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# Functional ultrasound imaging: A useful tool for functional connectomics?

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ABSTRACT

Functional ultrasound (fUS) is a hemodynamic-based functional neuroimaging technique, primarily used in animal models, that combines a high spatiotemporal resolution, a large field of view, and compatibility with behavior. These assets make fUS especially suited to interrogating brain activity at the systems level. In this review, we describe the technical capabilities offered by fUS and discuss how this technique can contribute to the field of functional connectomics. First, fUS can be used to study intrinsic functional connectivity, namely patterns of correlated activity between brain regions. In this area, fUS has made the most impact by following connectivity changes in disease models, across behavioral states, or dynamically. Second, fUS can also be used to map brain-wide pathways associated with an external event. For example, fUS has helped obtain finer descriptions of several sensory systems, and uncover new pathways implicated in specific behaviors. Additionally, combining fUS with direct circuit manipulations such as optogenetics is an attractive way to map the brain-wide connections of defined neuronal populations. Finally, technological improvements and the application of new analytical tools promise to boost fUS capabilities. As brain coverage and the range of behavioral contexts that can be addressed with fUS keep on increasing, we believe that fUS-guided connectomics will only expand in the future. In this regard, we consider the incorporation of fUS into multimodal studies combining diverse techniques and behavioral tasks to be the most promising research avenue.

## 1. Introduction

Brain function requires a continuous flow of information between specialized regions. These regions communicate by forming complex networks extending throughout the entire brain. How do these large-scale networks participate in cognition or perception? How do they change across brain states or in the course of a disease? And what is the extent of the networks recruited by a specific neuronal population or during a precise task? The neuroimaging community is actively working on these questions to reveal fundamental principles that govern brain function and dysfunction at the macroscopic scale.

On the one hand, large-scale networks are constrained by their structural connections. The “comprehensive structural description” of these networks is the task of the field termed “connectomics”, as initially formulated almost two decades ago (Sporns et al., 2005). Structural connectomics describes how different brain regions are physically connected through axonal tracts and, at the microscopic level, how many synapses are locally formed between neurons. On the other hand, the spatiotemporal patterns of neuronal activity within and between those large-scale networks may be more informative about brain function than the mere description of the structural connections. The study of these activity patterns at the network level is often referred to as “functional connectomics” (Alivisatos et al., 2012; Biswal et al., 2010; Smith et al., 2013). Note that here we use this term very broadly to include any study measuring whole-brain activity to extract information about brain function at the network level. In this review, we offer a perspective on how “functional connectomics” can benefit from the recent development of functional ultrasound (fUS) imaging. Functional ultrasound is a relatively new player in the field of neuroimaging that relies on an ultrasound-based Doppler approach to detect cerebral blood volume changes induced by neuronal activity (Mace et al., 2011). This technique allows for imaging brain activity on a large scale – up to the entire brain in rodents – that is paramount to addressing questions about the function of large-scale networks in a holistic manner.

We explain in the first section of this review the unique advantages and limitations of fUS for functional connectomics studies. On top of a large field-of-view, fUS achieves an excellent spatial (~100 $\mu$m) and temporal (~10 Hz) resolution in small animals, and can be used during behavioral tasks with affordable and portable equipment. In contrast, one limitation of fUS is the attenuation of ultrasound by the skull, which calls for invasive surgical procedures to obtain the best imaging results. Because of the unique assets of fUS, we identify two main streams of research that promise to deepen our knowledge of functional connectomics. First, fUS can be used to map the intrinsic network orga-

*Abbreviations:* fUS, functional ultrasound imaging.

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nization of the rodent brain, using a so-called "functional connectivity" approach. We describe how the resulting functional connectivity matrices can be used to detect changes in large-scale networks across behavioral and pathological states or in a dynamic manner. Second, because fUS is compatible with circuit manipulation tools and behavioral tasks in animal models, we describe how fUS can be used to reveal causal interactions between distant brain regions and the functional role of these interactions in specific behavioral contexts. Finally, we discuss how we foresee future developments and applications of fUS technology taking place in the context of functional connectomics, as well as pitfalls to avoid when using this method.

## 2. Functional ultrasound imaging

### 2.1. Principles of fUS imaging

To fully appreciate the potential impact of fUS in the field of functional connectomics, it is crucial to first understand the principles of the technique. The method relies on the neurovascular coupling effect, i.e., the fact that changes in neuronal activity trigger a local hemodynamic response that can serve as an indirect readout of that activity (Girouard and Iadecola, 2006; Iadecola, 2004; Raichle and Mintun, 2006). Functional ultrasound measures these blood volume changes in the brain microvasculature by detecting the moving blood's ultrasonic echoes. Blood echoes can be separated from those of other brain tissues to obtain a temporal snapshot of the quantity of blood moving in each voxel of the field of view (Fig. 1a).

The idea of detecting blood movement through its ultrasonic echo, or "Doppler signal", is not new; in fact, the same principle has been applied for decades to image different organs in a clinical context (Rubin et al., 1994; Szabo, 2004). This imaging mode is called "power Doppler", and works as follows. (1) A series of short ultrasound pulses is sent into the body in the kHz range. (2) If a particle is moving within a voxel, the successive echoes reflected from that particle exhibit a phase change over time, producing a signal that oscillates at the Doppler frequency (which depends on the velocity of the particle, the ultrasound frequency, and the speed of sound). (3) Because both blood cells and tissues are usually moving in the same voxel, tissue motion must be filtered out of the Doppler readout (more details in the next paragraph). (4) The power of the filtered signal gives a value proportional to the total number of moving blood particles – related to the blood volume – within that voxel (Rubin et al., 1997, 1995). Importantly, note that the velocity information is present in the Doppler signal, but not in its power, which therefore is only proportional to blood volume (Mace et al., 2013; Rubin et al., 1995).

Power Doppler imaging was historically insensitive to very small vessels. Consequently, fUS imaging was specifically conceived to improve the detection of blood volume in the brain microvessels, where most of the hemodynamic response induced by neuronal activity lies (Stackhouse and Mishra, 2021). This principle is illustrated in more detail in Fig. 1a (see Mace et al. 2013, Mace et al. 2011 for detailed explanations). To increase sensitivity, fUS uses plane waves of ultrasound, which allow imaging rates of ~30 kHz. This high sampling rate is above that needed to correctly sample cerebral microvascular flow. Indeed, as an example, blood in mouse cerebral arterioles (~10 mm/s velocity) generates a Doppler frequency of ~200 Hz when imaged with 15 MHz ultrasound, meaning that 400 Hz is sufficient to correctly sample this signal (according to the Nyquist criterion). Functional ultrasound takes advantage of the extra time between emissions to combine plane waves sent at different angles in a process called "coherent compounding", which increases spatial resolution, contrast, and signal-to-noise ratio (for a theoretical and experimental demonstration, see Montaldo et al. 2009). The acquisition of compound (i.e., high-quality) ultrasound images occurs at the appropriate rate of 500 Hz. Compound images must then be filtered to remove tissue motion, and the chosen filter also affects the size of the vessels that are available for imaging. For example, a temporal high-pass filter can be enough to isolate the faster signal coming from the blood cells, albeit at the expense of losing information from those capillaries where blood axial velocity is below 2–4 mm/s, depending on the cut-off frequency (Mace et al., 2013). On the other hand, a singular value decomposition-based spatiotemporal filter solves that problem by also taking into account the higher spatial coherence of tissue signal, so that the filtered signal can include even blood flows as slow as 0.5 mm/s (Demené et al., 2015). After filtering, a series of at least 50 compound images is integrated to calculate the power Doppler value, proportional to blood volume, and thus produces a fUS image in 100 ms. Integrating more compound images increases the sensitivity, but slows down the imaging rate. Finally, the voxel size (i.e., the sampling of the data) is usually chosen to closely match the spatial resolution, measured to be ~100 μm × 100 μm (×300 μm plane thickness) for single plane imaging at 15 MHz (see Mace et al. 2018 for an experimental validation).

The final result of a fUS recording is a time-resolved readout of the cerebral blood volume variation within each voxel of the acquired image. This signal can be analyzed with similar techniques to those that are available for other hemodynamic recording technologies, such as functional magnetic resonance imaging (fMRI). In particular, these time courses can be compared to each other (to analyze global intrinsic connectivity) or regressed against external perturbations (to identify specific neural pathways).

