides a detailed display of the results for the three mice in longitudinal study. We hope that these adjustments will provide a more thorough validation of the longitudinal imaging.

Reviewer 2 (Recommendations For The Authors):

Minor comments:

- *The statistical analyses are not always explained: could they be stated briefly in the legends of each figure, or gathered in a statistical methods section with details for each figure? Be sure to use the appropriate test (e.g. student t-test is used in Fig 5 k whereas normality of distribution is not guaranteed.)*

Thank you for pointing this out. We acknowledge that the statistical analyses were not clearly explained in the original version. In the revised manuscript, we have ensured that the statistical methods are clearly described.

(Fig.4 caption) “b,c, Comparisons of vessel diameter (b) and flow velocity (c) for the selected arterial and venous segments. Statistical analysis was conducted using t-test at each measurement point along the segments.”

(Fig.6 caption) “b,c, Comparisons of vessel diameter (b) and flow velocity (c) for the selected arterial and venous segments. Statistical analysis was conducted using the two one-sided test (TOST) procedure, which evaluates the null hypothesis that the difference between the two weeks is larger than three times the standard deviation of one week.”

Additionally, we corrected an error in the previous comparison of the violin plots on flow velocities, where a t-test was incorrectly applied; this has now been removed.

- *The authors use early in the manuscript the term vascularity, e.g. in "vascularity reduction", it is not exactly clear what they mean by vascularity, and would require a proper definition at that moment. If I am correct, a quantification of that "vascularity reduction" (page 5 line 132), is then done in Figures 5 d e f and j.*

Thank you for highlighting this issue. We acknowledge that our initial use of the term “vascularity” may have been unclear and potentially confusing. In the revised manuscript, we have included a clear definition of “vascularity” in the Methods section under Quantitative Analysis of ULM Images (Line 534).

The following sentence shows the definition of vascularity:

(Line 547) “Vascularity was defined as the proportion of the pixel count occupied by blood vessels within each ROI, obtained by binarizing the ULM vessel density maps and calculating the percentage of the pixels with MB signal.”

We have also added an instant definition when it was firstly used in Results part:

(Line 161) “When comparing vessel density maps, ULM images that are acquired in the awake state demonstrate a global reduction of vascularity, which refers to percentage of pixels that occupied by blood vessels.”

• *There is very little motion in the images presented, except for the awake "Bregma -4.2 mm" (Figure 3, directional maps), especially in the area including colliculi and mesencephalon, while the cortical vessels do not move. Can you comment on that?*

Thank you for highlighting this important aspect of motion in awake animal imaging. Motion correction is indeed a critical factor in such studies. In the original version of our discussion, we briefly addressed this issue (from Line 342 to Line 346), but we agree that a more detailed discussion is needed.

To minimize motion artifacts, we conducted habituation to acclimate the animals to the head-fixation setup, which helps reduce anxiety during imaging. With thorough head-fixed habituation, the imaging quality is generally well-preserved. We also applied correlation-based motion correction techniques based on ULM images, which can partially correct for overall brain motion, as stated in the previous version. However, this ULM-images-based correction is limited to addressing only rigid motion.

In the revised discussion, we have expanded on the limitations of our current motion correction approach and referenced recent work about more advanced motion correction methods:

(Line 346) “While rigid motion correction is often effective in anesthetized animals, awake animal imaging presents greater challenges due to the more prominent non-rigid motion, particularly in deeper brain regions. This is evidenced in Supplementary Fig. 1 (Mouse 7), where cortical vessels remain relatively stable, but regions around the colliculi and mesencephalon exhibit more noticeable motion artifacts, indicating that displacement is more pronounced in deeper areas. To address these deeper, non-rigid motions, recent studies suggest estimating nonrigid transformations from unfiltered tissue signals before applying corrections to ULM vascular images(16,50). Such advanced motion correction strategies may be more effective for awake ULM imaging, which experiences higher motion variability. The development of more robust and effective motion correction techniques will be crucial to reduce motion artifacts in future awake ULM applications.”

• *Figure 1f maybe flip the color bar to have an upward up and downward down.*

Thank you for your suggestion. This display method indeed makes the images more intuitive. In the revised manuscript, all directional flow color bars have been flipped to ensure that upward flow is displayed as ‘up’ and downward flow as ‘down.’

