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Ultrasound matrix imaging for transcranial in-vivo localization microscopy

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Transcranial ultrasound imaging is usually limited by skull-induced attenuation and high-order aberrations. By using contrast agents such as microbubbles in combination with ultrafast imaging, not only can the signal-to-noise ratio be improved, but super-resolution images down to the micrometer scale of the brain vessels can be obtained. However, ultrasound localization microscopy (ULM) remains impacted by wave-front distortions that limit the microbubble detection rate and hamper their localization. In this work, we show how matrix imaging, which relies on the prior recording of the reflection matrix, can provide a solution to those fundamental issues. As an experimental proof-of-concept, an in-vivo reconstruction of deep brain microvessels is performed on three anesthetized sheeps. The compensation of wave distortions is shown to drastically enhance the contrast and resolution of ULM. This experimental study thus opens up promising perspectives for a transcranial and non-ionizing observation of human cerebral microvascular pathologies, such as stroke.

INTRODUCTION

Transcranial imaging is essential to understand the complex vascular mechanisms underlying pathologies. For cerebrovascular events, such as strokes, X-rays and computed tomography (CT) are currently used to observe hemorrhagic events (1) and magnetic resonance imaging (MRI) to diagnose and date ischemic stroke (2). Although these methods allow visualization of many neurovascular diseases, MRI or CT-scans require large and expensive equipment that is only primarily available in health care centers.

Alternatively, transcranial ultrasound is used in Doppler mode to monitor reperfusion after a stroke (3). This technique allows portable real-time imaging at low cost. However, it remains a major challenge as the complexity of the skull layer with its unpredictable porosity causes severe attenuation, strong aberrations and multiple scattering, leading to a drastic reduction in resolution and contrast of the ultrasound image.

Microbubbles (MB) are often used to improve the visualization of blood flow in ultrasound imaging (4–6). In the clinic, they are injected intravenously and are excellent contrast agents due to their high impedance mismatch compared to soft tissues. The use of ultrafast ultrasound scanners, i.e. with high frame rates, has also enabled organ perfusion dynamics to be studied with greater precision (7, 8).

In addition to enhancing the blood signal, microbubbles can also be used to generate super-resolved images. This is the principle of ultrasound localization microscopy (ULM) (9–12), which is based on a similar idea developed a few years earlier in fluorescence microscopy (13, 14). By separating the echoes of individual injectable microbubbles, their position can be localized with a sub-wavelength precision and tracked over time. This allows an accuracy that no longer depends on the diffraction limit but on the accuracy of detection of their center. As a consequence, ULM results in a significant improvement in resolution by a factor of ten (15)

compared to standard ultrasound images. ULM has already proved its worth in many organs in depth, both pre-clinically (9, 16–23) and clinically (24–32). The real-time information contained in the displacement of microbubbles enables complex vascular networks to be mapped at the micrometer scale, even going as far as the capillary-level reconstruction of functional units, such as glomeruli in the kidney (sULM) (27–29). ULM imaging has even been successfully used in transcranial clinical imaging of the adult human brain (26), where aberration correction is critical (33, 34).

Recently, 3D ULM (19, 35–44) has overcome many 2D limitations, such as the projection of 3D structures in 2D, suboptimal tracking of microbubbles, and out-of-plane probe movements that prevent vascular reconstruction. Nevertheless, aberrations caused by the skull remain a problem for ULM, especially in 3D. The sensitivity of bubble detection is lower, which affects the contrast of the super-resolved image, but also the accuracy of bubble localization, where the high sidelobes of a degraded PSF can cause artifacts such as vessel doubling (34, 45).

To solve the fundamental problem of aberrations, adaptive focusing was introduced years ago in astronomy to compensate for phase distortions undergone by starlight when it passes through the atmosphere (46–49). This idea was then transposed to ultrasound imaging by tailoring a focusing law that either optimizes an image metrics (50, 51) or maximizes the spatial correlations of back-scattered echoes (52–56). In the specific case of ULM, several methods have been proposed to correct the aberrations caused by the skull. A first strategy (45) is based on deep learning algorithms trained with numerical simulations. Although it gives impressive in-vivo results for small animals, it is hampered by the specificity of the training set, so its transfer to other experimental configurations (probe, organ, SNR) may require tedious training procedures. The second method (34) uses microbubbles as ultrasound guide stars but suffers several drawbacks. On the one hand, microbubbles should be detected and isolated enough, which can be extremely difficult to assess because of the strongly distorted focal spot across

the skull, On the other hand, the number of detected micro-bubbles should be large enough for self-averaging in each isoplanatic patch.

Interestingly, inspired by previous seminal works (57–59), a matrix approach of ultrasound imaging has been recently developed to overcome aberrations without any guide star (60, 61). Based on the recording of the reflection associated with the ultrasonic probe and the linearity of the wave equation, it allows the simulation of wave focusing in post-processing and an iteration of this process towards local adaptive focusing laws in ultrasonic speckle (62). An optimized contrast and close-to-ideal resolution is then obtained for each voxel of the ultrasound image. UMI has been successfully applied to transcranial 3D imaging of a head phantom (63).

The goal of this paper is to demonstrate the interest of UMI for in-vivo brain imaging. In particular, we will show how: (i) UMI can be used to quantify aberrations and multiple scattering in in-vivo transcranial imaging; (ii) UMI can exploit static speckle from brain tissue to tailor complex focusing laws; (iii) UMI can leverage these focusing laws to increase the detection rate of microbubbles in ULM and improve their localization; (iv) UMI can be fruitfully combined to ULM in order to provide a three-dimensional, artifacts-free, super-resolved and contrasted image of brain vessels.

To that aim, an experimental proof-of-concept will consist in brain imaging on three anesthetized sheeps. The first step of the experimental procedure consists in the acquisition of the reflection matrix and the estimation of local focusing laws by means of UMI. The second step consists in a microbubble injection and the beamforming of ultra-fast images using the focusing laws provided by UMI. The last part consists in the usual ULM post-processing that consists in localizing and tracking micro-bubbles. Final ULM image contrast will be shown to be drastically improved by the better detection rate of smaller micrometer-sized vessels provided by UMI. The comparison with MRI will show how UMI allows the removal of artifacts from which the initial ULM image suffered.

RESULTS

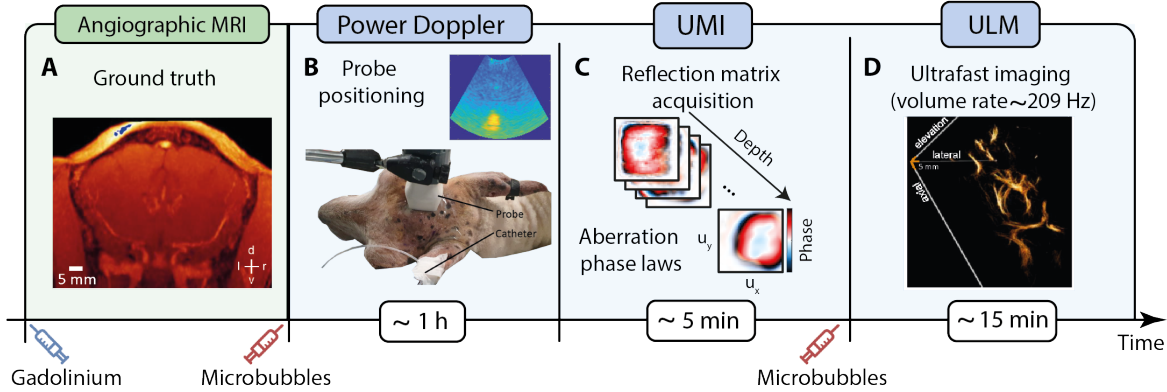


Figure 1: **Sheep experiment : A multi-sequence acquisition.** (A) Angiographic MRI. (B) Transcranial power Doppler for positioning the probe. (C) Acquisition of the reflection matrix to estimate local aberration laws with 3D-UMI. (D) Ultrafast imaging for super-resolved ULM images. The images shown in this figure are for illustrative purposes only and do not provide quantitative results.

Reflection matrix in a virtual source basis

In this paper, we demonstrate transcranial brain imaging by overcoming the major challenges of: (i) skull bone aberration with 3D UMI; (ii) diffraction-limited resolution with the super-resolution feature provided by ULM. As a proof-of-concept, the same experimental protocol (Fig. 1) has been applied to three anesthetized sheep (Methods). A 32×32 array of transducers is placed on the shaved head of each sheep directly over the crest of the frontal bone (Fig. 1B). The probe is driven in 1-2 frequency bandwidth in order to limit the attenuation by skull bone (64). The first ultrasound sequence consists in recording a high-dimension reflection matrix $\mathbf{R}_{\mathbf{u}\mathbf{v}}(\tau)$ (Fig. 1C) that contains the scattered wave-field $R(\mathbf{u}_{\text{out}}, \mathbf{v}_{\text{in}}, \tau)$ recorded by each transducer \mathbf{u}_{out} of the probe as a function of the echo time τ for a set of 324 incident diverging waves. This illumination basis is associated with a set of virtual transducers \mathbf{v}_{in} placed behind the probe (see Methods and Fig. S1). It maximizes the signal-to-noise ratio across the targeted

field-of-view (65).