### 2.2. State-of-the-art and methodological considerations

Functional ultrasound has specific advantages and limitations in comparison to other neuroimaging methods. We identify three technical points that are particularly important in the context of large-scale functional connectomics: (1) the brain coverage in different behavioral contexts and different species; (2) the spatiotemporal resolution; and (3) the invasiveness. We provide an overview of the current possibilities offered by fUS imaging based on those three technical points, with a focus on rodent imaging, in Fig. 1b.

#### 2.2.1. Brain coverage

Regarding brain coverage, fUS can be performed either in two spatial dimensions (single-plane imaging, or 2D fUS) or in three spatial dimensions (volumetric imaging, or 3D fUS). Traditional probes used in clinics are linear, i.e., based on a single line of piezoelectric elements, so that only single-plane imaging is possible. However, they also offer great imaging quality, and are still the most common type of probe used for imaging most species, including head-fixed rodents, monkeys, and humans (see Table 1 for an overview of studies using each type of probe). The initial proof-of-concept for fUS, performed in anesthetized rats, was developed using a commercially available linear probe (Mace et al., 2011). Because those clinical probes are quite bulky, some behavioral contexts require miniaturized probes that can be mounted on the head; for example, for freely-moving imaging of rats or mice (Tiran et al., 2017; Rabut et al., 2020; Sieu et al., 2015; Urban et al., 2015b).

A relevant advance in fUS imaging came recently with the introduction of matrix probes that allow for volumetric imaging and full mouse brain coverage in head-fixed configurations (Brunner et al., 2020a; Rabut et al., 2019; Sauvage et al., 2020). In this modality, two elements are important: the design of the matrix probe itself and the strategy used to control its piezoelectric elements for imaging. For example, a matrix probe based on a full array of 32 × 32 elements (10 × 10 $mm^2$ brain coverage) can have each of its 1024 elements working independently (Rabut et al., 2019). This enables full control, but the large number of independent electronic channels demands expensive scanners that can be out of reach for many neuroscience groups. Alteratively, the same matrix probe can be controlled by cheaper research systems (using typically 256 channels) by using a multiplexing approach (Brunner et al., 2020a). On the other hand, a different probe based on a 128 + 128 row-column array approach (Sauvage et al., 2020) achieves larger brain cov-

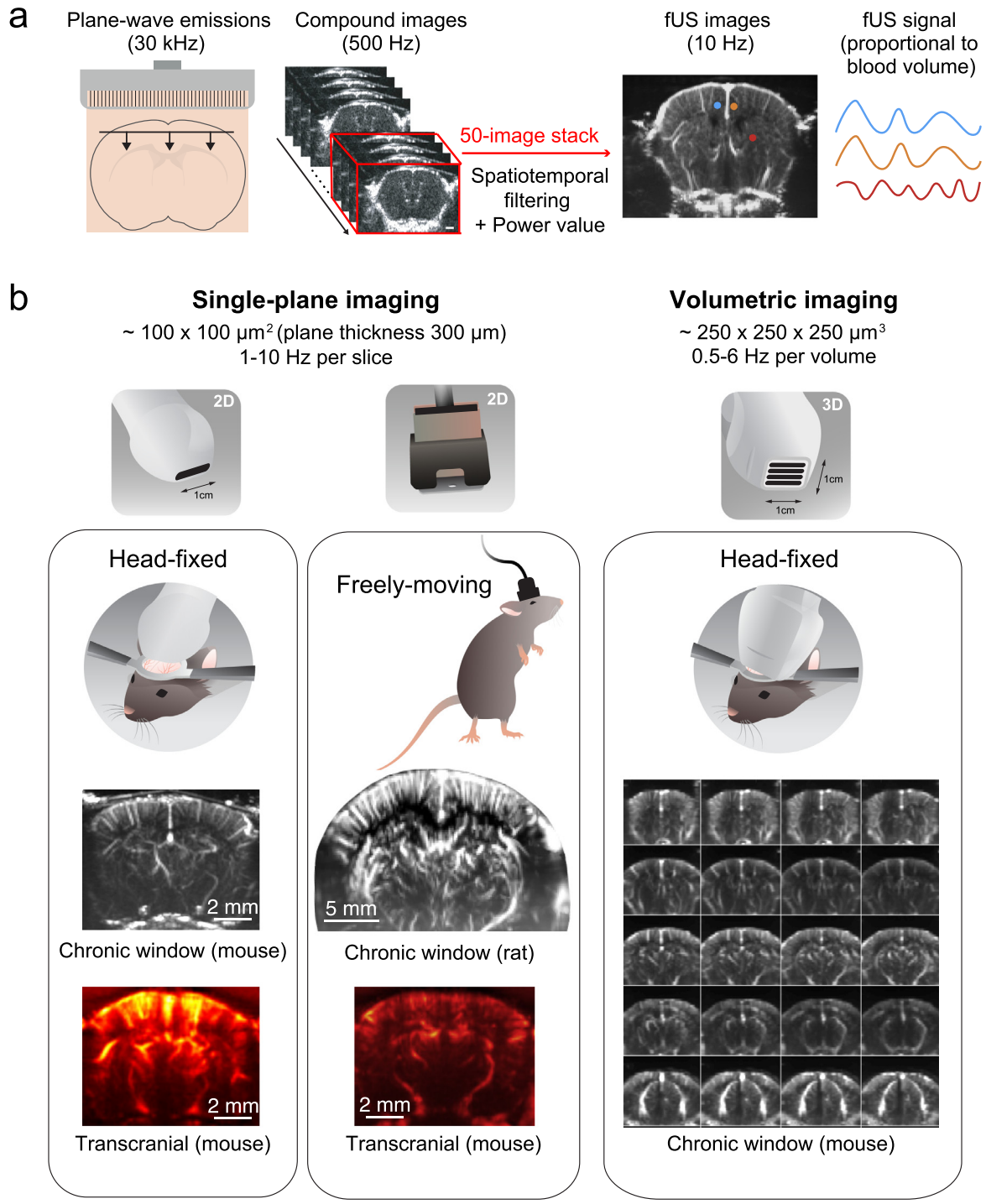


**Fig. 1.** Principles of fUS imaging and state-of-the-art for rodent imaging. a. fUS measures changes in blood volume in the brain microvasculature as an indirect readout of neuronal activity. Plane waves of ultrasound are emitted with different angles at ~30 kHz and their echoes are combined to produce high-quality ultrasound images ("compound images") at ~500 Hz. A stack of ~50 compound images, acquired in 100 ms, is filtered to remove tissue motion, and the power of each voxel filtered signal gives a value proportional to the blood volume within ("power Doppler" value). These fUS images can be acquired at up to ~10 Hz to follow changes in blood volume over time in each voxel of the image. b. Technical capabilities of fUS, illustrated in the context of rodent imaging. *Brain coverage:* fUS can be performed either in two spatial dimensions (single-plane imaging) or in three spatial dimensions (volumetric imaging). *Resolution:* Spatiotemporal resolution of single-imaging fUS is higher, but volumetric imaging allows for full brain coverage while keeping a fast acquisition rate. *Behavioral context:* Single-plane imaging is available for head-fixed and freely-moving animals using conventional or miniaturized probes, respectively, whereas volumetric imaging still requires head fixation. *Invasiveness:* fUS requires chronic cranial windows to reach its maximum depth-of-field; transcranial approaches are less invasive but the skull bone attenuates the ultrasound wavefront and decreases its penetrance, meaning that fewer brain areas are available for imaging. Single-plane brain images adapted with permission from Macé et al. (2018); Copyright 2018 Elsevier (top left image), Ferrier et al. (2020); Copyright 2020 National Academy of Sciences (bottom left image), Bergel et al. (2020); Copyright 2020 (CC-BY), (top right image) and Tiran et al. (2017); Copyright 2017 Elsevier (right bottom image). The volumetric fUS brain image is unpublished data provided by C. Brunner, acquired as in Brunner et al. (2020a).