- *Figure 2b the figure is a bit confusing in what is displayed between dashed lines, solid lines, dots... maybe it would be easier to read with*
- *bigger dots and dashed lines in color for each of the 4 series*
- *and so in the legend, thin solid lines in the corresponding color for the fit, but no solid line in the legend (to distinguish data/fit)*
- *no lines for FWHM as they are not very visible, and the FWHM values are not mentioned for these examples.*

Thank you for your detailed suggestions. We agree that the original Fig. 2b appeared messy and confusing. Based on this feedback and other comments, we decided to replace the FWHM-based vessel diameter measurement with a more stable binarization-based approach. In the revised version, we selected a specific segment of each vessel and measured the diameter by calculating the distance from the vessel's centerline to both side after binarization. Each point on the centerline of this segment provides a diameter measurement, which can be further used to calculate the mean and standard error. This updated method is more stable and reproducible, providing reliable measurements even for vessels that are not fully saturated. It also facilitates comparison across more vessels, helping to further demonstrate the generalizability of our saturation standard. We believe these adjustments make the revised Fig. 2b clearer and more readable.

- *Page 7, lines 144-147. This passage is not really clear when linking going up or down and going from the stem to the branches that it is specific to Figure 4a (and therefore to this particular location).*

Thank you for your insightful comments on our vessel classification method. We recognize the limitations of the previous approach and, in order to enhance the rigor of the study, we have opted not to continue using this method in the revised manuscript. We have removed all content related to vessel classification based on branchin and branch-out criteria. This includes the original *Classification of Cerebral Vessels* section in the Methods, the relevant descriptions in the Results section under "ULM reveals detailed cerebral vascular changes from anesthetized to awake for the full depth of the brain", limitation of this classification method in Discussion section, as well as related content in the original Figures 4 and 5.

In the revised analysis, for the comparison between arteries and veins, we focus solely on penetrating vessels in the cortex. For these vessels, it is generally accepted that downward-flowing vessels are arterioles, while upwardflowing vessels are venules. Accordingly, in the revised Figures 4 and 6, we analyze arterioles and venules exclusively in the cortex, without relying on the previous classification method that could be considered controversial.

- *Page 11 line 222 "higher vascular density" seems unprecise.*

Thank you for pointing this out. We have revised the sentence to more precisely convey our observations regarding changes in vascular diameter and vascularity within the ROI. We present these findings as evidence of the vasodilation effect under isoflurane, in alignment with existing research. The revised statement is as follows:

(Line 275) "Statistical analysis from Fig. 4 shows that certain vessels exhibit a larger diameter under isoflurane anesthesia, and the vascularity, calculated as the percentage of vascular area within selected brain region ROIs, is also higher in the anesthetized state. These findings suggest a vasodilation effect induced by isoflurane, consistent with existing research(20,40,41,43,44).

• *Discussion: page 12, lines 257-267: it is not exactly clear how 3D imaging will help for the differentiation of veins/arteries. However, some methods have already been proposed to discriminate between arteries and veins using pulsatility (Bourquin et al., 2022) or 3D positioning when vessels are overlapped (Renaudin et al., 2023). The latter can also help estimate the out-of-plane positioning during longitudinal imaging.*

Bourquin, C., Poree, J., Lesage, F., Provost, J., 2022. In Vivo Pulsatility Measurement of Cerebral Microcirculation in Rodents Using Dynamic Ultrasound Localization Microscopy. *IEEE Trans. Med. Imaging* 41, 782-792. <https://doi.org/10.1109/TMI.2021.3123912>

Renaudin, N., Pezet, S., Ialy-Radio, N., Demene, C., Tanter, M., 2023. Backscattering amplitude in ultrasound localization microscopy. *Sci. Rep.* 13, 11477. <https://doi.org/10.1038/s41598-023-38531-w>

Thank you for pointing this out. We have revised the relevant paragraph in the discussion to clarify the potential advantages of advances in ULM imaging methods, such as those based on pulsatility (as described by Bourquin et al., 2022) or backscattering amplitude (as demonstrated by Renaudin et al., 2023). These established methods could be helpful for longitudinal imaging. Below is the revised text in the discussion section:

(Line 370) “Advances in ULM imaging methods can benefit longitudinal awake imaging. For instance, dynamic ULM can differentiate between arteries and veins by leveraging pulsatility features(51). 3D ULM, with volumetric imaging array(52,53), enables the reconstruction of whole-brain vascular network, providing a more comprehensive understanding of vessel branching patterns. Meanwhile, 3D ULM also helps to mitigate the challenge of aligning the identical coronal plane for longitudinal imaging, a process that requires precise manual alignment in 2D ULM to ensure consistency. Additionally, this alignment issue can also be alleviated in 2D imaging using backscattering amplitude method, which may assist in estimating out-of-plane positioning during longitudinal imaging(54).”

Reviewer 3 (Public Review):

• *It is unclear whether multiple animals were used in the statistical analysis.*

Thank you for bringing this to our attention. We acknowledge that the original version did not clearly indicate the use of animals in the statistical analysis. In the revised manuscript, we have added Supplementary Figure 1 to specify the mice used, and we have labeled each mouse accordingly in the figures or captions. In the revised Figures 4 and 6, we have ensured that each quantitative analysis figure or its caption clearly indicate the specific mice.

• *Generalizations are sometimes drawn from what seems to be the analysis of a single vessel.*

Thank you for pointing this out. To enhance the generalizability of our conclusions, we have expanded our analysis beyond single vessels in several parts of the study. For instance, in Figure 2, we analyzed three vessels at different depths within the same brain region of a single mouse, and we have included additional results in the Supplementary Figure 2 to further support these findings. Additionally, we have revised the language in the manuscript to ensure that conclusions are appropriately qualified and avoid overgeneralization.

In Figures 4 and 6, we extended the analysis from single vessels to larger region-of-interest (ROI) analyses across entire brain regions. Unlike single-vessel measurements, which are susceptible to bias based on specific measurement locations, ROI-based analyses are less influenced by the operator and provide more objective, generalizable insights.

- *The description of the statistical analysis is mostly qualitative.*

We recognize that some aspects of the original statistical analysis (Figures 4 and 5 in the previous version) lacked rigor and description is more qualitative. The revised version of statistical analysis (Figure 4 and Figure 6) presents our findings from multiple dimensions, ranging from individual vessels to individual cortical ROI of arteries and veins, and ultimately to broader brain regions. For instance, as illustrated in the revised Figure 4f, the average cortical arterial flow speed decreases by approximately 20% from anesthesia to wakefulness, while venous flow speed decreases by an average of 40%, with the reduction in venous flow speed being significantly greater than that of arterial flow. We believe that this kind of description offers more quantitative analysis.

For more examples, please refer to the Results section where Figure 4 (Line 169 to Line 207) and Figure 6 (Line 224 to Line 258) are described. These sections have been extensively rewritten to emphasize quantitative interpretation of the data. Each part of the analysis now focuses more heavily on quantitative analyses that consistently show similar trends across all animals.

- *Some terms used are insufficiently defined.*
- *Additional limitations should be included in the discussion.*
- *Some technical details are lacking.*

Thank you for highlighting these issues. In response, we have made several improvements in the revised manuscript to address these issues. We have clarified terms such as “vascularity” (Line 547) and “saturation point” (Line 112) to ensure precision and prevent ambiguity. We have expanded the discussion (Line 310 to Line 377) to include limitations such as motion correction challenges and advances in ULM imaging methods, including dynamic ULM and backscattering amplitude techniques. We have added further details on interleaved sampling (Line 494 to Line 497), ULM tracking (Line 517 to Line 529), and quantitative analysis (Line 535 to Line 551) in the Methods section to provide a clearer understanding of our approach.

Please refer to our other responses for more specific adjustments.