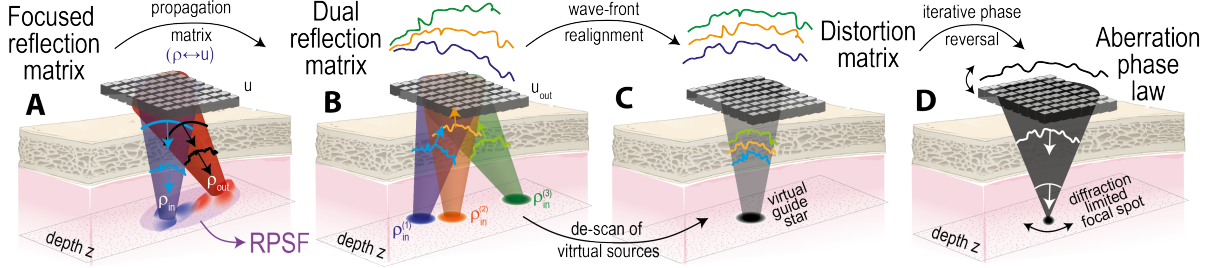


Figure 2: **Principle of ultrasound matrix imaging.** (A) Focused reflection matrix contains the time-gated response between virtual source (ρ_{in}) and detector (ρ_{out}) located at the same depth z . (B) An output projection of the focused reflection matrix in the transducer basis (\mathbf{u}_{out}) provides the reflected wave-fronts induced by each virtual source (ρ_{in}) at depth z . (C) Those wave-fronts are realigned to extract the wave-front distortions induced by the mismatch between the real speed-of-sound distribution and the wave velocity model. Seen from the focused basis, this operation leads to an angular de-scan of each virtual source at the same position, leading to the synthesis of a virtual guide star. (D) Exploiting the correlations between each distorted wave-front, an iterative phase reversal algorithm extract an aberration phase law that can be used to compensation for wave distortions induce by the skull and ideally retrieve a diffraction-limited focal spot across the field-of-view.

Quantifying skull-induced aberrations and scattering

In contrast with standard beamforming that relies on the principle of confocal imaging, the cornerstone of UMI consists in decoupling the input and output focal spots in post-processing. Mathematically, such a beamforming procedure can be written as follows:

$$R(\rho_{out}, \rho_{in}, z) = \sum_{\mathbf{v}_{in}} \sum_{\mathbf{u}_{out}} R(\mathbf{u}_{out}, \mathbf{v}_{in}, \tau_{in}(\mathbf{v}_{in}, \rho_{in}, z) + \tau_{out}(\mathbf{u}_{out}, \rho_{out}, z)), \quad (1)$$

where $\mathbf{r}_{in} = (\rho_{in}, z)$ and $\mathbf{r}_{out} = (\rho_{out}, z)$ are the coordinates of the input and output focal points, respectively. τ_{in} and τ_{out} are the expected travel times for the wave to go from the source/detector to the input/output focusing point (Methods, Eq. 4). The result of Eq. 1 is a focused reflection matrix $\mathbf{R}_{\rho\rho}(z)$ whose coefficients $R(\rho_{out}, \rho_{in}, z)$ probe the cross-talk between a virtual source

and detector at lateral positions ρ_{in} and ρ_{out} , respectively, both located at the same depth z inside the medium (Fig. 2A). This matrix enables a local quantification of the focusing quality and multiple scattering by providing a local point spread function in reflection (RPSF) (61, 63, 66) (Methods, Eq. 7). Figures 3C and F display maps of RPSFs at different depths in sheep n°5 and 6. In the single scattering regime and in absence of aberrations, the RPSF would exhibit a confocal diffraction-limited peak. On the contrary, the various RPSFs here display a distorted focal spot induced by wave velocity heterogeneities on top of an incoherent background due to multiple scattering events induced by the skull. Strikingly, whatever the imaging depth, the RPSF looks more distorted and less contrasted for sheep n°6 (Fig. 3F) than for sheep n°5 (Fig. 3C).

On the one hand, the aberration level can be quantified by investigating the spatial extent $\delta\rho$ of the RPSF (60, 62) (Figure S2). The depth evolution of $\delta\rho$ is shown in Fig. 3D. A general trend is a degradation of the resolution with depth as predicted by diffraction theory but also a more important aberration level in sheep n°6. This difference of behavior can be understood by looking at the micro-CT of each skull (Figs. 3A,B). While the skull of sheep n°5 shows a regular thickness $L \sim 5.3 \pm 1$ mm, skull n°6 displays a more irregular shape: $L \sim 5.7 \pm 1.7$ mm (42). This difference of morphology between the two skulls can explain the higher degree of aberrations observed in the latter case.

On the other hand, the multiple scattering rate can be estimated from the incoherent background of the RPSF (Methods, Eq. 9). It appears that the multiple scattering rate is far from being negligible even after the beamforming process since it reaches a value of 30 % in the $z = 35$ mm-region for sheep n°6 ((Fig. 3E). As the aberration level, the multiple scattering rate is larger for this sheep over almost the whole depth range. This feature can be understood by looking at the internal structure of the bone. Indeed, the diploë volume ratio ν is much higher in sheep n°6 ($\nu = 57\%$) than in sheep n°5 ($\nu = 29\%$) (42). This high degree of heterogeneity

seems to account for the higher multiple scattering rate observed in sheep n°6.

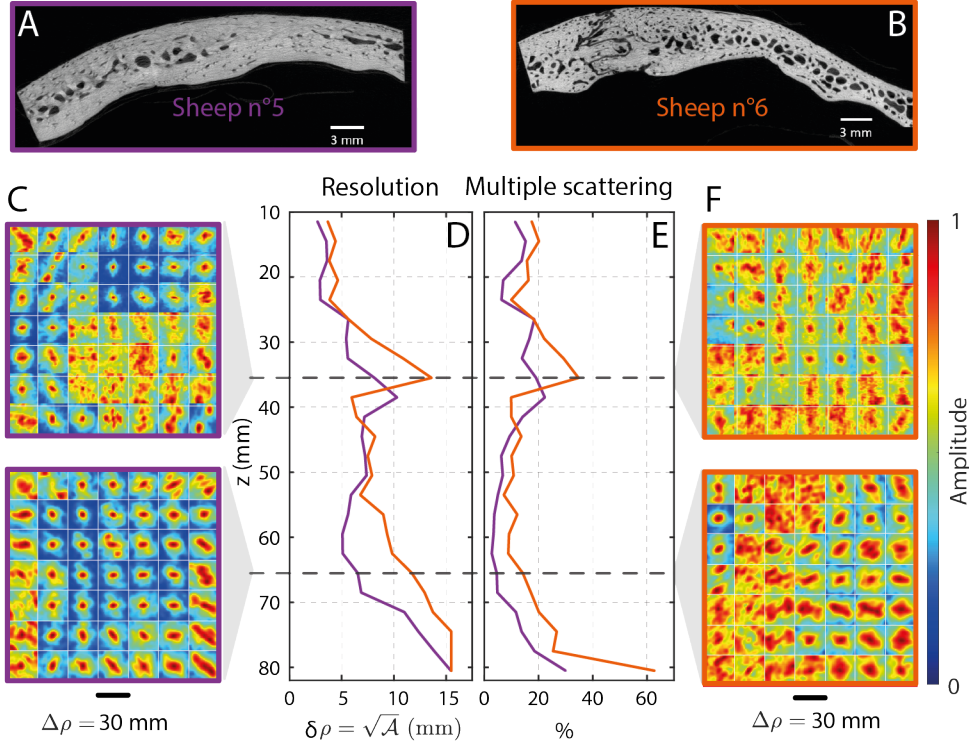


Figure 3: **Influence of cranial heterogeneities on aberration and multiple scattering.** (A and B) Micro-CT of sheep n°5 and 6, respectively. (C) RPSF maps at depth $z = 35$ mm and $z = 65$ mm for sheep n°5. (D and E) RPSF extension and multiple scattering rate as a function of depth, extracted by UMI, for sheeps n°5 (purple line) and 6 (orange line). (F) RPSF maps at depth $z = 35$ mm and $z = 65$ mm for sheep n°6.

Overcoming wave distortions

The complex speed-of-sound distribution highlighted by Fig. 3B hampers ultrasound brain imaging. The confocal image of the brain, that can be extracted from the diagonal coefficients of $\mathbf{R}_{\rho\rho}(z)$ (Methods, Eq. 5), is blurred by the skull heterogeneities (Fig. 4A). Fortunately, an optimized contrast and diffraction-limited resolution can be recovered using the distortion matrix concept (61, 63, 66). This matrix contains the wave distortions exhibited by each reflected

wave-front in the transducer basis (Fig. 2C). The choice of this basis is dictated by the fact that most aberrations are induced by the skull heterogeneities, *i.e* in the vicinity of the probe. An iterative phase reversal process is then applied to the distortion matrix to provide an estimation of an aberration phase law, $\phi(\mathbf{u}_{\text{out}}, z)$, at each depth (Methods, Fig. 2D and Movie S1). Examples of aberration phase laws are displayed in Fig. 4C. They show a complex feature, as quantified by the Strehl ratio \mathcal{S} that provides the ratio between the energy at focus with and without aberrations (67) (Methods, Eq. 18). For instance, the aberration law measured at depth $z = 50$ mm exhibits a Strehl ratio $\mathcal{S} = 0.03$. This extremely low value illustrates the detrimental impact of skull heterogeneities on the focusing process. Those aberration phase laws can be leveraged to compensate for the wave distortions exhibited by the focused reflection matrix (Methods). The resulting confocal image is displayed in Fig. 4E. It exhibits a clear improvement compared to the initial confocal image (Fig. 4A), with a contrast gain of 8 dB for the deepest bright scatterers ($z = 50$ and 60 mm). The efficiency of the correction process can be assessed by comparing the maps of RPSFs before (Fig. 4B) and after (Fig. 4D) aberration compensation. The spatial resolution is improved by a factor between 2 and 3 and the multiple scattering background is decreased by more than 10 dB beyond $z = 50$ mm (Figure S3). Nevertheless, the obtained RPSFs remain imperfect in Fig. 4D. High-order aberrations subsist because of their anisoplanicity. Last but not least, even though the confocal image shows a clear improvement, the diffraction-limited resolution $\delta\rho_0$ remains limited by the probe size D : $\delta\rho_0 \sim \lambda z/D$.