**Table 1**
Overview of the protocols used in example fUS papers related to connectomics.

| | | Brain coverage | | |
|---|---|---|---|---|
| | | Single planes (linear probe) | Large brain volume (scanned with linear probe) | Large brain volume (synchronous with volumetric probe) |
| **Behavioral context** | **Anesthetized** | **Rat:** Mace et al., 2011 (**ac**); Osmanski et al., 2014a (**ts**); Osmanski et al., 2014b (**ac**); Urban et al., 2014 (**ts**); Rideau Batista Novais et al., 2016 (**t**); Brunner et al., 2018 (**ts**); Mairesse et al., 2019 (**t**); Nayak et al., 2021 (**ac**); Rahal et al., 2020 (**ts**); Tang et al., 2020 (**ac**); Vidal et al., 2020 (**ts**); Claron et al., 2021a (**ac**); Provansal et al., 2021 (**ac**)<br>**Mouse:** Boido et al., 2019 (**cw**) | **Rat:** Gesnik et al., 2017 (**ac**) | **Rat:** Rabut et al., 2019 (**ac**), Sauvage et al., 2020 (**ac**) |
| | **Awake head-fixed** | **Mouse:** Ferrier et al., 2020 (**t**) | **Mouse:** Mace et al., 2018 (**cw**); Sans-Dublanc et al., 2021 (**cw**) | **Mouse:** Brunner et al., 2020a (**cw**) |
| | | **Ferret:** Bimbard et al., 2018 (**cw**); Landemard et al., 2020 (**cw**)<br>**Primate:** Dizeux et al., 2019 (**cw**); Blaize et al., 2020 (**cw**); Claron et al. 2021a (**cw**); Norman et al., 2021 (**cw**)<br>**Human:** Imbault et al., 2017 (**ac**); Soloukey et al., 2020 (**ac**) | **Pigeon:** Rau et al., 2018 (**ac**) | |
| | **Freely-moving** | **Rat:** Urban et al., 2015b (**cw**); Bergel et al., 2018 (**cw**); Bergel et al., 2020 (**cw**)<br>**Mouse:** Rabut et al., 2020 (**t**) | **Rat:** Sieu et al., 2015 (**cw**) | |
| | | **Human neonate:** Demene et al., 2017 (**t**) | **Human neonate:** Baranger et al., 2021(**t**) | |

Invasiveness (**t**: transcranial, **ts**: thinned-skull, **ac**: acute craniotomy, **cw**: chronic window).

erage (14 × 14 $mm^2$ mm) with the same cheaper research system and without the multiplexer, but at the expense of resolution and sensitivity (see Section 1.2.2). Finally, miniaturized matrix probes for volumetric imaging during freely-moving behavior have still not been developed, although such an advance is expected in the near future. Fig. 1b depicts examples of images offered by the most common types of probes currently available.

Importantly, it should be noted that the ability to record in behaving animals, either head-fixed or freely-moving, comes with specific challenges. Some specific types of motion are imperfectly separated from the blood motion and can create an artificial increase in the fUS signal, termed motion artifact (see a discussion on these aspects in Landemard et al. 2020). Trial averaging and various filtering methods, such as spatiotemporal filtering (Baranger et al., 2018; Demené et al., 2015), help alleviate this issue, but elimination of motion artifacts can be further improved.

### 2.2.2. *Spatiotemporal resolution*

The spatial resolution of fUS depends on the chosen ultrasound frequency, along with the physical parameters of the probe (distance between piezoelectric elements, acoustic lens, etc.). As a rule of thumb, the maximal spatial resolution that can be achieved is in the order of a single wavelength (i.e., ~100 µm for 15 MHz ultrasound). Increasing the frequency enhances the resolution, but decreases the penetration depth, whereas lower frequencies allow for deeper imaging at the cost of spatial resolution. Consequently, an optimal frequency exists for every brain size. For rodents, 15 MHz ultrasound is theoretically optimal to image a ~1 cm deep brain at ~100 µm within-plane resolution (Mace et al., 2011). The probes currently used for single-plane imaging offer experimental resolutions close to this theoretical limit. In contrast, matrix probes have not yet reached their corresponding theoretical limit, with the maximal spatial resolution currently used being only ~250 µm at 15 MHz due to the technological challenge of producing higher-density matrix probes (Brunner et al., 2020a). In that regard, the row-column array strategy performs less well with a spatial resolution of ~450 µm (Sauvage et al., 2020)

On the other hand, the maximal temporal resolution of fUS depends on the number of compound images used to produce a fUS image (i.e., 10 Hz for a stack of 50 compound images, 5 Hz for 100 images, etc.). In practice, the actual temporal resolution is determined by the capacity to acquire, compute, and save fUS images without any dead time. Thanks to fast GPU processing for beamforming the ultrasound images (Yiu et al., 2011), reaching the theoretical maximum is now possible for single-plane imaging, even for recording sessions that last multiple hours (Mace et al., 2018). Volumetric imaging is slower due to the computational constraints linked to the very high data load (1024 channels to compute for a standard matrix probe versus 128 channels for a linear probe), although the most recent volumetric fUS study already reached 6 Hz per volume using 80 compound images and the multiplexing approach (Brunner et al., 2020a). Importantly, even the lower spatiotemporal resolution of volumetric fUS (250 µm, 6 Hz) compares well with that of widely-used whole-brain imaging methods, such as fMRI.

Additionally, it should be noted that the spatiotemporal resolution of fUS is intrinsically limited by the hemodynamic signal itself, which is less localized and slower than the underlying neuronal activity (for a general review, see Hillman 2014; for a review including fUS, see Urban et al. 2017). Mathematically, fUS output can be thought of as the result of convolving the fast neural activity with a slower hemodynamic function (Aydin et al., 2020; Nunez-Elizalde et al., 2021). Consequently, coherence between the fUS signal and spontaneous local spiking rate, measured as multi-unit activity with Neuropixels probes, is only maximal in the frequency range below ~0.3 Hz (Nunez-Elizalde et al., 2021), which corresponds to the 'infraslow', 'slow', or 'delta' oscillatory bands of electrophysiological recordings, depending on the definition used (Buzsáki and Draguhn, 2004; Newson and Thiagarajan, 2019). Infraslow oscillatory activity (< 0.5 Hz) seems to reflect changes in cortical excitability across behavioral and physiological states (Goede and Putten, 2019; Hughes et al., 2011; Putten et al., 2015; Vanhatalo et al.,

2004) and could also underlie the resting-state fluctuations that will be the focus of Section 2 (Helps et al., 2007; Hiltunen et al., 2014; Mantini et al., 2007). Consequently, although fUS cannot reach the higher-frequency bands that are only available in electrophysiological studies, the slow oscillations that it does detect are of great relevance for functional connectomics. On top of that, different studies have found that fUS output is very strongly correlated across brain regions, stimulus parameters, and individuals with the outputs of calcium imaging (Aydin et al., 2020; Boido et al., 2019), local field potentials (Mace et al., 2011; Urban et al., 2014), and multielectrode arrays (Macé et al., 2018; Nunez-Elizalde et al., 2021; Sans-Dublanc et al., 2021), thus providing increased confidence in the usefulness of fUS imaging as a complementary brain-wide screening tool.

In addition to resolution, it is also important to highlight the excellent signal-to-noise ratio of fUS, which makes it capable of tracking the propagation of transient events like epileptic seizures (Mace et al., 2011; Rabut et al., 2019; Sieu et al., 2015) and subtle brain responses to single, brief sensory (Bimbard et al., 2018; Urban et al., 2014) or cognitive (Dizeux et al., 2019) stimuli. Functional ultrasound is sensitive enough to detect brain activation after a single 200 $\mu$s electrical stimulation in rats (Urban et al., 2014) and low-intensity olfactory stimulation in mice (Boido et al., 2019) on a trial-to-trial basis, which has also enabled motor intention in primates to be predicted from single-trial fUS outputs (Norman et al., 2021).

#### 2.2.3. *Invasiveness*

The invasiveness of fUS is one main limitation that requires specific consideration. Skull replacement is crucial for high-quality and deep imaging with fUS, because the bone attenuates and causes aberrations in the ultrasound wavefront (Pinton et al., 2012). Chronic imaging windows can now be routinely implanted for imaging across weeks (Bergel et al., 2018; Brunner et al., 2020a; Macé et al., 2018; Sieu et al., 2015; Urban et al., 2015b), as is commonly done for optical methods such as calcium imaging (Ghanbari et al., 2019; Kılıç et al., 2020; Kim et al., 2016). However, the impact of such a procedure cannot be ignored and interpreting results requires caution, for example when trying to observe the effect of a disease or a drug. To mitigate potential negative effects on brain physiology, thinned skull and transcranial approaches have been developed in rodents (Brunner et al., 2017; Tiran et al., 2017; Urban et al., 2014), but reduced invasiveness results in a tradeoff with imaging quality. Imaging through a thinned skull offers a diminished yet comparable depth-of-field, while causing less neuroinflammation than craniotomies (Urban et al., 2014), which is why this has been the chosen strategy in various fUS studies (Osmanski et al., 2014b; Rahal et al., 2020; Vidal et al., 2020). Nevertheless, bone regrowth degrades imaging quality over time, which makes it inappropriate for stable, chronic imaging over days. As for transcranial imaging, usually performed by placing the probe directly on the bone after removing the skin (Ferrier et al., 2020; Rabut et al., 2020; Tiran et al., 2017), it eliminates potential problems linked to cranial surgeries, but bone attenuation remains: only a shallower part of the brain can be imaged (see examples in Fig. 1b). A minimally-invasive way to regain imaging depth in transcranial imaging is by injecting echogenic contrast agents to increase the blood signal (Errico et al., 2016; Maresca et al., 2020). This strategy has, however, not been widely adopted as these contrast agents are, to date, short-lived (in the order of minutes) and require intravenous injection.