- *Without information about whether the results obtained come from multiple animals, it is difficult to conclude that the authors generally achieved their aim. They do achieve it in a single animal. The results that are shown are interesting and could have an impact on the ULM community and beyond. In particular, the experimental setup they used along with the high reproducibility they report could become very important for the use of ULM in larger animal cohorts.*

We thank the reviewer for recognizing the impact of our work. We also acknowledge that there were some issues—specifically, we did not provide sufficient proof of reproducibility. In the revised version, we have included additional animal experiment results to ensure that the conclusions were not drawn from a single animal but are generally representative of our aim. (See supplementary figure 1 for detailed use of the animals)

Reviewer 3 (Recommendations For The Authors):

- *The manuscript would be more convincing by removing some of the superlatives used in the text. For instance, shouldn't "super-resolution ultrasound localization microscopy" simply be "ultrasound localization microscopy"? Expressions such as "first study", "essential", and "invaluable", etc could be replaced by more factual terms. The word "significant" is also used sometimes with statistics to back it up and sometimes without.*

Thank you for highlighting this issue. We have removed the superlatives throughout the manuscript to make the language more precise. For instance, we have simplified “super-resolution ultrasound localization microscopy” to “ultrasound localization microscopy” throughout the main text and removed expressions such as “first study” and “invaluable”. We also reviewed all uses of “essential” and “significant,” replacing “essential” with more modest alternatives where it does not indicate a strict requirement. Similarly, where “significant” does not refer to statistical significance, we have used other terms to avoid any ambiguity.

• The section “Microbubble count serves as a quantitative metric for awake ULM image reconstruction” had several issues that I think should be addressed. Mainly, the authors make the case that after detecting 5 million microbubbles, there is no clear gain in detecting more. The argument is not very convincing as we know many vessels will not have had a microbubble circulate in them within that timeframe, which will be especially true in smaller vessels. While the analysis in Figure 2 shows nicely that the diameter estimate for vessels in the 20-30 μm range is stable at 5 million microbubbles, it is not necessarily the case for smaller vessels. A better approach here might be to select, e.g., a total of 5 million detected microbubbles for practical reasons and then to determine which vessel parameters estimation (e.g., diameter, flow velocity) remain stable. In addition:

a. Terms such as ‘complete ULM reconstruction’, ‘no obvious change’, ‘ULM image saturation’ are not well defined within the manuscript.

Thank you for pointing out these issues and for offering a more rigorous approach. We completely agree with your suggestion. While our analysis demonstrated stable diameter estimates for vessels with diameter around 20 μm at 5 million microbubbles, this does not necessarily ensure stability for smaller vessels. Therefore, the choice of 5 million microbubbles was primarily for practical reasons. In the revised version, we have provided a more objective description and clarification of this limitation. We also recognize that terms such as “complete ULM reconstruction,” “no obvious change,” and “ULM image saturation” were not well defined and may have caused confusion, reducing the rigor of this manuscript. Based on your feedback, we have clearly defined “ULM image saturation” within the context of our study, removed absolute and ambiguous terms like “complete ULM reconstruction” and “no obvious change”. We revised the entire section accordingly:

(Line 109) “To facilitate equitable comparison of brain perfusion at different states, a practical saturation point enabling stable quantification of most vessels needs to be established. Our observations indicated that when the cumulative MB count reached 5 million, ULM images achieved a relatively stable state. Accordingly, in this study, the saturation point was defined as a cumulative MB count of 5 million. There are also possible alternatives for ULM image normalization. For example, different ULM images can be normalized to have the same saturation rate. However, the proposed method of using the same number of cumulative MB count for normalization enables the analysis of blood flow distribution across different brain regions from a probabilistic perspective. The following analysis substantiates this criterion.

Fig. 2a compares ULM directional vessel density maps and flow speed maps generated with 1, 3, 5, and 6 million MBs, using the same animal as shown in Fig. 1. To quantitatively confirm saturation, multiple vessel segments were selected for further analysis. Fig. 2b presents the measured vessel diameter for a specific segment at various MB counts. After binarizing the ULM map, the vessel diameter was measured by calculating the distance from the vessel centerline to the edge. Each point along the centerline of the segment provided a diameter measurement, enabling calculation of the mean and standard error. At low MB counts, vessels appeared incompletely filled, leading to inaccurate estimation of vessel diameter due