Matrix imaging for ultrasound localization microscopy

To overcome diffraction, ULM is then performed by injecting boluses of microbubbles and tracking them using an ultra-fast imaging sequence (Methods). To that aim, an hybrid transmit basis is used with three cylindrical waves emitted by the entire probe and two spherical

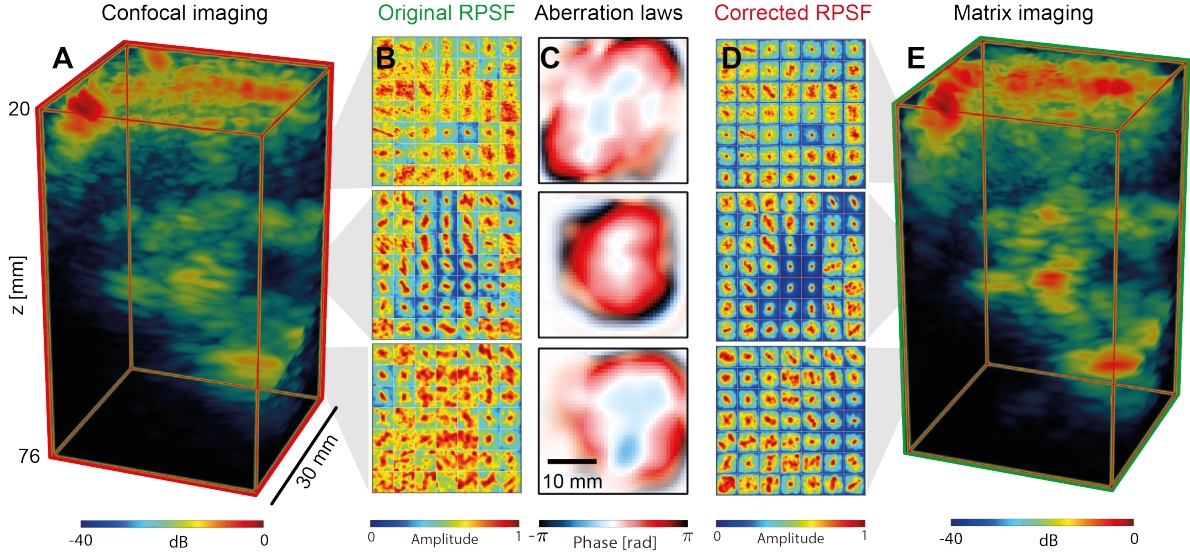


Figure 4: **Ultrasound matrix imaging in the sheep brain.** (A) Confocal volume extracted from the diagonal of the focused \mathbf{R} -matrices (Methods, Eq. 5). (B, C, and D) Maps of initial RPSFs, aberration phase laws, and corrected RPSFs, respectively, at three different depths $z = 32$ mm (top), $z = 50$ mm (middle) and $z = 68$ mm (bottom). Each RPSF is displayed in a scan range $\Delta\rho = \rho_{\text{out}} - \rho_{\text{in}}$ (see Methods) that varies from -15 to $+15$ mm in both x and y directions. (E) Confocal image after aberration correction. The results shown here correspond to ultrasound data acquired on sheep n°6

diverging waves transmitted successively by each probe panel (68). This sparse emission basis, referred to as \mathbf{s}_{in} , yields a volume rate of 209 Hz (Methods). For each incident wave-field, the raw data is stored in a set of new reflection matrices $\mathbf{R}'_{\text{us}}(\tau, t_m) = [R'(\mathbf{u}_{\text{out}}, \mathbf{s}_{\text{in}}, \tau, t_m)]$ recorded at different times t_m . A set of three-dimensional images is then obtained by beamforming the reflection matrix rephased by the conjugate of the aberration law to compensate for skull-induced aberrations:

$$\mathcal{I}'(\mathbf{r}, t_m) = \sum_{\mathbf{s}_{\text{in}}} \sum_{\mathbf{u}_{\text{out}}} \exp[-j\phi(\mathbf{u}_{\text{out}}, z)] \times R'(\mathbf{u}_{\text{out}}, \mathbf{s}_{\text{in}}, \tau_{\text{in}}(\mathbf{s}_{\text{in}}, \boldsymbol{\rho}, z) + \tau_{\text{out}}(\mathbf{u}_{\text{out}}, \boldsymbol{\rho}, z), t_m) \quad (2)$$

After filtering out the static component in the image series (Methods), the dynamic component of the brain can be highlighted. A two-dimensional cross-section of the resulting image is displayed in Fig. 5B at a given time t_m . This image is compared to its raw counterpart (Fig. 5A) obtained without aberration compensation. Thanks to UMI, isolated microbubbles show less distorted focal spots, as shown by two examples in the purple and red squares in Figs. 5A,B. Just above (~ 50 mm), a halo of microbubbles in the blue square seem to indicate the presence of a vessel but the individual detection of each microbubble is complicated by their high concentration.

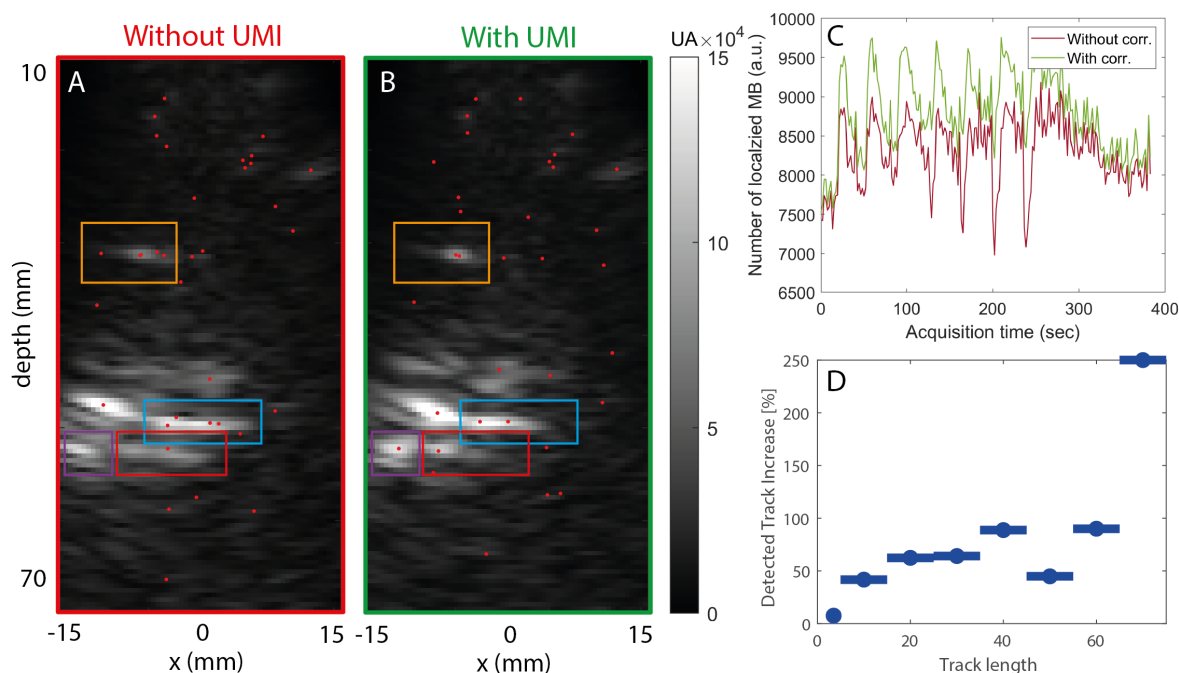


Figure 5: Enhancing micro-bubble localization accuracy with UMI. (A and B) Coronal middle slice of the ultrasound image without and with aberration correction at a given time, respectively. The position of the detected microbubbles is highlighted by red dots. The blue and orange boxes show areas where false alarms are reduced by reducing the side lobes of the PSF. The purple region shows a region where aberration compensation allows the detection of a microbubble. The red square highlights a zone where a microbubble is reassigned at a new location. (C) Number of localized microbubbles as a function of the acquisition time t with (green) and without (pink) UMI. (D) Increase of the microbubble detection rate with UMI for different track lengths. The data shown here correspond to sheep n^o 6 (acquisition 4).