In addition to the aforementioned three points, fUS is a versatile method that has already been used in multiple species: mice (Macé et al., 2018), rats (Mace et al., 2011; Urban et al., 2014), rabbits (Demené et al., 2018; Kohlhauer et al., 2015), ferrets (Bimbard et al., 2018; Landemard et al., 2020), pigeons (Rau et al., 2018), macaques (Blaize et al., 2020; Dizeux et al., 2019; Norman et al., 2021), and humans (Baranger et al., 2021; Demene et al., 2017; Imbault et al., 2017; Soloukey et al., 2020; Urban et al., 2015a). There are also ongoing studies, to our knowledge, on reptiles, tree shrews and marmosets. All of these studies are methodologically similar, most often using single-plane imaging with commercial probes as described above, with the only difference being that lower frequencies are typically used for bigger brains to image deeper (for example, a probe frequency of 6.4 MHz for neonates (Baranger et al., 2021) and 6 MHz for non-human primates (Dizeux et al., 2019)). Volumetric imaging has not yet been applied to larger brains, but we expect such applications when bigger matrix probes are developed for this purpose in the near future. Interestingly, although the invasiveness of fUS deep imaging intrinsically limits its use in humans, specific applications that are not available for other imaging modalities, namely neonatal and intraoperative imaging, are possible and very promising. Indeed, neonate brain networks can be imaged non-invasively through the fontanel (Baranger et al., 2021; Demene et al., 2017) and, in adults, fUS can be used to identify functional brain regions directly in the operating room (Imbault et al., 2017; Soloukey et al., 2020).

In summary, fUS is a relatively new neuroimaging method that is most developed for rodent brain imaging. It possesses the unique ability to record neuronal activity in mice at the whole-brain scale, under challenging behavioral conditions, and with a portable device. These technical advances are penetrating other model species, and even being applied to humans. Functional ultrasound has reached its maximum capabilities for single-plane imaging, and volumetric fUS is emerging for whole-brain recordings. On the other hand, fUS is limited by the skull, the nature of the hemodynamic signal, and the presence of motion artifacts. To give an overview of the field, we have summarized in Table 1 the methodological conditions of the majority of fUS publications related to functional connectomics. In the next sections, we review what advances have been made in understanding large-scale neural networks using fUS thus far.

## 3. Mapping intrinsic functional connectivity with fUS

Neuroimaging studies have revealed the existence of intrinsic patterns of strongly-correlated activity between distant brain regions (Biswal et al., 1995; Horwitz et al., 1984). Such patterns, or functional networks, manifest spontaneously across subjects during so-called “resting” time periods, and differ across tasks, arousal states, and pathophysiological conditions (Chuang and Nasrallah, 2017; Khadka et al., 2013; Rosenberg et al., 2020; van Dijk et al., 2010), so that they are often used as a proxy to study how behavior, cognition, or pathology impact brain function. Despite initially being discovered in humans (Damoiseaux et al., 2006; Fox et al., 2005; Raichle et al., 2001), some of them have also been identified in rodents (Coletta et al., 2020; Grandjean et al., 2020; Mandino et al., 2021), although comparisons across species should be taken with caution (for a detailed review about this matter, see Gozzi and Schwarz, 2016). Moreover, they can be observed either using whole-brain mapping techniques, such as fMRI (Biswal et al., 1995), positron emission tomography (PET) (Horwitz et al., 1984; Raichle et al., 2001; Shulman et al., 1997) and electro- and magnetoencephalography (EEG/MEG) (Helps et al., 2007; Mantini et al., 2007; Stam, 2004), or at the local scale using invasive electrophysiological recordings (He et al., 2008; Jerbi et al., 2010; Pan et al., 2011) and optical methods such as calcium imaging (Matsui et al., 2016; Schwalm et al., 2017). Because of this consistency, the study of correlated activity patterns on the brain-wide level is often used for brain phenotyping, to identify biomarkers of behavior or pathology (Biswal et al., 2010; Grandjean et al., 2020; van Dijk et al., 2010). However, controversy still exists within the neuroimaging community about the interpretation, origin and implications of intrinsic activity pattern, as well as about their usefulness for understanding brain function and diseases (Cole et al., 2010; Lu et al., 2019; Lurie et al., 2020).

When studying spontaneous brain activity, a common way to reveal intrinsic patterns is to extract the activation time-course of all voxels or groups of voxels of interest and to search for correlations amongst them. While it is possible to select a single voxel or group of voxels

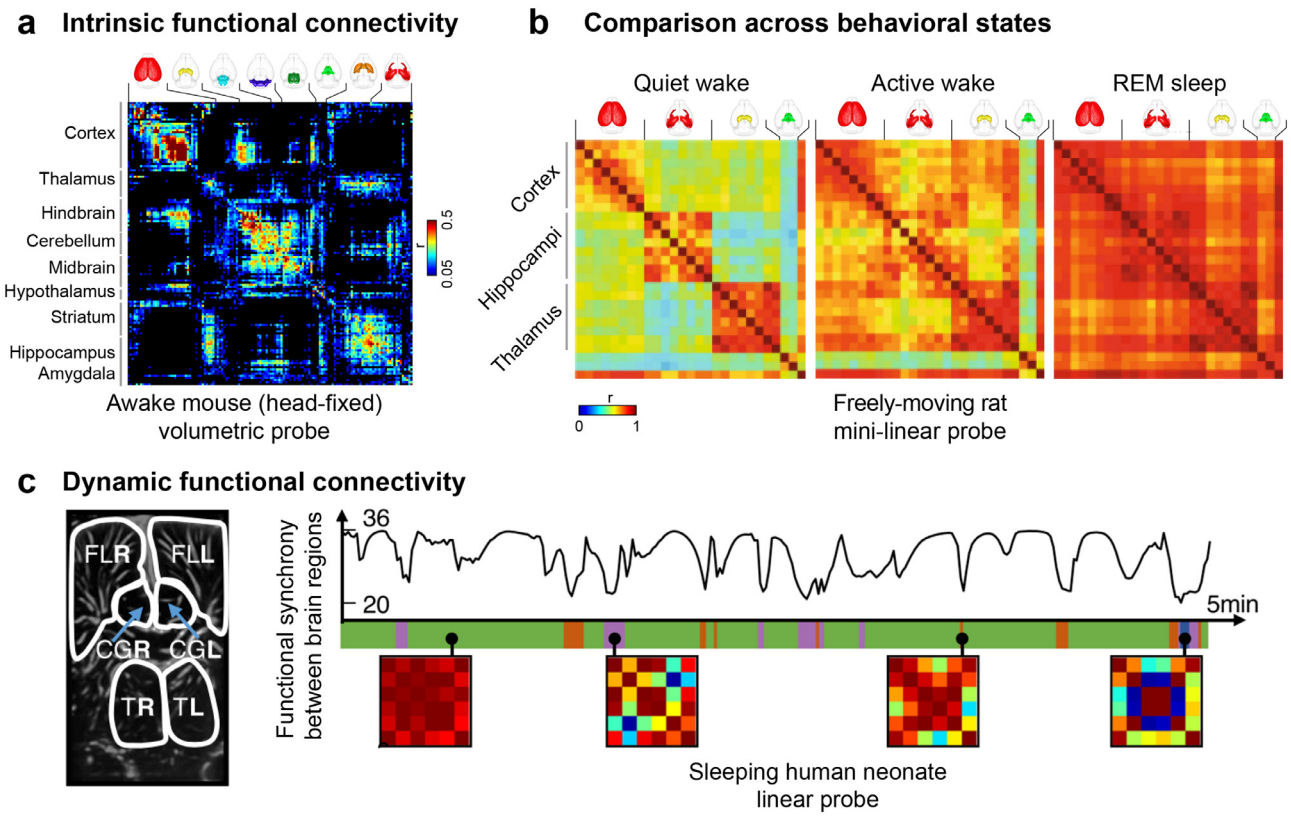


**Fig. 2.** Intrinsic functional connectivity as observed with fUS a. Example of a functional connectivity matrix obtained with fUS in awake head-fixed mice (60 min recording): the resulting pattern is called static or time-averaged connectivity. Figure adapted with permission from Brunner et al. (2020b); Copyright 2020 (CC-BY). b. Example of changes in functional connectivity observed with fUS in freely-moving rats across 3 behavioral states. Functional connectivity matrices can be used to identify brain networks that underlie behavioral changes. Figure adapted with permission from Bergel et al. (2018); Copyright 2019 (CC-BY), modified by adding 3D representations of the brain subdivisions using the Allen Brain Institute atlas. c. Example of a dynamic functional connectivity approach using fUS in the neonate brain during sleep. Left: fUS image of the neonate brain with the six regions of interests. Right: Time-resolved connectivity matrices (here based on instantaneous phase shifts) can be clustered into different groups or "brain states" . The curve shows a global metric of synchrony and the color ribbon shows the "brain states", also depicted as matrices below. The occurrence of these states may serve as a diagnostic tool: for example, they differed between preterm and full-term neonates. Figure adapted with permission from Baranger et al. (2021); Copyright 2021 (CC-BY), y label removed for clarity.

