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Large-scale optoacoustic microscopy image of cerebral vasculature in the mouse brain.

A sound solution for deep-brain imaging

Ultrasound-based modalities are revealing the brain's inner workings with steadily increasing speed, resolution and depth.

By Michael Eisenstein

Armed with a confocal or multiphoton microscope, researchers can extract gloriously detailed images of cellular- and molecular-scale events in the living brain, including fluctuations of calcium ions and voltage associated with neuronal activity. Indeed, even super-resolution imaging is accessible in vivo, revealing dynamic changes in dendritic spine organization with sub-diffraction-limit levels of detail.

There are stringent limits, however, on how far one can probe with light alone, and the physics of light microscopy swiftly becomes unforgiving as one delves even superficially into the brain. "Conventional optical imaging has a problem because beyond a millimeter light diffuses," says Lihong Wang of the California Institute of Technology. And given that even the tiny mouse brain encompasses a volume of roughly 500 mm^3, that means light microscopists are largely poking around the edges of this complex organ — and missing critical information as a consequence.

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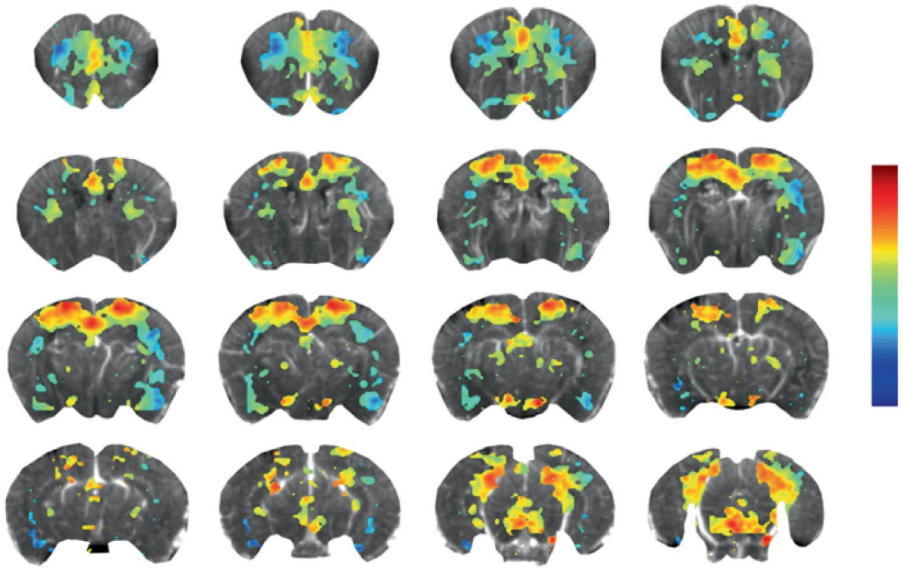

fUS imaging of brain-wide activity elicited by innate mouse behavior during an assay that tests behavior while the mouse explores a simulated burrow.

"That is like looking at a house from a helicopter view, but never going inside the house to know how the people live," says Alan Urban at Neuro-Electronics Research Flanders in Leuven, Belgium.

In contrast, ultrasound can glide through tissues where light collides, sidestepping the photon-scattering effects that confound optical imaging. "When sound comes out of tissue, it goes through essentially a transparent medium," says Wang. Accordingly, the past 20 years have seen rapid progress in the use of ultrasound to access the interior of the brain. For example, Urban is among a cohort of researchers using a method called functional ultrasound (fUS) to track changes in the flow of blood through the cerebral vasculature with 100-micrometer resolution. These data can provide a near-real-time readout for brain-wide changes in neuronal activity associated with diverse behaviors and disease states. "There's no other competing method that exists that gives you the whole-brain activity in a head-fixed, fully behaving mouse – for example, running on a treadmill" says Emilie Macé, a neuroscientist at the University Medical Center Göttingen in Germany who helped develop fUS.

Wang's team helped establish another powerful technique known as photoacoustic or optoacoustic imaging, which leverages light-based excitation and ultrasound detection to image specific molecules within the brain. And although it cannot match the ultra-deep penetration of fUS, it can monitor processes that go well beyond blood flow. "Any material in nature has an optoacoustic signal because it arises from absorption of light," says Daniel Razansky of ETH Zürich. "So the main advantage is this a label-free way of investigating tissues." This technique can also be extended with a myriad of exogenous labels. In one study, Razansky and colleagues used photoacoustic imaging of a β-amyloid-specific dye to visualize individual plaques deep within the brain of an Alzheimer's model mouse[1].