A deconvolution of the dynamic image with a theoretical PSF and the application of a detection threshold provides a localization of bubbles in the field-of-view (Methods). The position of the detected bubbles are highlighted with red disks in Fig. 5A and B. While several microbubbles are wrongly assigned to the high side-lobes of each distorted focal spot in Fig. 5A, UMI drastically reduces this number of false detection in Fig. 5B by restoring a satisfying PSF, in particular in the colored squares. Despite this reduction of false alarms, the number of localized microbubbles is increased. Figure 5C illustrates this assertion by showing, for sheep n°6, the higher number of detected bubbles over time with UMI. The different peaks corresponds to each injection of microbubbles.

To further reduce the false alarm rate and better appreciate the effect of UMI, microbubbles can be tracked over time (Methods). Fig. 5D shows the increase of detected tracks provided by UMI as a function of their length. UMI significantly increases the number of detected tracks by a factor ranging from 40 to 250% according to the track length. Table 1 shows the same observable for different minimum path lengths. The general trend is the same for each acquisition, thereby showing the robustness of UMI and its benefit in each imaging configuration.

Sheep number	Acq. number	Additional tracks detected with UMI correction [%]				
		≥ 2 frames	>3 frames	>5 frames	>10 frames	>30 frames
S4	1	24.0	41.7	49.1	63.3	121.7
S5	1	14.1	26.6	35.7	54.5	76.6
	2	7.5	13.7	16.2	28.5	42.8
S6	1	15.4	16.3	19.2	23.5	43.5
	2	8.7	13.3	15.3	19.1	36.1
	3	6.7	11.5	13.2	17.3	18.3
	4	6.9	22.3	32.5	47.2	66.2
	5	8.7	27.9	32.5	53.5	77.1
Mean		11.5	21.7	26.7	38.4	60.3
Std		6.0	9.0	11.2	16.1	28.5

Table 1: **Quantification of UMI benefits on ULM tracks.** Percentage of additional tracks provided by UMI for various minimal tracks length expressed in frames.

ULM density maps are built by counting the number of tracks crossing each voxel of the

field of view. Such volumes are shown from two different angles in Fig. 6. The typical structure of the cerebral vessels can already be recognized before aberration correction (Figs. 6A and C). This structure is known as the Willis circle (69) and can be seen as the main cross-road of the main vessels in the brain.

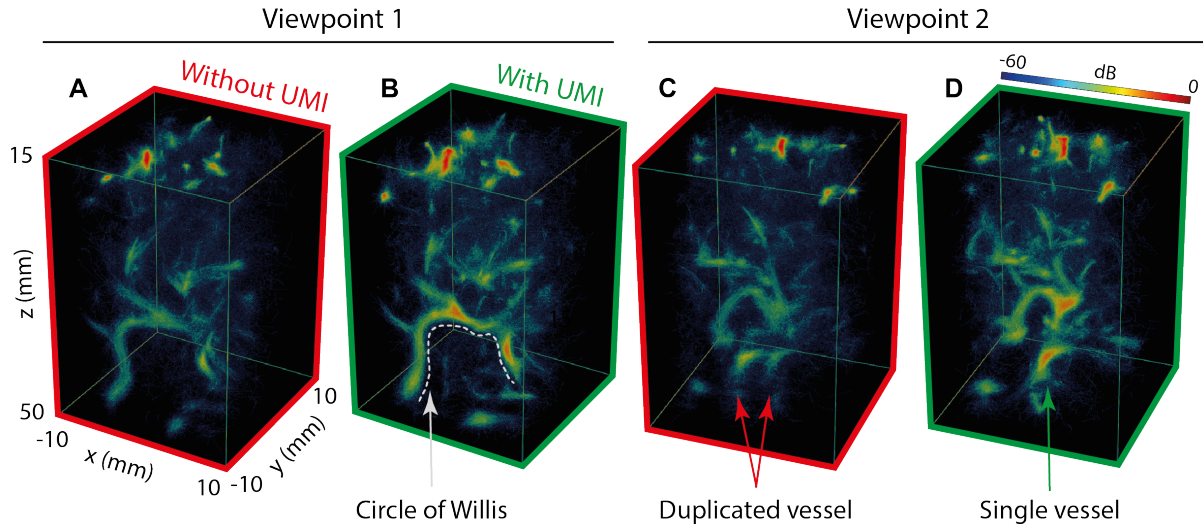


Figure 6: Correcting ULM images with UMI. 3D images shown as maximum intensity projections from two different points of view. ULM volumes (A, C) before and (B, D) after aberration compensation. The circle of Willis is located around $z = 40 - 45$ mm. The same dynamic range is displayed for all images. The data shown here correspond to sheep n° 6, acquisition 4.

However, the skull heterogeneities also induce artifacts such as the duplication of one artery that can be noticed at the bottom of the conventional ULM image (Figs. 6A and C). This type of artifact has already been observed in transcranial imaging in both mouse (45) and human (34). Strikingly, the focusing laws extracted by UMI (Fig. 4C) allow the removal of such artifacts as highlighted by the corrected ULM images in Figs. 6B and D. More generally, the higher detection rate and correct repositioning of micro-bubbles lead to a better contrast for ULM as highlighted by the vessel network on top of the Willis circle ($z = 25-35$ mm) that appears much brighter (+6 dB) and better resolved. The same resolution and contrast improvement can be observed for the other 3D ULM images shown in Figure S4 and corresponding to other

acquisitions on the three sheeps.

To appreciate more quantitatively the benefit of UMI, the ULM density maps are superimposed on MRA, which is considered as the gold standard for observing large vascular structures in the brain. After UMI, the ULM density maps match more closely with the vessels revealed by MRA. In the coronal slice (Fig. 7A,C), UMI allows a more complete and brighter reconstruction of the vascular network (Fig. 7C) that matches in depth with the Willis polygon revealed by MRA, especially in the anterior cerebral arteries that deviate from the polygon (green and blue arrows). In the sagittal section (Fig. 7B,D), ULM provides a better adequation between MRA and ULM images of venous system (yellow arrows). Besides the repositioning of large vessels, the increase in the total SNR of the ULM volume and the appearance of small vessels provided by UMI are noteworthy compared to the initial ULM image but also with respect to MRA that only provides an image of the largest vessels.

DISCUSSION

Compared to a previous study on a static brain phantom (63), we have here demonstrated the benefit of UMI for transcranial in-vivo imaging. Besides improving the contrast and resolution of standard ultrasound brain images, we also showed that it can be fruitfully coupled to other imaging modalities such as ULM. While the gain in contrast provided by UMI leads to increase the overall number of detected microbubbles, it also drastically increased the length of the microbubble tracks, which is affected by SNR, point-source separability and localization precision. Because UMI reduces the side lobes of the imaging PSF, the localization of microbubbles is actually much sharper. Doing so, UMI provides a more detailed ULM image and the removal of common artifacts such as duplication of vessels. The success of UMI has been confirmed by confronting ULM images with gold standard MRA.

More fundamentally, UMI also showed some correlation between the aberration level and

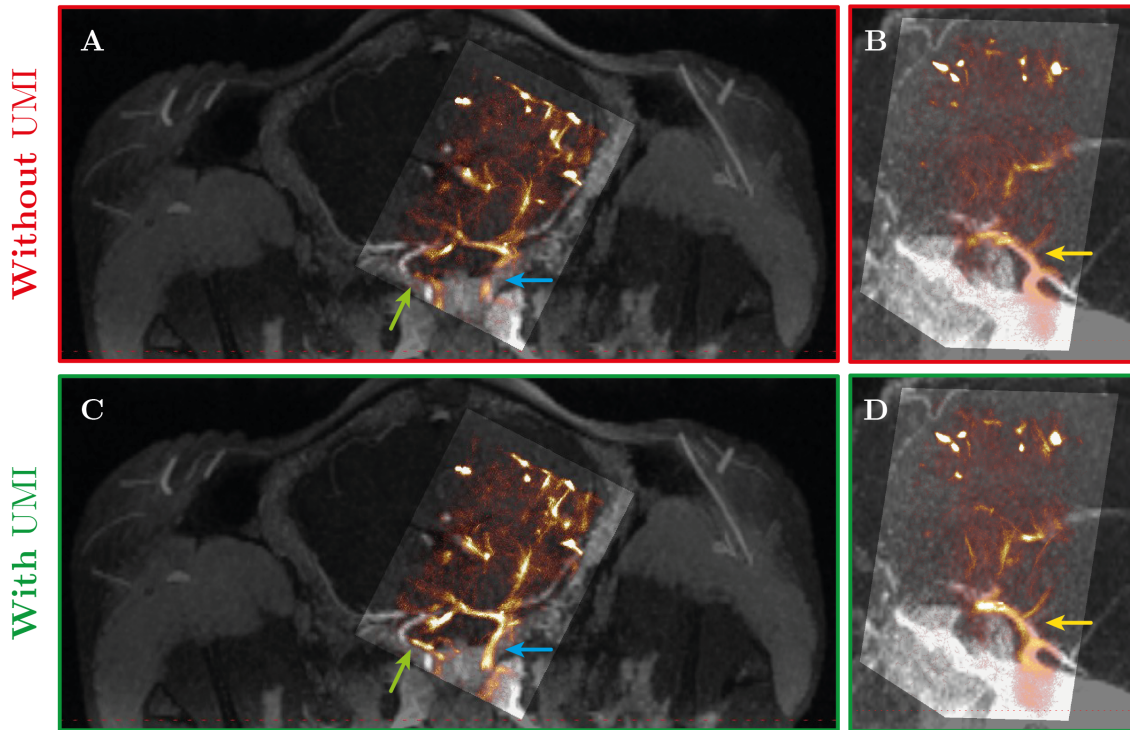


Figure 7: **Confronting ULM density maps to MRA images.** **a, b** Coronal slices and sagittal slices, respectively: The ULM sections are shown as maximum intensity projections of 5 mm (coronal) and 1 cm (sagittal) thickness. Arbitrary colormaps are used with fixed dynamic range for the ULM (hot colormap) and MRA (B&W scale) images to facilitate their comparison. **c, d** Same images as in **a, b** with ULM images corrected by UMI. Green and blue arrows show a better reconstruction of the posterior cerebral arteries in deviation from the Willis polygon. Yellow arrows show the correction in the venous system. The data shown here correspond to sheep number 6 (acquisition 4).

the skull thickness variations. It also highlighted the impact of diploë volume fraction on the multiple scattering level. The striking result of UMI on sheep n°6 is promising for applications of UMI on human brain imaging, since this skull heterogeneity is in line with what is expected for human brain imaging across the temporal window (70, 71).