and correlate it with all the others to identify only the brain networks that include it (a seed-based analysis), for whole-brain analyzes it is more common to compute every possible pairwise correlation in an unbiased way and then to display the resulting correlation coefficients as a matrix, namely a "connectivity matrix", for visualization purposes (for comprehensive reviews about this procedure, see Lv et al. (2018), centered in humans, and Gorges et al. 2017, focusing on animal models). We provide example connectivity matrices obtained by fUS in Fig. 2. Thus, a connectivity matrix is a graphical representation of a global correlation pattern, which is commonly referred to as "functional connectivity". For simplicity, we will also use that term in this review, but it must be noted that we refer to networks of regions with temporally correlated activity, without assuming whether or not they are anatomically connected (Friston, 2011; Reid et al., 2019). Importantly, metrics of temporal association other than correlation (synchronization likelihood, mutual information, spectral coherence, etc.) sometimes appear in functional connectivity studies but have not been applied to fUS data yet, and therefore lie outside the scope of this review. In contrast, an alternative to connectivity matrices that is starting to show in some fUS work is to use data-driven approaches, such as independent component analysis (Ferrier et al., 2020; Vidal et al., 2020) and clustering analyzes (Macé et al., 2018; Sans-Dublanc et al., 2021), to spatially decompose the field of view into different networks of voxels in an unsupervised fashion (Bajic et al., 2017; McKeown et al., 1998).

Of note, current whole-brain imaging methods (fMRI, PET, EEG) are to date the most common approach for studying intrinsic activity patterns, but some of their characteristics unavoidably limit the number of experiments where they can be applied. For example, work with rodents potentially offers the possibility to expand intrinsic activity studies by including behavioral information and invasive complementary tools, but the footprint of MRI and PET scanners constrains the repertoire of behaviors that can be performed and hinders the ability to perform multimodal imaging to independently confirm observations. Moreover, specific limitations exist in terms of spatiotemporal resolution or sensitivity, and these machines require dedicated facilities that entail additional issues associated with expense, lack of portability, and need of specialized maintenance. In contrast, fUS in rodents offers a whole-brain view with good spatiotemporal resolution, and facilitates recording rich behavioral contexts with a comparatively cheaper and more portable machine at the cost of more invasiveness. Consequently, despite being a relatively new player in the neuroimaging field, fUS can be a valuable alternative to other brain-wide imaging modalities to explore functional connectivity, especially under behavioral conditions inaccessible to other methods.

### 3.1. *Resting-state functional connectivity*

Can fUS identify those well-known intrinsic connectivity patterns from rodents that are apparent with, for example, fMRI? One way to achieve this is by mimicking resting-state fMRI functional connectivity experiments, i.e., by recording the brain during an extended resting state period (in the order of minutes) and calculating the correla-

tions between voxels or regions of interest. It is important to point out that, whereas humans can be instructed to lie still during recording, resting state in rodent fMRI is more commonly induced through anesthesia (Mandino et al., 2020). The first fUS-based functional connectivity study adapted the approach used in fMRI for fUS by recording single planes from anesthetized rats with thinned skull (Osmanski et al., 2014a). This work identified distinct cortical functional parcellations that exhibited some patterns apparently analogous to well-known networks that are visible in mouse fMRI studies, namely the default mode and the lateral sensorimotor networks. However, the correspondence of the networks across modalities is difficult to prove quantitatively. A refined and updated version of this protocol has since been published (Bertolo et al., 2021).

A major advancement for the study of intrinsic brain activity with fUS was the recent development of whole-brain imaging in rodents using volumetric probes (described in Section 1). Single-plane imaging is very useful in a limited range of experiments, but fUS will become much more relevant for functional connectivity if distributed large-scale correlations can be simultaneously investigated across a substantial volume of the brain. Two studies have pioneered this effort. First, a proof-of-concept experiment demonstrated the feasibility of obtaining whole-brain connectivity matrices using volumetric fUS probes at 0.66 Hz on anesthetized rats after an acute craniotomy (Rabut et al., 2019). The second study used the same ultrasound matrix probe (thus reaching the same spatial coverage and resolution) in a computationally more powerful workstation: this achieved real-time fUS imaging at up to 6 Hz in awake behaving mice with a chronic window (Brunner et al., 2020a). A whole-brain connectivity matrix from awake mice was presented in the preprint version of this study (Fig. 2a, Brunner et al., 2020b).

Notably, this last study (Brunner et al., 2020a) and previous work in freely-moving rats (Urban et al., 2015b) demonstrate the feasibility of fUS-based functional connectivity measures during wakefulness. Recording in awake rodents is an improvement over anesthesia since anesthetics can affect the hemodynamic response (Masamoto and Kanno, 2012) and thus attenuate or confound the functional connectivity signal (Chuang and Nasrallah, 2017; Gao et al., 2017; Grandjean et al., 2014; Jonckers et al., 2014; Mandino et al., 2020; Xie et al., 2020). It has been argued that connectivity matrices detected using fUS under light sedation are comparable to those measured when the animals are awake (Ferrier et al., 2020), but achieving stable and reproducible results under sedation is challenging. In awake recordings, resting-state data can be extracted from the spontaneous periods of rest in between movements or tasks. Nonetheless, it should be kept in mind that spontaneous resting in awake animals might still differ from instructed resting in humans, as they may switch between active and quiet states. However, we believe this potential confound actually opens new research paths on behavioral states. Our view is that the general trend for fUS will be to avoid the use of anesthetics.

With advances on two fronts - the synchronous recording of a larger brain volume, and compatibility with awake states - the fUS technology slowly becomes mature for standardized functional connectivity studies.

### 3.2. Changes across clinical conditions and behavioral states

When data from an entire recording session are used to generate a single connectivity matrix, the resulting connectivity pattern is referred to as static or time-averaged connectivity. Static connectivity matrices act as a sort of “fingerprint” for the general state of the brain under the physiological conditions at the time of the recording, and are used to test differences across groups or individuals (Finn et al., 2015; Gratton et al., 2018). Keeping in mind the controversy around such approach, multiple fMRI studies have shown that the properties of these static connectivity matrices (i.e., how strongly different regions correlate) vary significantly across individual personality traits (Bergmann et al., 2020; Gratton et al., 2018), genetic backgrounds (Fu et al., 2015; Glahn et al., 2010), or pathologies (Karbasforoushan and Woodward, 2013; Khadka et al., 2013; Menon, 2011; Zhang et al., 2010); they may even be used to predict cognitive and attentional abilities (Rosenberg et al., 2020). Along that line, several studies have used fUS to compare connectivity matrices between specific experimental groups, either focusing on diseases or the effects of drugs with a preclinical scope, or addressing more fundamental scientific questions, such as the effect of behavioral states on these brain-wide patterns.