Neither method has been fully embraced by the neuroscience mainstream just yet, but these and related techniques are steadily evolving to a stage where their potential has become impossible to ignore. "I know diehard optics people and diehard MRI people who are buying ultrasound systems – so it's happening," says Caltech researcher Mikhail Shapiro.

Going with the flow

The brain demands constant fuel, and any time activity surges in a given region, so too does the demand for oxygen and nutrients. To address this, the vasculature surrounding active neuronal circuits will transiently dilate, allowing more blood to reach the cells that need it. This 'neurovascular coupling' effect is well established and is the basis for the widely used functional magnetic resonance imaging (fMRI) technique. However, fMRI instruments are bulky and expensive, offer only modest spatial resolution, and are generally of limited utility for studies with non-anesthetized animals.

Mickael Tanter at the École Supérieure de Physique et de Chimie Industrielles (ESPCI) Paris recognized an opportunity to exploit the same physiological phenomenon using ultrasound. His plan involved bombarding circulating red blood cells with ultrasound and then measuring the frequency change in the reflected sound, using this Doppler shift to determine the cells' trajectory and velocity. This required the development of an extremely fast transducer that produced ultrasound as a planar wave rather than a focused beam, enabling the system to scan much more terrain in a single pass.

In 2011, a team led by Tanter, which included Macé – then a PhD student in his lab – and collaborator Gabriel Montaldo, described a first-generation fUS system based on this principle[2]. It was slow, requiring eight seconds to generate a single image, but got the job done. "In our first recording, we saw this beautiful propagation of epileptic seizures," says Macé. "You could see activity deep in the brain and resolve one cortical column, for example."

In the ensuing decade, fUS has matured rapidly, thanks to parallel advances in both transducer hardware and the computers needed to process ever-growing amounts of image data. "We typically have 100-micrometer resolution in space, and in time it can go down to 20 to 30 milliseconds," says Tanter. "So it's even already better for these basic parameters than fMRI." He estimates that this level of performance can be sustained at depths of up to a few centimeters, which is already larger than the typical mouse brain. But fUS can go even deeper, enabling image in larger animals like nonhuman primates, although this requires the use of lower-frequency ultrasound and brings trade-offs in terms of spatial resolution.

Several groups have also demonstrated the feasibility of capturing 3D data from the brain with probes that incorporate arrays of planar ultrasound transducers. In 2020, for example, Urban, Macé and colleagues described a system that could image blood flow across virtually the entire mouse brain with roughly 200- to 300-micrometer resolution, capturing data at rates of up to six frames per second[3]. Such capabilities will be essential for studying brain-wide networks that coordinate behavior in a naturalistic fashion, according to Urban, who says their 3D fUS system took minutes to complete experiments that required hours with a 2D system. "If you put an animal seven hours in a machine, you don't have the same

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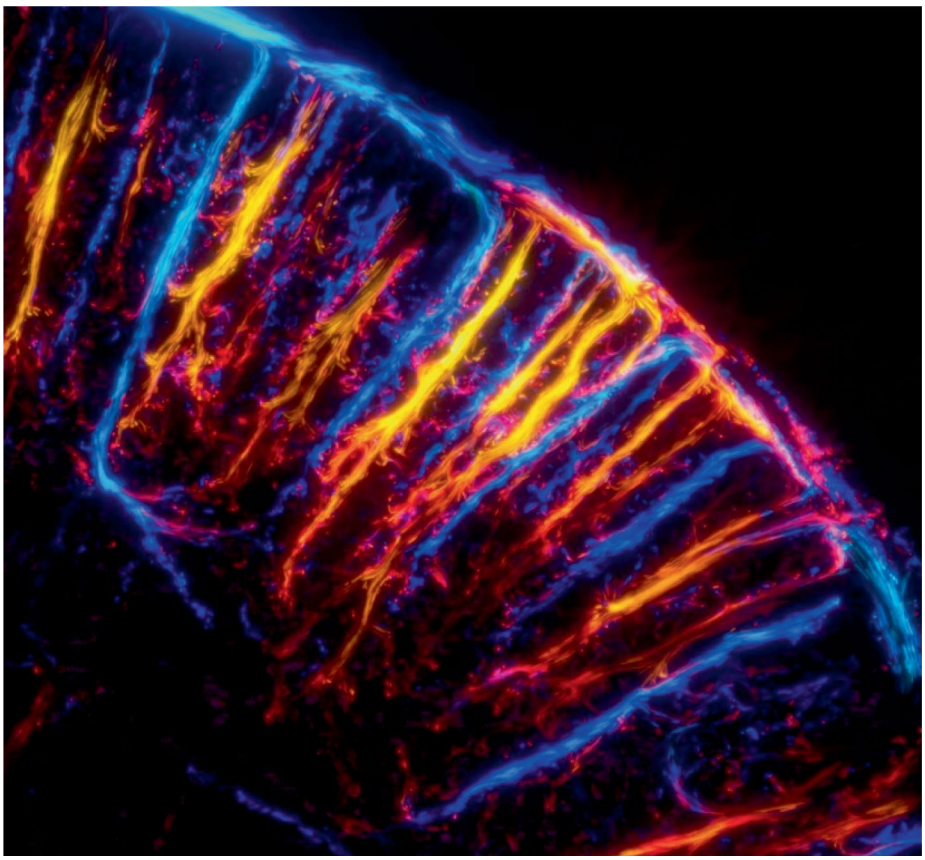
animal anymore," he says. "You have another animal that is tired, afraid, bored or whatever."