UMI approach for aberration compensation is perfectly complementary with the alternative route proposed recently by Robin *et al.* (34) that exploited bubbles as ultrasound guide stars for trans-cranial adaptive focusing. To some extent, UMI is more robust since it directly synthe-

sizes virtual guide stars from speckle (61) and can work whatever the concentration of bubbles. However, in the current realization, UMI also showed some limits. First, it requires the prior recording of a high-dimension reflection matrix. Second, in the present case, it only provided the depth-dependence of aberration phase laws and was not able to capture the lateral variations of aberrations. Last but not least, it did not compensate for the multiple reverberations inside the skull that prevented us from brain imaging right below the skull.

In future works, these limits will be addressed by exploiting the dynamics of blood vessels. Indeed, rapid decorrelation of speckle may offer an opportunity as it provides numerous speckle realizations for a single voxel. Therefore, the aberration phase laws could in principle be extracted at a higher spatial resolution, since UMI would no longer have to rely on the isoplanicity assumption (72, 73). In other words, time averaging will replace spatial averaging, and the aberration phase laws can be extracted directly from the ultrafast sequence [Fig. 1D] and not from a prior static sequence [Fig. 1C]. This access to a large number of disorder realizations can also be leveraged for tailoring complex spatio-temporal focusing laws capable of harnessing multiple reverberations.

In the future, we therefore expect that aberration and scattering compensation provided by UMI could help ULM in the detection and classification of cerebrovascular accidents in humans. An important goal will be to distinguish between ischemic and hemorrhagic strokes in the early phase, as has been done in the rat brain (36). Therefore, one perspective of this work is to conduct a pilot study with patients who have recently suffered a stroke.

In this paper, we have shown the great advantages and relative simplicity of the UMI and ULM combination. 3D transcranial in-vivo imaging of sheep brain has been performed and shown to be in excellent agreement with gold standard MRA. Although we have proven this claim for the specific case of ultrasound data acquired with a multiplexed matrix probe, these results are much more general. Indeed, UMI can be applied to any other ultrasound probe

(e.g. sparse or RCA array), any other modality (e.g. conventional Doppler or shear wave elastography) and in either 2D or 3D configurations.

Beyond the specific case of ultrasound, this study paves the way towards the combination of matrix imaging (74, 75) with super-resolution localization techniques (13) in all fields of wave physics, ranging from optical microscopy with fluorescent molecules (76) to seismology for dynamic imaging of glaciers (77, 78).

MATERIALS AND METHODS

Experimental procedure

All experiments were performed between October 2022 and January 2023 in accordance with ARRIVE guidelines, the European Directives and the French Legislation on Animal Experimentation (#32738) and approved by the local ethics committee (CENOMEXA, No54). A total of 6 female domestic sheep were included in the study (*Ovis aries* weighing 35-40 kg and aged 15-16 months). Three of them were used to optimize the experimental protocol, the other three are presented here (Table S1).

The sheep were sedated with ketamine (12 mg/kg) and xylazine (1 mg/kg) for placement of the venous catheter. Propofol (100 μ g/kg) was then administered and endotracheal intubation was performed. During the surgical and imaging procedures, anesthesia was maintained by administration of sevoflurane (1.5-2.0 %) in air. Cardiac activity, oximetry and arterial pressure (between 80 and 120 mm Hg) were monitored throughout the procedure. Respiratory rate and tidal volume were adjusted to maintain physiologic limits, and body temperature was maintained at 38 °C with a warming blanket.

At the end of the ultrasound examination, i.e. after 5 hours, the anesthesia was terminated so that the animal could wake up and be returned to its box. Three imaging sessions were performed within one to two weeks. At the end of the last imaging session, the sheep was eu-

thanized with a barbiturate overdose (pentobarbital 0.2g/kg) after 2 minutes of 8% sevoflurane inhalation. A controlled arterial pressure of less than 20 mmHg and no cardiac activity were used to assess death.

After the last imaging session and the death of the animal, the part of the skull below the probe was removed for cleaning and preserved in saline and bleach. This subject was then fixed in a bath of formalin-free fixative F13 (Morphisto, Germany) for one day, then dried and placed under vacuum for micro-CT imaging (Skyscan 1176, Bruker, Kontich, Belgium, voxel size 18 μm) (42).

Ultrasound sequences

A low-frequency matrix array probe (Vermon, Tours, France), as described in Tab. 2, is placed on the shaved head of the sheep directly over the crest of the frontal bone. 3D ultrasound sequences were then recorded with a Vantage 256 4-to-1 multiplex system (Verasonics UTA 1024-MUX adapter, Kirkland, USA). The ultrasound waves were generated synthetically in this way so that the reflected wavefronts corresponding to each individual ultrasound wave emission could be reassembled in post-processing (42, 68). The successive ultrasound imaging sequences of the entire sheep experiment are summarized in Fig. 1.

Number of transducers	$32 \times 32 = 1024$ (with 5 dead elements)
Geometry (y-axis)	3 inactive rows between each block of 32×8 elements
Pitch	$\delta u = 0.55 \text{ mm}$ ($\approx \lambda/2$ at $c = 1540 \text{ m/s}$)
Aperture	$\Delta u = \begin{pmatrix} \Delta u_x \\ \Delta u_y \end{pmatrix} = \begin{pmatrix} 17.6 \text{ mm} \\ 19.3 \text{ mm} \end{pmatrix}$
Central frequency	$f_c = 1.56 \text{ MHz}$
Bandwidth	60% $\rightarrow [1 - 2] \text{ MHz}$
Transducer directivity	$\theta_{max} = 64^\circ$ at $c = 1540 \text{ m/s}$

Table 2: **Matrix array datasheet.**

Initial real-time power Doppler imaging is used to correctly position the probe before acquiring longer ultrasound data. This sequence is performed with the injection of microbubble contrast agent (Sonovue, Bracco, Italy) (79) to obtain SNR-enhanced images of the cerebral vasculature. Once a sufficiently good signal is obtained, the probe is fixed, and we wait until the microbubbles has disappeared, i.e. 10 minutes.

A UMI sequence is then performed. It consists in the acquisition of the reflection matrix using a set of 324 synthetic spherical waves whose associated virtual sources are located along a sphere radius $\mathcal{R} = 39.5$ mm: $\mathbf{R}_{\mathbf{u}\mathbf{v}}(t) = [R(\mathbf{u}_{\text{out}}, \mathbf{v}_{\text{in}}, t)]$, where the vector \mathbf{v}_{in} indicates the position of the corresponding virtual sources (see Supplementary S1) and \mathbf{u}_{out} refers to the position of the transducers. The insonification of the medium is made with diverging waves associated with virtual sources located above the probe (65) such that:

$$\mathbf{v} = \begin{pmatrix} v_x \\ v_y \\ v_z \end{pmatrix} = -\mathcal{R} \begin{pmatrix} \sin \theta_x \\ \sin \theta_x \\ \sqrt{1 - \sin^2 \theta_x - \sin^2 \theta_y} \end{pmatrix}, \quad (3)$$

with $\boldsymbol{\theta} = (\theta_x, \theta_y)$ the angle associated with each virtual source. The angular range spans from -30° to 30° with an angular pitch $\delta\theta = 3^\circ$.

Finally, a hybrid ultrasound localization microscopy (ULM) sequence is performed. Three cylindrical waves were emitted simultaneously from the entire probe and two spherical waves were decomposed by panels to achieve a volume rate of 209 Hz (42, 68). Since only a few insonifications are used, the ULM acquisition consists of a partial reflection matrix that is acquired at several points in time t_m so that the reflected wavefronts are stored in a second matrix $\mathbf{R}' = [R'(\mathbf{u}_{\text{out}}, \mathbf{i}_{\text{in}}, \tau, t_m)]$. During this acquisition, microbubble injection boluses (Sonovue, Bracco, Italy) were injected, i.e. from 1.5 to 2 ml/min, during 2.5 to 6.5 minutes.