Concerning preclinical studies, a couple of studies applied the same paradigm as in Osmanski et al., 2014a to anesthetized rat pups to examine the hereditary effects of maternal protein deficiency during gestation (Mairesse et al., 2019; Rideau Batista Novais et al., 2016). The authors observed that the low-protein diet decreased both inter- and intra-hemispheric functional connectivity in the pups, which led to the identification of underlying microstructural impairments of possible neuroinflammatory origin (Rideau Batista Novais et al., 2016). Follow-up work confirmed that an oxytocin agonist with anti-inflammatory properties could partially prevent the loss of functional connectivity, underscoring the neuroprotective role of oxytocin on the developing brain (Mairesse et al., 2019). Taken together, both papers exemplify how to use functional connectivity “fingerprints” to generate hypotheses that can guide further experiments. Likewise, two other studies have used fUS-measured functional connectivity matrices as a tool to investigate pharmacologically-induced changes in brain functional architecture (Rabut et al., 2020; Vidal et al., 2020). The first study showed that scopolamine, a cholinergic modulator, leads to time- and dose-dependent changes in brain activation and connectivity in awake freely-moving mice (Rabut et al., 2020), whereas the second found that atomoxetine, a norepinephrine reuptake inhibitor, reduces connectivity at low doses in anesthetized rats (Vidal et al., 2020). In addition, fUS has been used to show altered functional connectivity in a rat model of arthritis (Rahal et al., 2020) and in preterm babies (Baranger et al., 2021), providing a potential endophenotype that could be used in diagnosis. On the methodological side, it should be noted that all these works utilized single-plane transcranial fUS imaging, and therefore focused on correlations between 5 and 7 dorsal brain regions within each brain plane. The exception is the study in neonates (Baranger et al., 2021), performed through the anterior fontanel, which could reach deep structures and record from a large fraction (40%) of the neonate brain using a scanning approach. Taken together, these studies demonstrate the potential of fUS as a screening tool for drugs or preclinical models.

Concerning fundamental research, functional connectivity matrices have been used to compare brain activity in freely-moving rats during different behavioral states, including locomotion and different phases of natural sleep (Fig. 2b, Bergel et al., 2018). Brain-wide connectivity was found to be similar between wakefulness, rest, and non-REM sleep, but stronger during locomotion and even stronger during REM sleep. Another study in head-fixed mice confirmed the nonspecific increase of functional connectivity during locomotion (Ferrier et al., 2020). Note that both natural sleep and locomotion are behavioral states that have hardly ever been studied in whole-brain imaging experiments so far due to the inherent difficulty of scanning rodents with traditional whole-brain methods without anesthesia. In particular, recordings of freely-moving animals during spontaneous period of rest is a more naturalistic way to get insights on basal brain activity than the classical combination of anesthesia and head fixation. For that reason, we consider that these studies spotlight fUS as a noteworthy neuroimaging tool for researching functional connectivity in rodents during behavioral states that would otherwise be inaccessible.

### 3.3. Towards dynamic functional connectivity

Despite their utility, time-averaged connectivity matrices may ignore important dynamical aspects of intrinsic brain activity. Indeed, unlike fingerprints, functional connectivity patterns fluctuate over time, with several network configurations appearing and disappearing in a

dynamic fashion along the time of the recording (Deco et al., 2011; Hutchison et al., 2013; Preti et al., 2017). This effect has been observed at several timescales and when using different methods, including fMRI, EEG, invasive electrophysiological recordings, and calcium imaging (Chang et al., 2013; MacDowell and Buschman, 2020; Matsui et al., 2019; Thompson et al., 2013). Accumulating evidence indicates that transient connectivity patterns may reflect ongoing changes in the internal state of the individual, implying that dynamic, or time-resolved, functional connectivity might be a more accurate proxy for the study of cognition, emotion, and arousal than static functional connectivity (Fong et al., 2019; Gonzalez-Castillo and Bandettini, 2018; Vidaurre et al., 2021). In addition, various fMRI studies have indicated that, with sufficient data and unsupervised machine learning techniques, such as clustering, it may be possible to extract a sequence of different functional connectivity matrices, labeled "brain states", that can then be associated to behavioral, cognitive, or physiological states (Preti et al., 2017; Song and Rosenberg, 2021).

Two studies began exploiting this idea using fUS. The first one imaged variations in the brain functional connectivity of a rat model of arthritis and clustered the resulting time courses in up to seven "brain states", finding that the occurrence probability of at least four of them was significantly correlated to different pathophysiological markers, such as pain sensitivity or inflammation, that differed between arthritic and healthy animals (Rahal et al., 2020). Notably, this study used a phase-difference matrix rather than the more common correlation-based matrix (for a comparison between correlation- and phase-based dynamic analyzes, refer to Preti et al., 2017). The results achieved are encouraging, in spite of the limitations of using anesthetized rats and a transcranial approach discussed earlier. More recently, an almost identical protocol was applied to sleeping human newborns, which found four different brain states whose occurrence frequency varied in preterm neonates (Fig. 2c, Baranger et al., 2021). These studies establish fUS as a promising tool for describing, detecting, and understanding transient brain states, especially considering its high temporal resolution. Although this approach is still in its infancy, extending the fUS-based dynamic functional connectivity approach to cognition and behavior could bring new insights into these complex functions.

In summary, fUS has started to successfully incorporate analysis methods originally developed for the fMRI field to identify intrinsic large-scale brain networks. In particular, fUS has been used to establish either global (i.e., whole-brain) or restricted (i.e., to a few regions) functional connectivity matrices that include both time-averaged and time-resolved characteristics. Beyond identifying networks, which can be performed with fMRI, fUS seems particularly promising for tracking how these networks, represented as connectivity matrices, change across conditions, across states, or dynamically on a short timescale.

## 4. Mapping brain-wide pathways through evoked activity with fUS

Intrinsic functional connectivity studies can reveal networks of regions with coordinated activity and how they change over time or across states. However, the information on what specific brain pathway within the network is implicated in a particular behavior, task, or function can be difficult to extract from connectivity matrices. For example, a visual stimulus will transiently increase local activity across the visual system without changing the fact that visual regions have correlated activity even at rest. Along these lines, fMRI studies at the systems level have generally reported subtle effects of task-evoked activity on resting-state functional networks (Gonzalez-Castillo and Bandettini, 2018; Gratton et al., 2018). By contrast, a classic approach to mapping brain pathways associated with a specific brain function is to transiently activate them through experimental manipulations.

Experimental manipulations can target the environment, the behavior of the individual, or specific neural circuits (Fig. 3). Sensory stimuli can be controlled and parametrized to map how they are processed in sensory systems. Behavioral variables can be manipulated in the context of a task to reveal the brain pathways they engage. Finally, optogenetics, chemogenetics, and electrical stimulation provide unique alternatives for evoking brain activity in specific pathways with genetic, spatial, and temporal precision. All three types of manipulations can be repeated many times and the responses averaged to reveal the implicated pathways. An additional advantage is that, in contrast to intrinsic activity imaging, they create the highly reproducible experimental conditions needed to draw causal relationships between networks and behavior.

### 4.1. Networks activated by sensory manipulations

The majority of fUS studies published so far have focused on sensory mapping. We have identified two reasons for that. First, sensory mapping is experimentally practical, as sensory stimuli can vary in a controlled manner. Second, sensory systems have already been very well characterized using other neuroimaging methods. Therefore, sensory mapping was often used as a benchmark to validate the fUS method itself. For instance, the first ever fUS paper exploited the robust topography of the barrel cortex in single- or multi-whisker stimulation tasks to highlight the technique's high spatiotemporal resolution (Mace et al., 2011). Later, whisker stimulation was also used among other validation experiments in the first works on volumetric imaging (Brunner et al., 2020a; Rabut et al., 2019) and on behaving rodents (Ferrier et al., 2020; Urban et al., 2015b). In parallel, other works have focused on activations of the somatosensory system to finely characterize spatiotemporal features of the hemodynamic response measured by fUS (Urban et al., 2014) or to evaluate the effect of stroke on brain activity (Brunner et al., 2018).

One of the areas where fUS imaging has been most commonly applied is the mapping of visual circuits. Beyond general activation maps (Gesnik et al., 2017; Rau et al., 2018), fUS has been used to image ocular dominance columns in the deep layers of the visual cortex of primates (Blaize et al., 2020) or the retinotopy of the visual cortex and superior colliculus of mice (Fig. 3a, Macé et al., 2018). These studies produced fine-grained maps of the deep structures of the visual system that could not have been achieved at the same level of detail by other methods. Now, volumetric fUS makes it possible to obtain such whole-brain retinotopic maps in awake mice (Brunner et al., 2020a) up to 10 times faster than with a linear probe.

Functional ultrasound has also been applied to image brain responses to other sensory stimuli. In the olfaction domain, fUS was able to confirm that different odorants activate different regions of the olfactory bulb, but a similar portion of the anterior piriform cortex of anesthetized rats (Osmanski et al., 2014b). Other studies have sequentially used fUS and two-photon calcium imaging in the mouse olfactory bulb to precisely characterize neurovascular coupling across odors and odor intensities, highlighting the efficiency of fUS for following transient odor-evoked response dynamics (Aydin et al., 2020; Boido et al., 2019) even at very low odorant concentrations (Boido et al., 2019). Finally, in the auditory domain, fUS imaging has been used in awake ferrets exposed to different sounds to disentangle the fine-grained tonotopic organization of the auditory cortex and other subcortical structures (Bimbard et al., 2018). A subsequent study further advanced the analysis of the ferret auditory system by comparing the responses to natural versus synthetic sounds across regions, revealing a marked difference to human auditory processing (Landemard et al., 2020). This illustrates the growing interest in the method to uncover new aspects of sensory processing in ethologically relevant contexts.