to incomplete profiles. For example, at 1–2 million MBs, the binarized ULM map displayed a width of only one or two pixels along the segment. As a result, the measurements always yielded the same diameter values (two pixels, ~10µm) with a consistently low standard error of the mean across the entire segment. With increased MB counts, the measured vessel diameter gradually rose, ultimately reaching saturation. The plots in Fig. 2b show that vessel diameter stabilized at 5 million MB count. Additionally, Fig. 2c illustrates the changes in flow velocity measured at different cumulative MB counts. The violin plots display the distribution of flow speed estimates for all valid centerline pixels within the selected segment. At low MB counts (1–3 million), flow velocity estimates fluctuated, but they stabilized as the MB count increased (4–6 million MBs). At 5 million MBs, flow velocity estimates were nearly identical to those at 6 million MBs, corroborating previous findings that vessel velocity measurements stabilize as MB count grows (39). To assess the generalizability of the 5 million MB saturation condition, vessel segments from three different mice across various brain regions were examined. The results, shown in Supplementary Fig. 2, confirm that this saturation criterion applies broadly. Although the 5 million MB threshold may not ensure absolute saturation for all vessels, it is generally effective for vessels larger than 15 µm. This MB count threshold was therefore adopted as a practical criterion.”

b. The choice of 10 consecutive tracking frames is arbitrary and should be described as such unless a quantitative optimization study was conducted. Was there a gap-filling parameter? What was the maximum linking distance and what is its impact on velocity estimation?

Thank you for your comment. We acknowledge that the choice of 10 consecutive tracking frames was based on our common practice rather than a specific quantitative optimization. Additionally, with the *uTrack* algorithm, we set both the gap-filling parameter and maximum linking distance to 10 pixels. Setting these parameters too high could potentially overestimate velocity. These details have now been added to the Methods section for clarity:

(Line 517) “The choice of 10 consecutive frames (10 ms) was based on established practice but can be adjusted as needed. For the *uTrack* algorithm, two additional key parameters were specified: the maximum linking distance and the gap-filling distance, both set to 10 pixels (~50 microns). This configuration means that only bubble centroids within 10 pixels of each other across consecutive frames are considered part of the same bubble trajectory. Additionally, when the start and end points of two tracks fall within this threshold, the gap-filling parameter merges them into a single, continuous track. It is important to select these parameters carefully, as overly large values could lead to an overestimation of flow velocity. By setting the maximum linking distance to 10 pixels, we effectively limited the measurable velocity to 50 mm/s, under the assumption that no bubble would exceed a 50-micron displacement within the 1 ms interval between frames. After determining bubble tracks with the specified parameters for *uTrack* algorithm, accumulating the MB tracks resulted in the flow intensity map. Considering the velocity distribution across the mouse brain, this 50 mm/s limit ensures that the vast majority of blood flow is captured accurately.”

c. 'The plots (Figure 2b) clearly indicate that the vessel diameter stabilized beyond 5 million MB count.' This is true for one vessel. To generalize that claim, the analysis should be performed quantitatively on a larger sample of vessels in various areas of the brain, across multiple animals.

Thank you for pointing out this limitation. We agree that conclusions drawn from a single vessel cannot be generalized across all regions. Following your suggestion, we have added *Supplementary Figure 2*, where we analyzed multiple vessels from different brain regions across three mice. This expanded analysis further confirms that a 5 million MB count is sufficient to stabilize vessel diameter measurements across various samples.

(Line 133) “To assess the generalizability of the 5 million MB saturation condition, vessel segments from three different mice across various brain regions were examined. The results, shown in Supplementary Fig. 2, confirm that this saturation criterion applies broadly. Although the 5 million MB threshold may not ensure absolute saturation for all vessels, it is generally effective for vessels larger than 15 μm . This MB count threshold was therefore adopted as a practical criterion.”

• *“Statistical analysis validates the increase in blood flow induced by anesthesia” is a very interesting section but even though a quantitative analysis was conducted in Figure 5, the language used remains mostly qualitative. I think this section should include quantitative conclusions from the statistical analysis to increase the impact of this work.*

Thank you for your valuable feedback. We recognize that some aspects of the original quantitative analysis (Figures 4 and 5 in the previous version) lacked rigor, such as the classification of arteries, veins, and capillaries, and that the data presented in each row of Figure 5 represented only one mouse per coronal section, limiting the generalizability of statistical conclusions.