A total of 1 to 6 acquisitions were performed per sheep. The experimental conditions are summarized in Table S1. It should be noted that, for some acquisition, the UMI sequence is

performed after the ULM sequence and not before, but this does not change the principle of the method.

Focused reflection matrix

A general procedure to build each focused reflection matrix, noted $\mathbf{R}_{\rho\rho}(z)$, is to use a delay-and-sum (DAS) algorithm, which consists of applying appropriate time delays to create a double synthetic focusing procedure at both input and output (Bureau 2023) (63). Mathematically, such a beamforming procedure is described by Eq. 1. The input and output times-of-flight, τ_{in} and τ_{out} , are given by:

$$\tau_{\text{in/out}}(\mathbf{w}, \mathbf{r}) = \frac{|\mathbf{w} - \mathbf{r}|}{c_0} = \frac{\sqrt{(x - w_x)^2 + (y - w_y)^2 + (z - w_z)^2}}{c_0}. \quad (4)$$

with $\mathbf{w} = \mathbf{v}_{\text{in}}$ at input and $\mathbf{w} = \mathbf{u}_{\text{out}}$ at the output.

The confocal volume shown in Fig. 4e can be extracted from the diagonal elements of the reflection matrix ($\rho_{\text{in}} = \rho_{\text{out}}$), so that :

$$\mathcal{I}(\boldsymbol{\rho}, z) = |R(\boldsymbol{\rho}, \boldsymbol{\rho}, z)|^2 \quad (5)$$

Reflection point spread function

The off-diagonal points in $\mathbf{R}_{\rho\rho}(z)$ can be exploited for a quantification of the focusing quality at any pixel of the ultrasound image. To that aim, the spreading of energy is investigated along each antidiagonal of $\mathbf{R}_{\rho\rho}(z)$. Mathematically, this can be done by means of a change of variable in order to express the focused \mathbf{R} -matrix in a common mid-point basis (63):

$$R(\Delta\boldsymbol{\rho}, \boldsymbol{\rho}_m, z) = R(\boldsymbol{\rho}_{\text{out}}, \boldsymbol{\rho}_{\text{in}}, z) \quad (6)$$

with $\boldsymbol{\rho}_m = (\boldsymbol{\rho}_{\text{in}} + \boldsymbol{\rho}_{\text{out}})/2$, the mid-point between $\boldsymbol{\rho}_{\text{in}}$ and $\boldsymbol{\rho}_{\text{out}}$, and $\Delta\boldsymbol{\rho} = \boldsymbol{\rho}_{\text{out}} - \boldsymbol{\rho}_{\text{in}}$, their relative position. A local average of the back-scattered intensity is then performed needed to smooth out

the speckle reflectivity and provide an estimator of the reflection point spread function (RPSF):

$$RPSF(\Delta\boldsymbol{\rho}, \mathbf{r}_p) = \langle |R(\Delta\boldsymbol{\rho}, \boldsymbol{\rho}_m, z)|^2 \mathcal{P}(\boldsymbol{\rho}_m - \boldsymbol{\rho}_p, z - z_p) \rangle_{\boldsymbol{\rho}_m, z} \quad (7)$$

with $\mathbf{r}_p = (\boldsymbol{\rho}_p, z_p)$. The symbol $\langle \dots \rangle$ denotes a spatial average over the variables in the subscript. $\mathcal{P}(\boldsymbol{\rho}_m - \boldsymbol{\rho}_p, z - z_p) = 1$ for $|\boldsymbol{\rho}_m - \boldsymbol{\rho}_p| < p_\rho/2$ and $|z_m - z_p| < p_z/2$, and zero otherwise. The dimensions of \mathcal{P} used for Figs. 4c,d and 3e-h are $\mathbf{p} = (p_\rho, p_z) = (4.3, 3)$ mm.

As shown by Figs. 4c,d and Figs. 3e-h, the RPSF displays the following typical shape: A distorted confocal spot associated with singly-scattered echoes on top of a flat background induced by multiple scattering events. On the one hand, the extension $\delta\rho$ of the confocal peak is directly related to the local resolution of the ultrasound image. $\delta\rho$ can be obtained by considering the de-scanned area \mathcal{A}_{-3dB} over which the normalized RPSF is larger than -3dB (Supplementary Fig. S2), such that

$$\delta\rho = \sqrt{\mathcal{A}_{-3dB}/\pi}. \quad (8)$$

On the other hand, the multiple scattering rate α_M can be estimated by averaging the RPSF beyond the confocal peak, *i.e* for $4\delta\rho_0 < \|\Delta\boldsymbol{\rho}\| < 6\delta\rho_0$:

$$\alpha_M(\mathbf{r}_p) = \frac{\langle RPSF(\Delta\boldsymbol{\rho}, \mathbf{r}_p) \rangle_{4\delta\rho_0 < \|\Delta\boldsymbol{\rho}\| < 6\delta\rho_0}}{RPSF(\Delta\boldsymbol{\rho} = \mathbf{0}, \mathbf{r}_p)} \quad (9)$$

Aberration phase law

An aberrations law is extracted using the distortion matrix framework (61, 63) following these different steps:

- (i) Projecting $\mathbf{R}_{\rho\rho}(z)$ onto the transducer basis (\mathbf{u}) either at input or output using a ho-

homogeneous propagation model:

$$\mathbf{R}_{\mathbf{u}\rho}(z) = \mathbf{G}_0(z) \times \mathbf{R}_{\rho\rho}(z), \quad (10)$$

and

$$\mathbf{R}_{\rho\mathbf{u}}(z) = \mathbf{R}_{\rho\rho}(z) \times \mathbf{G}_0(z)^\top \quad (11)$$

with $\mathbf{G}_0 = [G_0(\mathbf{u}, \boldsymbol{\rho}, z)]$, the transmission matrix between the probe and the set of voxels at depth z for an ideal homogeneous medium:

$$T_0(\mathbf{u}, \boldsymbol{\rho}, z) = \frac{z \exp\left(jk_0 \sqrt{\|\mathbf{u} - \boldsymbol{\rho}\|^2 + z^2}\right)}{4\pi(\|\mathbf{u} - \boldsymbol{\rho}\|^2 + z^2)} \quad (12)$$

- (ii) Isolate the distorted part of each wavefront by subtracting the geometric component that would be ideally obtained in absence of aberrations (i.e. constant speed of sound c_0).

The result is the input and output distortion matrices which are expressed as follows:

$$\mathbf{D}_{\mathbf{u}\rho}(z) = \mathbf{R}_{\mathbf{u}\rho}(z) \circ \bar{\mathbf{T}}_0 \quad (13)$$

and,

$$\mathbf{D}_{\rho\mathbf{u}}(z) = \mathbf{R}_{\rho\mathbf{u}}(z) \circ \bar{\mathbf{T}}_0^\top \quad (14)$$

with $\bar{\mathbf{T}}_0$, the normalized transmission matrix such that

$$\bar{T}_0(\mathbf{u}, \boldsymbol{\rho}, z) = T_0(\mathbf{u}, \boldsymbol{\rho}, z) / |T_0(\mathbf{u}, \boldsymbol{\rho}, z)|.$$

- (iii) Exploit the angular memory effect by computing the correlation matrices of wavefront distortions,

$$\mathbf{C}_{\text{in}}(z) = \mathbf{D}_{\mathbf{u}\rho}(z) \times \mathbf{D}_{\mathbf{u}\rho}^\dagger(z), \quad (15)$$

and

$$\mathbf{C}_{\text{out}}(z) = \mathbf{D}_{\rho\mathbf{u}}^T(z) \times \mathbf{D}_{\rho\mathbf{u}}^*(z). \quad (16)$$

- (iv) A numerical iterative phase reversal algorithm is performed to extract an estimation of aberration laws $\Phi_{\text{in/out}}(z) = [\phi_{\text{in/out}}(\mathbf{u}_{\text{in/out}}, z)]$ by solving the following equation:

$$\Phi_{\text{in/out}}(z) = \arg \{ \mathbf{C}_{\text{in/out}} \times \exp(i\Phi_{\text{in/out}}(z)) \}. \quad (17)$$

The complexity of aberration laws is quantified by the Strehl ratio that can be computed as follows (67):

$$\mathcal{S}(z) = |\langle \exp[i\phi(\mathbf{u}, z)] \rangle_{\mathbf{u}}|^2. \quad (18)$$

The aberration phase laws yield an estimator of the input/output transmission matrix between the probe and each focal plane:

$$\mathbf{T}_{\text{in/out}}(z) = \exp(i\Phi_{\text{out}}) \circ \mathbf{T}_0 \quad (19)$$

- (vi) Aberrations are compensated by applying the phase conjugate of the transmission matrices to provide an updated focused reflection matrix:

$$\mathbf{R}_{\rho\rho}(z) = \mathbf{T}_{\text{in}}^\dagger(z) \times \mathbf{R}_{\mathbf{u}\rho}(z), \quad (20)$$

and

$$\mathbf{R}_{\rho\rho}(z) = \mathbf{R}_{\rho\mathbf{u}}(z) \times \mathbf{T}_{\text{out}}^*(z), \quad (21)$$

The whole process is repeated two times at input and output to improve gradually the focusing process. The confocal volume (Eq. 5) displayed in Fig. 4f and the map of RPSFs (Eq. 7) shown in Fig. 4d are extracted from the diagonal coefficients of this corrected focused reflection matrix.