### 4.2. Networks activated by behavioral manipulations

It has been frequently argued in recent years that discoveries from systems neuroscience cannot be fully understood unless in the light of behavior (Gomez-Marin et al., 2014; Krakauer et al., 2017), an opinion

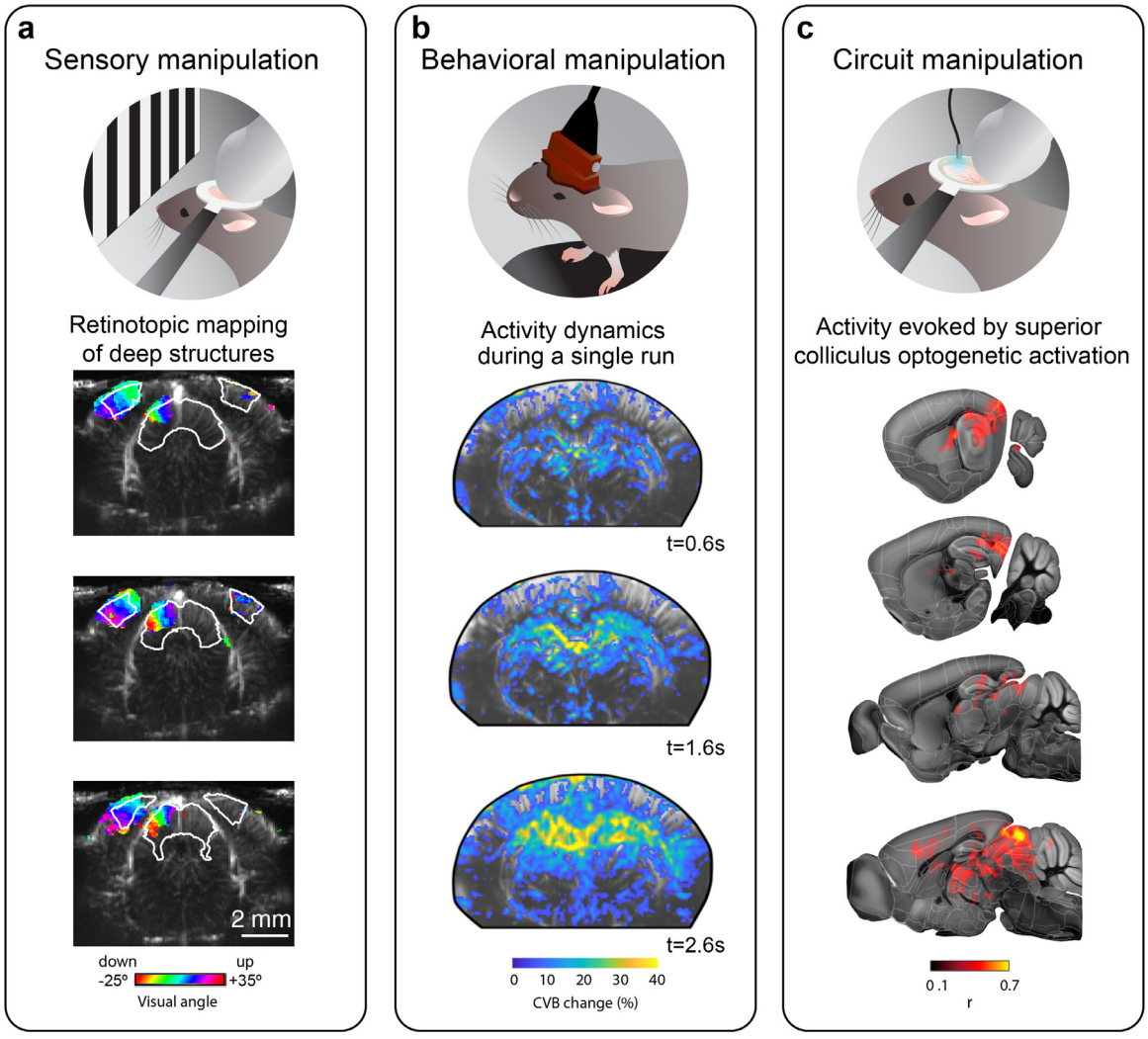


**Fig. 3.** Mapping large-scale activated pathways with fUS. Transient activity patterns evoked by extrinsic manipulations can uncover the pathways implicated in a particular behavior, task, or function.
a. Sensory manipulation. Example of the use of fUS to produce retinotopic maps of both cortical and deeper structures in head-fixed mouse. Figure adapted with permission from Macé et al., 2018; Copyright 2018 Elsevier. b. Behavioral manipulation. Example of the ability of single-plane fUS to track spatiotemporal patterns of activity in the context of a behavioral task, here the stereotyped run of a freely-moving rat ($t$ = time from run onset). Figure adapted with permission from Bergel et al., (2020); Copyright 2020 (CC-BY). c. Circuit manipulation. Example of an opto-fUS experiment with volumetric imaging in head-fixed mice. Maps show the voxels activated by optogenetic activation of a specific cell population in the superior colliculus (Grp-Cre). Figure adapted with permission from Brunner et al. (2020a); Copyright 2020 Elsevier.

that has expanded in parallel with the popularity of behavioral quantification tools (Anderson and Perona, 2014; Datta et al., 2019; Mathis and Mathis, 2020; Musall et al., 2019). One unique asset of fUS compared to alternative whole-brain imaging methods available in mammals is its compatibility with behavior. Indeed, active tasks can be implemented with fUS as long as they are physically compatible with miniaturized probes or head-fixation paradigms, thus providing the experimental conditions necessary to understand the brain-wide circuits underlying behavior.

A field that is exploiting this fact is the study of locomotion. Widespread hyperemia was observed in freely-moving rats during a locomotion task with fUS (Sieu et al., 2015). A later study also reported a significant vascular amplification in active states compared to rest (Bergel et al., 2018). Recently, a follow-up study used fUS to explore the spatiotemporal dynamics of locomotion-evoked brain hyperactivity, describing an activation sequence that propagates across retrosplenial and parietal cortices, the dorsal thalamus, and the hippocampus (Fig. 3b, Bergel et al., 2020).

Another relevant area where fUS can complement other neuroimaging techniques is the analysis of high-order, “cognitive” pathways. This field generally involves non-human primates performing complex tasks. Two recent attentional experiments focused on the supplementary eye field: one demanding different saccade responses to different cues (Dizeux et al., 2019) and the other involving varying reward magnitudes (Claron et al., 2021b). In both cases, fUS imaging was capable of tracking transient changes in brain activity that correlated with rule modifications or pupil size, respectively, thus shedding light on the cortical correlates of attention and arousal. A third study imaged the posterior parietal cortex during a complex task involving delayed eye saccades and hand reaches: it found that the fUS signal during the preparation phase was sufficient to predict hand movements or the direction of the eyes (Norman et al., 2021). Because of the bigger brain size of monkeys, all three studies had to limit the imaging to specific parts of the cortex. In the future, this field of research would greatly benefit from the development of larger matrix ultrasonic probes with a broader field of view

to study cognitive signals at a larger scale and without prior knowledge of the region of interest in non-human primates.

In parallel, other studies have focused on obtaining an unbiased whole-brain view during behavior in mice, a model where numerous genetic tools are available. For example, a systematic screening of behavior-related activity was performed for a visuomotor behavior, the optokinetic reflex, and revealed unexpected regions displaying oculomotor-related activity, such as the amygdala (Macé et al., 2018). A similar approach was applied to a reaching task using volumetric imaging (Brunner et al., 2020a). These works indicate that unbiased screening of brain activity at the spatiotemporal resolution offered by fUS may be of great value in uncovering networks involved in a behavior that may have been missed by conventional neuroimaging approaches. Indeed, fMRI studies involving human and primate behavior often have a cortical bias due to, among other reasons, the combination of decreased signal in depth and the small size of subcortical regions in these species (Keuken et al., 2018; Parvizi, 2009; Tetereva et al., 2020). Whole-brain fUS recordings during behavior in rodents could counter this bias and help expand our knowledge of the role of sub-cortical structures in various brain functions.