The fUS readout is indirect – a delayed response to actual neuronal activation event, and one which cannot be directly tied to specific neurons. But preliminary studies have supported the validity of this approach. In one, Urban teamed up with Matteo Carandini at University College London and colleagues to compare fUS against direct electrophysiological measurements obtained with the state-of-the-art Neuropixels technology at multiple sites in the cortex and hippocampus[4]. This electrophysiological system uses specially designed probes to continuously record the firing patterns of hundreds of neurons at multiple sites in the brain, and it is widely used to document behavior-related neurological activity in animal models. Results from the two methods correlated closely, with a delay of a few seconds for the fUS relative to the action potential data. Macé reports similar results from her own work. "Each time I checked, there was a good correspondence," she says.



Primary sensory cortex activation in the rat brain during whisker stimulation as seen with functional ultrasound localization microscopy (fULM).

In stark contrast

A few years after the development of fUS, Tanter's team devised a riff on this method that makes it possible to zoom even deeper into fine details of the brain vasculature. The method was inspired by super-resolution optical microscopy techniques that rely on the sequential excitation of individual fluorophores to reconstruct images, achieving sub-diffraction-limit spatial resolution. To mirror this achievement, Tanter turned to an existing tool: tiny gas-filled, lipid-based microbubbles that were already in use as an ultrasound contrast agent.

"Ultrasound happens to be very sensitive to these tiny spheres," explains Pengfei Song of the University of Illinois Urbana-Champaign, noting that these microbubbles have distinct mechanical properties relative to other particles in the bloodstream. Once injected intravenously, they remain in the bloodstream for a few minutes before rupturing and being cleared from the body. But during that span, they can generate a crisp image of the circulatory system. In his team's initial 2015 demonstration of this 'ultrasound localization microscopy' (ULM) approach, Tanter's team demonstrated brain imaging in mice with ten-micrometer spatial resolution at depths of up to a centimeter in the brain[5]. Subsequent work has shown that ULM can even access the deepest regions of the far-larger human brain, although this brings compromises in spatial resolution.

ULM makes it possible to visualize capillaries – the narrowest tributaries of the vascular network – which are invisible to fUS. These capabilities could be valuable for studying a range of disorders including stroke and neurodegenerative disease, allowing researchers to zoom in on ever-smaller subunits of the neuronal circuitry. However, the first iterations of ULM were restricted to static snapshots of the brain because the data acquisition process was so slow. "It takes at least 20 or 30 seconds to acquire images because it takes time for bubbles to go in very tiny vessels," says Tanter. This may be sufficient to reveal the presence of an aneurysm or delineate the damage from a brain injury. For example, Song's team has used ULM to map vascular damage arising from a model of Alzheimer's in mice, confirming that they can observe pathology in vivo that previously required post-mortem histological analysis. However, such snapshots yield only limited functional data.