Ultrasound localization microscopy

From the reflection matrix \mathbf{R}' recorded using the hybrid sequence, a ULM image can be built by performing the following post-processing steps.

- (i) High volume rate ultrasound images are constructed using a conventional delay-and-sum algorithm into a $300 \times 300 \times 600 \text{ mm}^3$ volume with $0.5 \times 0.5 \times 0.5 \text{ mm}^3$ voxels (68):

$$\mathcal{I}'(\boldsymbol{\rho}, z, t_m) = \sum_{\mathbf{s}_{\text{in}}} \sum_{\mathbf{u}_{\text{out}}} R'(\mathbf{u}_{\text{out}}, \mathbf{s}_{\text{in}}, \tau_{\text{in}}(\mathbf{s}_{\text{in}}, \boldsymbol{\rho}, z) + \tau_{\text{out}}(\mathbf{u}_{\text{out}}, \boldsymbol{\rho}, z), t_m)$$

- (ii) A high-pass time filter is applied to the set of images by means of a two frames sliding average window. Thereby, the static component of the ultrasound image (*i.e.* tissues) is removed while the dynamic part of the image (moving microbubbles) is enhanced.
- (iii) The microbubbles were then detected in each volume by localizing regional maxima on each image \mathcal{I}' . The center of each PSF was sub-localized using the radial symmetry method (80–82), *i.e.* by assuming a PSF Gaussian shape and by applying a gradient algorithm. Microbubbles with a signal-to-noise ratio (SNR) below 10.5 were discarded. This SNR was calculated as the intensity at the center of the microbubbles divided by the mean intensity of the neighboring voxels over a 5-mm-wide patch. The detected bubbles and their localization are superimposed to one cross-section section of the ultrasound image in Figs. 5a,b.
- (iv) Tracking algorithm has been performed using the Hungarian method (83) to build the histogram displayed in Fig. 5d. A maximum linking distance between two microbubbles is fixed to 1.5 mm. A one frame gap is allowed and no restriction on track duration is imposed.
- (v) A 3D ULM density map $\mathcal{U}(\mathbf{r})$ is obtained by accumulating and projecting all tracks of microbubbles onto a three-dimensional grid whose spatial sampling is $\lambda/10$. The result is displayed in Fig. 6a,c.

The same process is applied to the ultrasound images \mathcal{I}' corrected by UMI (Eq. 2). The result-

ing ULM maps are displayed in Figs. 6b,d.

Angiographic MRI sequences

Angiographic MRI sequences (MRA) were performed with a 3T MRI scanner (GE SIGNA PREMIER) in a thermoregulated room, i.e. at approximately 20°C (axial propeller, TR=5539 ms, TE=165.744 ms, slice thickness 2 mm, 31 slices, in-plane resolution 0.375×0.375). The superimposition of MRA and ULM images in Fig. 7 has been performed manually using Amira software (Thermofischer, v2019.4).

SUPPLEMENTARY MATERIALS

Section S1. Virtual source basis.

Section S2 Resolution enhancement.

Section S3 Multiple scattering rate.

Section S4 ULM images for each dataset.

Fig. S1. Virtual source basis.

Fig. S2. Quantifying the ultrasound image resolution.

Fig. S3. Single and multiple scattering rates.

Fig. S4. Impact of UMI on different ULM acquisitions.

Tab. S1. Experimental conditions for each acquisition.

Movie S1. Result of UMI at each depth for sheep n°6, acquisition 4.

Movie S2. ULM images for different acquisitions before and after UMI correction.

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Competing interests: A.A. and M.F. are inventors on a patent related to UMI held by CNRS

(no. US11346819B2, published May 2022). O.C. holds patents in ultrasound super-resolution imaging. O.C. is co-founder and shareholder of the startup ResolveStroke, which holds several patents. O.C. is the co-founder of the Resolve Stroke company that commercializes a ULM platform. All authors declare that they have no other competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from L.D. and O.C.

Supplementary Materials

S1 Virtual source basis

The distribution of virtual sources considered for the acquisition of the reflection matrix (UMI acquisition) is displayed in Figure S1a. As described in the Methods Section, the virtual sources are located on a sphere centered on the probe midpoint. The time delays applied to each transducer to create the virtual source associated with the white spot in Fig. S1A are shown in Fig. S1B.

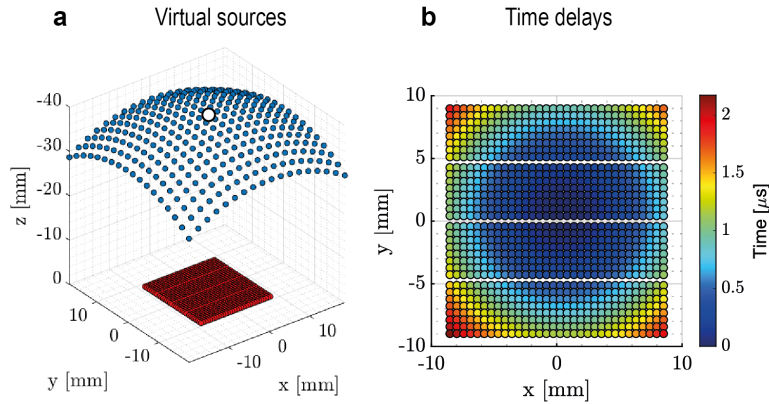


Figure S1: **Virtual source basis.** (A) The \mathbf{R} -matrix is acquired with a set of spherical diverging wave associated with virtual sources whose spatial distribution is displayed with blue dots. (B) Time delay applied to transducers to generate the diverging wave associated with the virtual source highlighted by a white disk in panel A.

S2 Resolution enhancement

The method used for the resolution estimation is illustrated in Fig. S2 by considering an example of RPSF for a specific data set (sheep 6, acquisition n°5) and at a specific depth ($z = 36$ mm). The RPSF map before and after the correction is shown in Figs. S2A,D. An example of RPSF is displayed in Fig. S2B,C. The -3 dB-area, noted \mathcal{A} , is evaluated for each RPSF. An

estimate of the resolution length $\delta\rho$ is then deduced using Eq. 8 (Fig. S2E). The depth evolution of the resolution $\delta\rho$ is shown for each data set in Fig. S2F. The benefit of UMI in terms of resolution is clear whatever the acquisition. The gain in resolution displayed in Fig. S2G increases from a factor 2 at shallow depths to 3 at large depths.

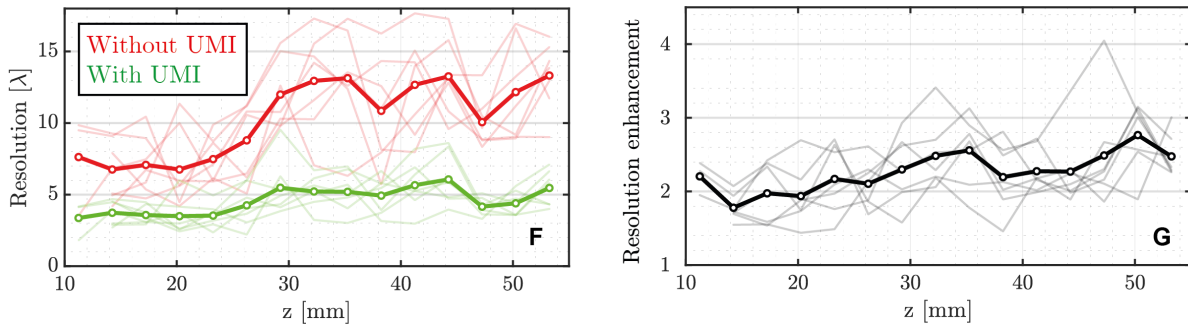
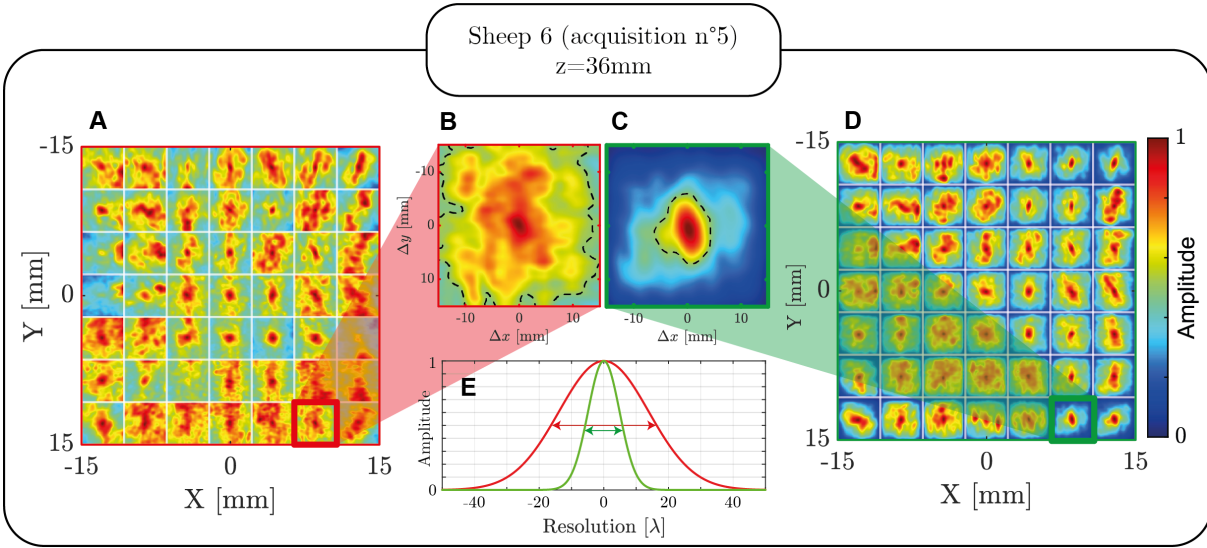


Figure S2: **Quantifying the ultrasound image resolution.** (A,D) RPSF map at $z = 36$ mm before and after correction, respectively. (B,C) Example of one RPSF at a specific location with the -3 dB-area surrounded by a dashed contour, before and after correction, respectively. (E) Radial average of the RPSFs displayed in panels C (red line) and D (green line). (F) Resolution $\delta\rho$ as a function of depth before (red) and after UMI correction (green). The transparent curves refer to the seven individual data sets, while the opaque curves represent their average. (G) Resolution enhancement provided by UMI. The gray lines refer to each individual data set, the black line shows their average value.