In response to the reviewers’ feedback, the revised version incorporates a new approach by merging the previous Figure 4 and Figure 5 into a single, consolidated figure (now Figure 4). This updated figure aims to present our findings from multiple dimensions, ranging from individual vessels to individual cortical ROI of arteries and veins, and ultimately to broader brain regions. We have focused on quantitative analyses that consistently show similar trends across all animals. For instance, as illustrated in the revised Figure 4f, the average cortical arterial flow speed decreases by approximately 20% from anesthesia to wakefulness, while venous flow speed decreases by an average of 40%, with the reduction in venous flow speed being significantly greater than that of arterial flow. We believe that this approach offers more insightful analysis and enhances the overall impact of the study.

For more examples, please refer to the revised Results section where Figure 4 are described (from Line 169 to Line 212). These sections have been extensively rewritten to emphasize quantitative interpretation of the data. Each part of the analysis now focuses more heavily on quantitative analyses that consistently show similar trends across all animals.

• *In the methods, it is claimed that 6 healthy female C57 mice were used in the study, but it is hard to tell whether more than one animal is shown in the figures. It is also unclear whether the statistics were performed within or across animals. Since one of the major strengths of the manuscript is that it shows the feasibility of performing reproducible measurements using ULM, most figures should be repeated for each individual animal and provided in supplementary data and statistics should be performed across animals.*

Thank you for bringing this to our attention. We acknowledge that the original version did not clearly indicate the use of individual animals. In the revised manuscript, we have added Supplementary Figure 1 to specify the mice used, and we have labeled each mouse accordingly in the figures or captions. Additionally, we included statistics across animals in the revised Figures 4 and 6, and detailed data for each individual mouse are now provided in Supplementary Figures 3 and 4.

• *The effect of aliasing should be discussed given that 1) a high-frequency probe is used along with a correspondingly relatively low frame rate (1000 fps) and 2) Doppler filtering is used to separate upward from downward-moving microbubbles. There will be microbubbles that circulate faster than the Nyquist limit, which will thus appear as moving in the opposite direction in the Doppler spectrum. It would be important to*

double-check that the effect is not too important and to report this as a limitation in the discussion.

Thank you for highlighting this important point. Aliasing is indeed a relevant issue to consider, especially for higher flow velocities in large vessels. We have added a discussion on this limitation in the revised manuscript:

(Line 359) “Based on the maximum linking distance and gap closing parameters outlined in the Methods section, blood flow with velocities below 50 mm/s can be detected. However, the use of a directional filter to estimate flow direction may introduce aliasing. MBs moving at higher velocities may be subject to incorrect flow direction estimation due to aliasing effects. Given that the compounded frame rate is 1000 Hz, with an ultrasound center frequency of 20 MHz and a sound speed of 1540 m/s, the relationship between Doppler frequency and the axial blood flow velocity⁽¹²⁾ indicates that aliasing will not occur for axial flow velocities below 19.25 mm/s. In all flow velocity maps presented in this study, the range is limited to a maximum of 15 mm/s, remaining below the critical threshold for aliasing. Additionally, all vessels analyzed in the violin plots for arteriovenous flow comparisons fall within this range. While cortical arterioles and venules generally exhibit moderate flow speeds, aliasing remains a factor to consider when combining directional filtering with velocity analysis.”

• The method used to classify vessels may be incorrect and may not be needed. I would recommend the authors not use it and describe the vessels as vessels that branch in or out, etc. Applying an arbitrary threshold of 2 to detect capillaries is also not very convincing. I understand that the authors might decide to maintain this nomenclature, in which case I would recommend clearly explaining it at the beginning of the manuscript along with some of the caveats that are already reported in the discussion.

Thank you for your comments on our vessel classification method. We recognize the limitations of the previous approach and, in order to enhance the rigor of the study, we have opted not to continue using this method in the revised manuscript.

In the revised analysis regarding artery and vein, we focus solely on penetrating vessels in the cortex. For these vessels, it is generally accepted that downward-flowing vessels are arterioles, while upward-flowing vessels are venules. Accordingly, in the revised Figures 4 and 6, we analyze arterioles and venules exclusively in the cortex, without relying on the previous classification method that could be considered controversial.

Additionally, we agree that classifying vessels with values below 2 as capillaries was not a robust approach. Thus, we have removed all related analyses from the revised manuscript.