Newer iterations of ULM are introducing hacks that bring functional imaging within reach. In 2022, for example, Tanter's team published a method in which they integrate data collected at various time points from multiple replications of the same behavioral experiment and then use that to reconstruct a single high-resolution video of blood flow[6]. "If you do it 20 times, you can do ULM at one or two images per second and you begin to see the increase of activity at microscopic scale over the whole brain," says Tanter. As an initial demonstration, his team showed that they could reconstruct the brain activity triggered by visual stimuli or a brush of the whiskers, and they are now looking into the use of this 'functional ULM' method to study the pathology of congenital vascular disorders as well as drug development research. However, these studies are also limited by the need to continually re-administer the microbubble contrast agent, as each dose dissipates within minutes.

Best of both worlds

At the end of the day, there is one fundamental constraint on both fUS and ULM: the only thing

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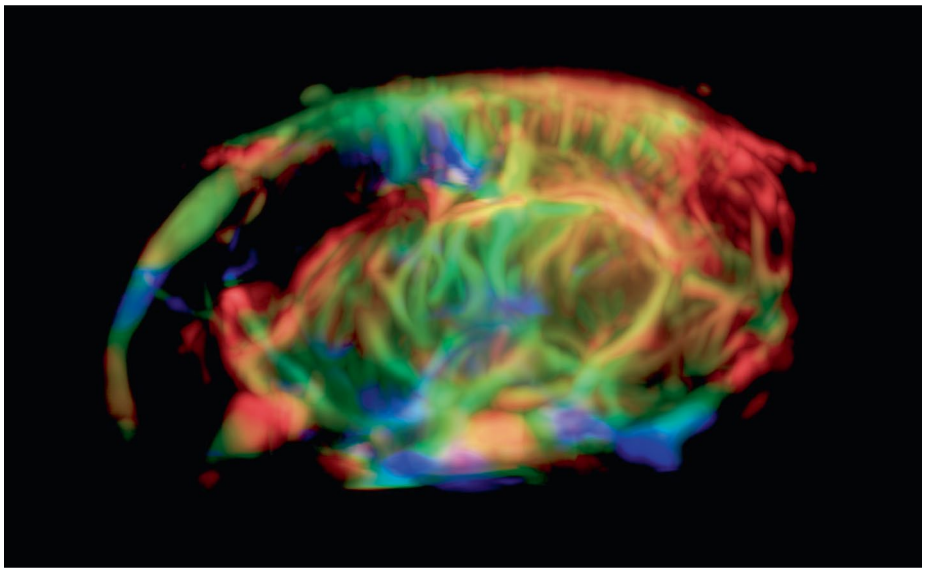

Photoacoustic tomography is capable of mapping the whole-brain oxygenation of a mouse with ischemic stroke.

you can see is blood flow. And as informative as this can be, it only reflects one dimension of brain structure and function.

"There are many processes in the brain that occur without any hemodynamic changes," says Razansky, and photoacoustic imaging offers a powerful complementary method for visualizing these. As with optical imaging, this approach relies on laser illumination to stimulate specific molecules of interest. But rather than documenting the subsequent emission of photons, photoacoustic imaging uses ultrasound to detect sonic 'echoes' arising from the heat and pressure generated by molecules after photon absorption.

The photoacoustic phenomenon was first identified nearly 150 years ago by Alexander Graham Bell, but high-resolution imaging only became possible with the advent of powerful pulsed lasers and sophisticated computer algorithms for data analysis. And by using lasers in the near-infrared range of the spectrum, one can elicit the photoacoustic effect deep in the brain, producing a clear and molecule-specific ultrasound signal that can be targeted based on the laser wavelength. "The background is very clean," says Junjie Yao of Duke University. "If there is no optical absorption of the light, there is no ultrasound signal."

Wang and colleagues published the first demonstration of this method in a live animal in 2003, exploiting the strong photoacoustic response of hemoglobin[7]. This allowed their group to document the in vivo response to whisker stimulation in mice, producing hemodynamic data similar to the results derived from fMRI — or fUS, although this would not be described for another eight years — with ~200-nanometer resolution. Importantly, this approach could also discriminate between oxygenated and deoxygenated hemoglobin, providing details about brain metabolic activity that are inaccessible even to fMRI.