S3 Multiple scattering rate

Figure S3A displays the depth evolution of the single and multiple scattering rates (Methods) before and after UMI for the acquisition 4 on sheep n°6. Corresponding RPSF maps and confocal volumes are displayed in Fig. 4. The expression of the multiple scattering rate α_M is provided in the Methods (Eq. 9) and the single scattering rate α_S is deduced from α_M as follows: $\alpha_M = 1 - \alpha_S$. The reduction of the multiple scattering rate provided by UMI is displayed in Fig. S3B.

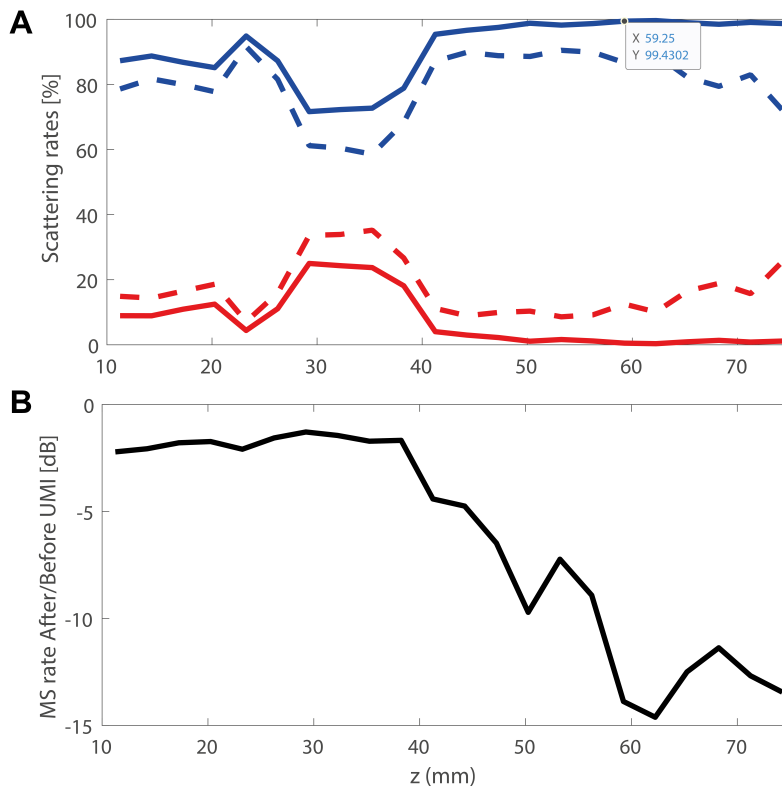


Figure S3: **Single and multiple scattering rates.** (A) Depth evolution of the single scattering (blue) and multiple scattering (red) rates without (dashed line) and with UMI (continuous line). (B) Ratio between the multiple scattering rate measured after and before UMI. The y-scale is in dB. The ultrasound data shown here corresponds to sheep n°6, acquisition 4.

S4 ULM images for each dataset

Figure S4 compares the ULM images obtained with and without UMI for different acquisitions on the three sheeps (Tab. S1). In all cases, a clear resolution and contrast improvement is observed for the different viewing angle (i.e. different probe position) in each acquisition. To better visualize such data in 3D, corresponding films in which the viewing angle changes are available in Movie S2.

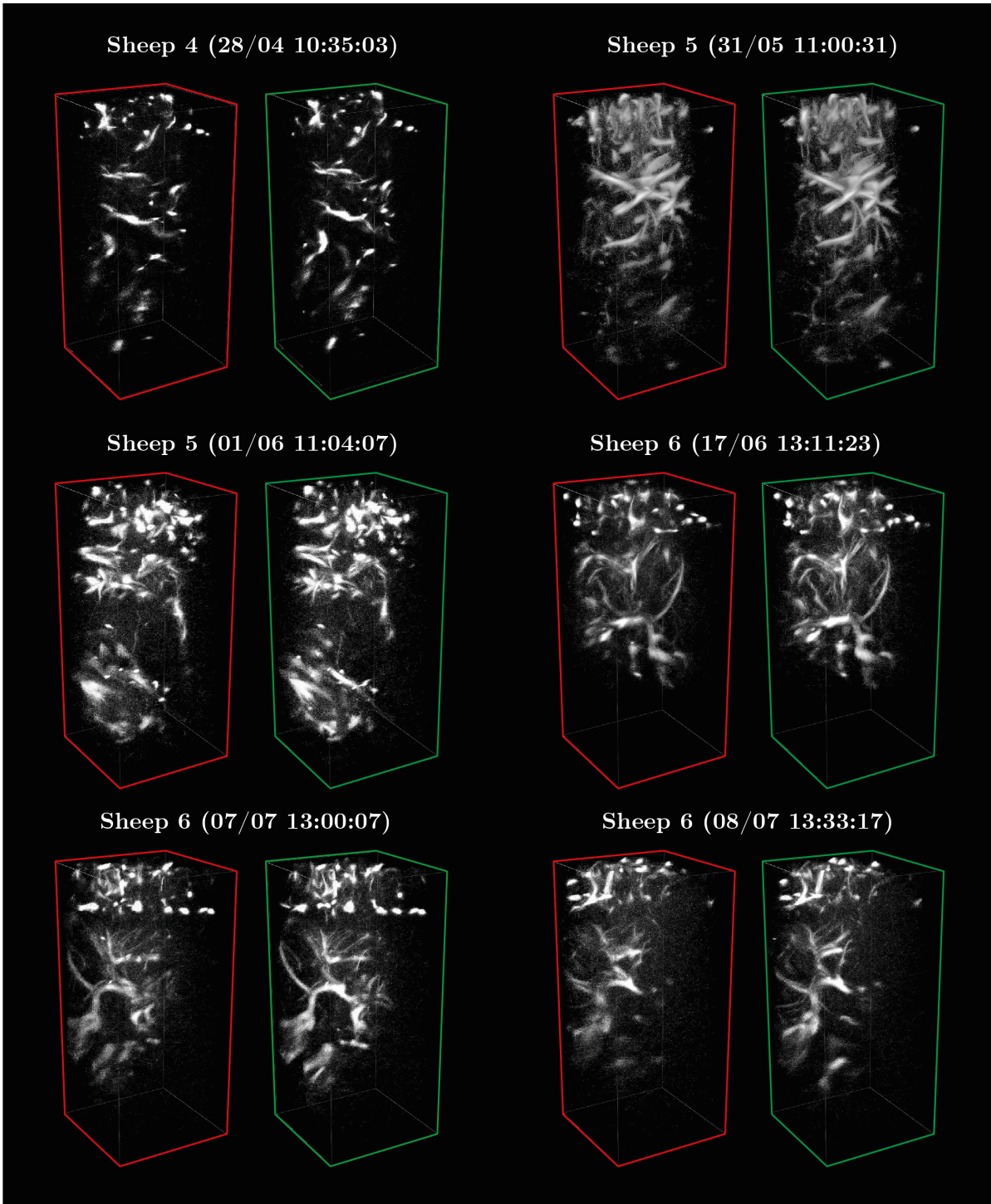


Figure S4: **Impact of UMI on different ULM acquisitions.** ULM volumes with (green) and without (red) application of the UMI correction. Several data acquisitions on three different sheeps are presented here (see also Movie S2).

Sheep number	Acq. number	Date (day/month)	UMI	ULM			Figures
			Time	Time	Injection volume (mL/min)	Nb of injections	
S4	1	28/04	11:27	10:35	2	6	S2
S5	1	31/05	11:21	11:00	2	3	S2, S4
	2	01/06	11:43	11:04	1.5	6	3, S2,S4
S6	1	17/06	10:33	10:40	1.5	5	S2
	2	17/06	10:33	10:58	2	5	S2
	3	17/06	13:33	13:11	2.5	5	S4
	4	07/07	11:49	13:00	1.75	7	4,6,5,7, S2, S4
	5	08/07	12:36	13:33	1.5	10	3,S2, S4

Table S1: Experimental conditions for each acquisition.