Minor comments:

• Line 16: "resolves capillary-scale ..."; it is not clear that the resolution that is achieved in this work is at the capillary scale.

Thank you for your valuable feedback. We understand that “capillary-scale” may overstate the achieved resolution in our work. To clarify, we have revised the sentence as follows:

(Line 18) “Ultrasound localization microscopy (ULM) is an emerging imaging modality that resolves microvasculature in deep tissues with high spatial resolution.”

This adjustment more accurately reflects the resolution capabilities of ULM as used in our study.

• Line 22: 'vascularity' is not well defined in the manuscript. Consider defining or using another term.

Thank you for pointing out the need for clarification on vascularity. We acknowledge that our initial use of the term “vascularity” may have been unclear and potentially confusing. In the revised manuscript, we have included a clear definition of “vascularity” in the Methods section under Quantitative Analysis of ULM Images (Line 534).

The following sentence shows the definition of vascularity:

(Line 547) “Vascularity was defined as the proportion of the pixel count occupied by blood vessels within each ROI, obtained by binarizing the ULM vessel density maps and calculating the percentage of the pixels with MB signal.”

We have also added an instant definition when it was firstly used in Results part:

(Line 161) “When comparing vessel density maps, ULM images that are acquired in the awake state demonstrate a global reduction of vascularity, which refers to percentage of pixels that occupied by blood vessels.”

| • Line 30: *I'm not convinced the first two sentences are useful.*

Thank you for pointing out this issue. The opening sentence of the article lacked focus and was too broad. We have rewritten the sentence as follows:

(Line 34) “Sensitive imaging of correlates of activity in the awake brain is fundamental for advancing our understanding of neural function and neurological diseases.”

| • Line 37: *'micron-scale capillaries': this expression is unclear. Capillaries are typically micron-scaled, so it gives the impression that ULM can image ULM at the one-micron scale, which is not the case.*

Thank you for your helpful comment. We agree that “micron-scale capillaries” could be misleading, as it might imply a resolution at the single-micron level. To clarify, we have revised the sentence as follows:

(Line 40) “ULM is uniquely capable of imaging microvasculature situated in deep tissue (e.g., at a depth of several centimeters).”

This revised wording more accurately describes ULM’s capability without implying single-micron level resolution.

| • Line 74: *I don't think motion-free imaging is possible in the context of awake animals. Consider 'limiting motion' instead.*

Thank you for pointing out the potential issue with the term “motion-free”. We agree that achieving entirely motion-free imaging is challenging, especially in the context of awake animals. In response to your suggestion, we have revised the sentence to better reflect this limitation:

(Line 76) “To achieve consistent ULM brain imaging while allowing limited movement in awake animals, a headfixed imaging platform with a chronic cranial window was used in this study.”

This revised wording more accurately conveys our approach to minimizing motion without implying that motion is completely eliminated.

| • Line 134: *'clearly reveals decreased vessel diameter' How was that demonstrated?*

- Line 153: 'significant' according to which statistical test?
- Line 167: 'slight increase', by how much, is it significant?
- Line 183: 'smaller vessels' the center of the distribution is not at 10mm/s, and velocity is not necessarily correlated with diameter.
- Line 184: 'more large vessels', see above. What is a large vessel, and how was this measured?
- Line 205: 'significantly lower', according to which statistical test?

We acknowledge that the original version did not properly use the terms of statistical analysis. In the revised manuscript, we have deleted the related points, and rewritten the statistical analysis part to ensure the terms are used correctly. Please refer to the revised part of “ULM reveals an increase in blood flow induced by isoflurane anesthesia” (From Line 169 to Line 209). In the revised Figures 4 and 6, we have also ensured that each quantitative analysis figure or its caption is clearly explained.

- Line 398: the interleaved sampling scheme should be described in more detail.

Thank you for pointing out this issue. The previous version did not clearly explain the details of interleaved sampling. We have now added the following paragraph to the *Ultrasound imaging sequence* section in Methods:

(Line 494) “Interleaved sampling is employed to capture high-frequency echoes more effectively. With the system’s sampling rate limited to 62.5 MHz, the upper limit of the center frequency of the transducer passband is 15.625 MHz. To mitigate aliasing, two transmissions are sent per angle, staggered in time. This approach effectively doubles the sampling rate, ensuring more accurate image reconstruction.”