Many photoacoustic imaging experiments in neuroscience continue to focus on hemodynamics and oxygenation, but this approach is applicable to diverse molecules, both endogenous and foreign. "We're really very versatile — in fact, much more even than fluorescent labels," says Wang, noting that light-absorbing proteins that emit too weakly for optical detection can still produce a clear and powerful ultrasound signal. Researchers have also developed useful infrared-absorbing dyes for photoacoustic experiments, such as the HS-169 and AOI987 chromophores that Razansky and colleagues used to label and visualize amyloid-β throughout the brain with eight-micrometer spatial resolution[1]. And critically, photoacoustic imaging is compatible with multispectral analysis, capturing data for different light-absorbing molecules in a single experiment. Photoacoustic methods also bring other advantages, including the ability to perform 3D whole-brain imaging with remarkable speed. "For high-speed, high-resolution imaging, we can get the whole cortical microvasculature of the mouse within one-tenth of a second," says Yao. Considerably higher speeds are possible if one is willing to compromise on resolution.

Conversely, photoacoustic imaging is generally more restricted than fUS in terms of depth owing to the inevitable attenuation of light in brain tissue, even at less-scattering infrared wavelengths. "There's a depth beyond which you don't really have any useful signal," says Vasilis Ntziachristos, director of the Institute for Biological and Medical Imaging at Helmholtz Munich. "For our practical implementations right now, this is about three centimeters." This is suitable for rodent studies, but insufficient for whole-brain coverage in larger animal models or humans.

The big picture

All of these various methods are now being employed with awake, behaving animals, where they are giving researchers the opportunity to reveal the large-scale networks that underlie both simple and complex behaviors.

"My personal interest is to look at the limbic system, emotional and arousal states," says Macé. "If you think about an emotion, it's something that obviously affects the whole brain, even your body — you can't just look in one region and say you understand it." But even fundamental physiological processes involve the entanglement of sites throughout the brain, such that individual 'parcels' of behavior-related neuronal circuitry will seldom tell the full story. "A lot of people would stick an electrode in one area of the brain, do a stimulus and say, 'Oh yeah, there are 3 neurons out of 60 billion that do this,'" says Urban. But through fUS experiments, his team has observed that whisker brushing can unexpectedly stimulate neurons in visual centers, for example, while visual stimuli provoke activity in somatosensory brain regions. "We want to say that the brain is not at all parcellized — it's a continuum," says Urban.

Most behavioral experiments are still done with head-fixed animals that are stably secured to the instrumentation for fUS or photoacoustic imaging while they move on a treadmill, perform memory tests, or simply eat and sleep. With photoacoustic techniques, imaging can be performed directly through the skull — at least in small animals like rodents — without introducing a cranial 'window'. "We try our best to avoid using the window because it does change the intracranial brain pressure, and so it does change the normal activities of the brain," says Yao. fUS experiments generally do not have that luxury; here, even a thin layer of mouse skull can create distortions in

CREDIT: JUNJIE YAO, DUKE UNIVERSITY

the ultrasound signal that heavily degrade imaging resolution. Several research groups are developing algorithms that can potentially remedy these skull-induced aberrations, but some in the field are skeptical. "We do not perform transcranial imaging in our lab," says Macé. "For me, the goal is to have the highest imaging quality possible and to see all brain regions well." She notes that such experiments are already standard practice in brain studies based on optical imaging or electrophysiology.

Experiments in freely moving animals remain a challenge. Moving tissue can easily distort the acoustic readout in ultrasound, and it has proven difficult to shrink the ultrasound package into a compact and energy-efficient unit that can be readily mounted onto an animal's head for extended durations. There are successful examples, but these inevitably entail compromises in performance. "Once you make it wearable, you can image just a small field of view very slowly," says Razansky. Tanter's team has done a lot of work on shrinking the fUS apparatus, including a 2020 experiment in which they used a head-coupled transducer tethered to a computer to analyze changes in brain activity as rats navigated a corridor structure. This allowed them to observe the hemodynamic activity changes associated with memory formation and consolidation in the cortex, hippocampus and thalamus as the animals became familiar with their environment over repeated trials – although they were limited to planar images[8].

But Song is optimistic about the prospect that advances in materials science might soon deliver flexible, lightweight sensors that entail fewer compromises in performance. "I think that's going to be the shining moment of ultrasound in the future," he says, "that we can have a wearable fMRI equivalent with optical imaging spatial resolution."

Sound and vision

Many other exciting opportunities await the ultrasound field. One is the development of genetically encoded reporters that can trigger a more direct readout of neuronal activity, such as the GCaMP proteins, which produce a fluorescent signal in response to the release of calcium ions.