- Figure 1: Which mouse is it? Are these results consistent across all animals?
- Figure 2: Which mouse is it? Are these results consistent across all animals?
- Figure 3: Which mouse is it? Are these results consistent across all animals?
- Figure 4: Which mouse is it? Are these results consistent across all animals?
- Figure 5: Is it a single mouse or multiple mice? Are these results consistent across all animals?

We acknowledge that the original version did not clearly indicate the numbers of animals in the statistical analysis. In the revised manuscript, we have added Supplementary Figure 1 to specify the mice used, and we have labeled each mouse accordingly in the figures or captions. In the revised Figures 4 and 6, we have ensured that each quantitative analysis figure or its caption clearly indicate the specific mice.

For original Figures 1 and 2, these are presented as case studies to illustrate the methodology. Since the anesthesia time required for tail vein injection for each animal varies slightly, it is challenging to have the consistent time taken for each mouse to recover from anesthesia across all mice. For instance, in Figure 1, the mouse took nearly 500 seconds to recover from anesthesia, but this duration is not consistent across all animals, which is a limitation of the bolus injection technique. We have noted this point in the discussion (discussion on the limitation of bolus injection), and we have also clarified in the results section and figure

captions that these figures represent a case study of a single mouse rather than a standardized recovery time for all animals.

We further clarified this point in the end of the Figure 2 caption:

(Fig.2 caption) “This figure presents a case study based on the same mouse shown in Fig 1. The x-axis for d-f begins at 500 seconds because, at this point, the mouse’s pupil size stabilized, indicating it had recovered to an awake state. Consequently, ULM images were accumulated starting from this time. It is important to note that not every mouse requires 500 seconds to fully awaken; the time to reach a stable awake state varies across individual mice.” We added the following statement before introducing Figure 1e:

(Line 93) “Due to differences in tail vein injection timing and anesthesia depth, the time required for each mouse to fully awaken varied. Although it was not feasible to get pupil size stabilized just after 500 seconds for each animal, ULM reconstruction only used the data that acquired after the animal reached full pupillary dilation, to ensure that ULM accurately captures the cerebrovascular characteristics in the awake state.”

We added the following statement before introducing Figure 2d:

(Line 139) “To further verify that the proposed MB bolus injection method can help to achieve ULM image saturation shortly after mice awaken from anesthesia, an analysis on the change in MB concentration over time was conducted once pupil size had stabilized (T = 500s).”

For Figures 3, 4, and 5 (in the revised version, Figures 4 and 5 have been combined into a single Figure 4), the data represents results from three individual mice, with each coronal plane corresponding to a different mouse. In the revised version, we have added labels to indicate the specific mouse in each image to improve clarity. We also recognize that some analyses in the original submission (original Figure 5) may have lacked sufficient statistical power due to the small sample size. Therefore, in the revised version, we have focused only on findings that were consistently observed across the three mice to ensure robust conclusions.

Minor corrections and typos from all reviewers:

We would like to sincerely thank the reviewers for their careful reading of our manuscript. We appreciate the time and effort taken to point out the minor typographical errors. We have carefully addressed and corrected all the identified typos, as listed below:

From Reviewer #1:

- Line 316: “insensate”: correct, please.

(Line 409) “After confirming that the mouse was anesthetized, the head of the animal was fixed in the stereotaxic frame.”

From Reviewer #3:

- Line 15: Super-resolution ultrasound localization microscopy -- consider removing super-resolution as it gives the impression that it is different from standard ULM.

(Line 18) “Ultrasound localization microscopy (ULM) is an emerging imaging modality that resolves microvasculature in deep tissues with high spatial resolution.”

- Line 39: typo: activities should be activity.

(Line 41) “ULM can also be combined with the principles of functional ultrasound (fUS) to image whole-brain neural activity at a microscopic scale.”

| • *Line 47: typo: over under.*

(Line 50) “Therefore, in neuroscience research, brain imaging in the awake state is often preferred over imaging under anesthesia.”

Once again, we are grateful for the reviewers’ thorough review and valuable input, which have helped us improve the clarity and precision of the manuscript.

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