In fact, the calcium-induced conformational change sufficiently alters the absorbance of fluorescent GCaMP proteins to an extent that they can be directly monitored using photoacoustic imaging. However, these reporters preferentially respond to blue-green light, which scatters heavily in brain tissue and is ill-suited to deep imaging. Several groups have already made meaningful headway in engineering alternative calcium reporters that efficiently absorb longer-wavelength near-infrared illumination, although these reporters have not yet demonstrated sufficient robustness for high-resolution photoacoustic imaging of calcium flux. Razansky and Yao are among those actively pushing development on this front. "We can now use even longer wavelengths in the 700–800 nanometer range to specifically label the neurons with a near-infrared protein or probe," says Yao. "Then we can look at the entire brain, not just the surface."

This solution won't work for fUS users, but a naturally occurring mechanism for regulating bacterial buoyancy in water could give these researchers a viable alternative for imaging neuronal function. Certain microbes express a suite of genes that enable them to synthesize nanoscale protein-encapsulated gas vesicles, and Shapiro's group has learned how to engineer and repackage these genes into a cassette that can be expressed in mammalian cells. By hitching this cassette to various promoters, his team demonstrated that they could produce a high-resolution, ultrasound-compatible reporter of gene expression – analogous to the microbubbles used in ULM, but far smaller[9]. "They don't produce as much contrast, but they're also able to get into places where microbubbles cannot get into," says Shapiro. "They can actually get out of the vasculature and get into organs." This work is still at an early stage, but Shapiro says that his team is already collaborating on a number of projects that could demonstrate the utility of genetically encoded gas vesicles as a reporter for fUS in the brain.

All of these ultrasound methods can also be combined – both with each other and with other modes of imaging, considerably boosting the amount of information that can be derived in a single study. In one recent example, Song and Yao collaborated to perform photoacoustic imaging and ULM simultaneously, which made it possible to extract the blood oxygenation data exposed by the first modality with the depth of penetration and resolution of the second. "In both imaging technologies, we use the same detection system, and so it's almost like a free lunch to do both at the same time and get complementary information," says Yao. And Macé says that her group is now using fUS in conjunction with two-photon imaging, electrophysiology, and even optogenetics, thereby enabling simultaneous manipulation and observation of neuronal function in live animals.

And of course, there is tremendous interest in translating these approaches to humans for both clinical research and medical diagnostics. Our thick skulls make this a considerable challenge, but a few early demonstrations have shown that ultrasound can perform remarkably well even within the murky depths of the human brain. Last year, Wang demonstrated that photoacoustic imaging in a patient who had undergone a partial craniotomy for a surgical procedure was highly consistent with the results from blood oxygen level-dependent (BOLD) fMRI[10]. "We can see the total concentration and oxygen saturation, and we can actually see the changes slightly faster than BOLD fMRI," says Wang. The advent of effective algorithms for correcting bone-induced signal aberrations could make this a powerful and more versatile alternative for deep-brain imaging.

Investing in ultrasound's future

The ultrasound imaging field is growing – albeit slowly. Many pioneers are seeing rising interest in these technologies; for example, Macé says she knows of at least 20 neuroscience labs that have taken on fUS, and she routinely receives new inquiries. Many commercial systems are also available. In addition to the widely used Verasonics ultrasound systems, Tanter has founded a company called Iconeus that offers a platform for performing fUS and ULM experiments, and Wang says that more than 20 companies are developing photoacoustic imaging systems.

But getting started can be expensive, says Song, noting that the basic ultrasound system he uses for ULM costs $200,000, with another $50,000 for various probes. For photoacoustic imaging systems, there is also the added cost of lasers. And this is before getting into the computational demands, which can be intensive for users interested in pursuing real-time 3D imaging at any meaningful scale. "We're generating 200 terabytes of raw data every six months," says Song. To keep the flood at bay, most ultrasound brain imaging practitioners are finding ways to selectively filter and retain relevant data during experiments – for example, by using deep learning and other algorithmic methods – rather than attempting to capture and keep every single byte. Analysis also remains thorny, with no standardized pipelines or best practices.

All of these problems will inevitably be addressed as the first and second wave of adopters continue to refine and test ultrasound's capabilities and limits. But perhaps what the field needs more than anything else is a proper showcase, one that goes beyond validating findings and hypotheses generated by other means. "I'm waiting for the groundbreaking finding with the method – but it's also still early," says Macé. "I think it will come when we continue to explore."

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