### 4.3. Networks activated by circuit manipulations

Direct manipulation of brain areas or cell populations is a very precise way to interrogate the functional connections. Indeed, activating or silencing a certain neural circuit enables hypotheses about its spatial extent, activation dynamics, downstream targets, and general function to be tested (Mandino et al., 2020). Circuit manipulation techniques currently available include optogenetics, chemogenetics, and direct electrical stimulation. The small size of fUS probes facilitates the combination with optic fibers (Brunner et al., 2020a; Rungta et al., 2017; Sans-Dublanc et al., 2021) and electrodes (Bergel et al., 2020, 2018; Bimbard et al., 2018; Mace et al., 2011; Nayak et al., 2021; Sieu et al., 2015), so that genetic manipulations can benefit from the high spatiotemporal resolution of fUS to investigate the brain-wide activity patterns evoked.

Optogenetics stands out for its capacity to activate or inhibit specific cell populations with high temporal precision and adjustable frequencies (Deisseroth, 2015). Along this line, the combination of optogenetics with fMRI, called opto-fMRI, has proven its value for neuroimaging since it was tried for the first time one decade ago (Desai et al., 2011; Lee et al., 2010). Notably, the recent combination of optogenetics with fUS, likewise termed opto-fUS, has been shown to detect induced neural activity with higher sensitivity than standard opto-fMRI (Edelman et al., 2021). The feasibility of opto-fUS was first demonstrated in a study exploring the vascular effects of light stimulation (Rungta et al., 2017). Then, opto-fUS was applied to comprehensively dissect superior colliculus output circuits (Sans-Dublanc et al., 2021). This study revealed the distinct downstream pathways activated by genetically-defined neuronal populations known to contribute to different defensive behaviors; and was able to identify a new region important in collicular-mediated defensive behaviors in the thalamus. A companion study reproduced the same experiment much faster using volumetric imaging, accentuating the potential of opto-fUS to map whole-brain targets of specific circuits with high spatiotemporal resolution, versatility, and affordability (Fig. 3c, Brunner et al., 2020a). Opto-fUS was also recently applied in anesthetized rats to assess the potential of optogenetic activation of the visual cortex for vision restoration strategies (Provansal et al., 2021). In contrast, chemogenetics is comparable to optogenetics in terms of spatial precision, yet temporally less controllable and does not need an optic fiber (Sternson and Roth, 2014). It also provides the opportunity to block or activate specific circuits on much longer timescales and without the need of inserting a fiber. However, chemogenetics has not been combined with fUS to date.

Direct electrical stimulation is another way to efficiently evoke transient activity with high temporal precision when genetic cell targeting is not essential (Desmurget et al., 2013). Electrical stimulation can be combined with fUS, as shown in a study that stimulated the frontal cortex of ferrets while recording from the auditory cortex to study the top-down modulation of auditory areas (Bimbard et al., 2018). More recently, a similar approach was used in anesthetized rats to examine evoked activity in the motor cortex after deep-brain stimulation of the thalamus, finding that the transient activity patterns were strongly dependent on the stimulation frequency (Nayak et al., 2021). To end this section, we want to point out the potential of combining fUS and direct electrical manipulation for the study of the peripheral nervous system. Epidural electrical stimulation is possible in anesthetized rats and pigs to induce spinal-cord responses that could be tracked with fUS imaging (Song et al., 2019). Further studies applying this protocol revealed insights into spinal cord hemodynamics in anesthetized rats (Claron et al., 2021a; Tang et al., 2020), including how it is affected by inflammatory pain (Claron et al., 2021a). Even though most fUS studies carried out so far have focused on the brain, spinal-cord circuit mapping could also greatly benefit from the high spatiotemporal resolution and ease of use of fUS.

All these studies illustrate the potential of combining circuit manipulation techniques with fUS imaging to visualize brain-wide evoked activity patterns. Notably, optical fibers and electrodes do not degrade image quality (i.e. shadowing) because their diameter is usually smaller than fUS spatial resolution (Nunez-Elizalde et al., 2021). Thus, their main limitation is that inserting these probes requires careful positioning with respect to the ultrasound probe. We believe that this new option for quickly revealing the brain-wide functional connections of specific circuits is likely to interest many neuroscience groups in the near future.

## 5. Future directions

### 5.1. Technical developments

All the works discussed in this review illustrate the technical capabilities of fUS. However, as we have remarked throughout the text, such capabilities have not yet reached their maximum potential. The most obvious example of this is in regard to volumetric fUS probes, which are limited by two technological constraints: (1) the difficulty in packing enough piezoelectric elements to reach the maximum theoretical spatial resolution; and (2) the computational power required to process the data in real-time for thousands of channels. Concerning the first point, a new generation of ultrasound probes is emerging that is based on micromachined ultrasound transducers (C-MUT or P-MUT technology) and will likely eliminate these hardware roadblocks (Brenner et al., 2019; Jung et al., 2017). This technology offers extreme miniaturization, flexible design, a large range of possible ultrasound frequencies, and a large bandwidth per element. Beyond the ability to produce dense matrix probes to increase the spatial resolution of volumetric imaging, it supports the development of larger matrix probes adapted to animal models with bigger brains than rodents, and of miniaturized probes for whole-brain imaging during behavior. Concerning the computational load, the computational capabilities will only grow in the future, enabling more and more ultrasound channels to be processed simultaneously in real-time, which will increase temporal resolution and sensitivity for volumetric imaging. It is also possible to acquire all the raw ultrasound data and compute the compound and Doppler images off-line, but that strategy requires enormous storage capacity and write speed – so far, it has only been possible to save a few minutes of recording for volumetric imaging (Rabut et al., 2019). Moreover, real-time processing is crucial for quality control during the experiment, and for any closed-loop experimental design. In the meantime, the incorporation of deep-learning approaches to fUS compound image formation may be an efficient way to speed up the imaging process by decreasing the data load and computational capacity demands using pre-trained reconstruction models that can handle sparser data while potentially achieving similar image quality (di Ianni and Airan, 2020; van Sloun et al., 2020).

Another potentially interesting technical development is the hybridization of fUS imaging with other techniques for multimodal circuit dissection. We have reviewed several proofs of concept that show how fUS probes can be combined with optic fibers (Brunner et al., 2020a; Edelman et al., 2021; Rungta et al., 2017; Sans-Dublanc et al., 2021) and stimulation electrodes (Bimbard et al., 2018; Nayak et al., 2021) to evoke transient activity in specific brain pathways. Hybridization with other neuroimaging techniques has also been suggested to exploit the advantages of each one and validate results, for example with PET imaging (Tournier et al., 2020). On the other hand, the non-simultaneous combination of fUS and calcium imaging or other optical techniques is already feasible (Aydin et al., 2020; Boido et al., 2019; Rungta et al., 2017), since both techniques can share the same cranial window (Kılıç et al., 2020). With some more spatial constraints, the combination of fUS and EEG has been achieved by implanting electrodes in rats (Bergel et al., 2020, 2018; Mace et al., 2011; Sieu et al., 2015) and non-invasively in neonates (Baranger et al., 2021; Demene et al., 2017). Finally, the combination of fUS and dense multielectrode arrays, either successively (Macé et al., 2018; Sans-Dublanc et al., 2021) or simultaneously (Nunez-Elizalde et al., 2021), has proved essential for validating fUS findings at the cellular level. The future incorporation of fUS into multimodal neuroimaging protocols can bridge the single-neuron and network scales, thereby greatly enhancing our understanding of brain function.

### 5.2. Analysis tools for fUS data

One of the greatest challenges for fUS imaging research is to converge towards standardized procedures for acquiring and preprocessing data. It is well known from resting-state fMRI experiments that the order and choice of preprocessing steps impact the final functional connectivity or activity measures (Gargouri et al., 2018). However, different groups tend to use their own individualized analysis pipelines (Carp, 2012), which can lead to different conclusions even when analyzing the same dataset (Botvinik-Nezer et al., 2020). Functional ultrasound imaging is a new technique that is just starting to shift from proof of feasibility to truly contributing to systems neuroscience. Therefore, the ample variety of preprocessing steps displayed by the handful of papers published so far (i.e., the presence or absence of band-pass filtering, spatial smoothing, global signal regression, etc., and the extent thereof) is a necessary consequence of that exploratory phase, but attention should be paid in this regard to avoid an excessive proliferation of individualized pipelines. Moreover, the vast majority of those pipelines have not been publicly shared. For example, a recently published protocol for standardized fUS data acquisition and analysis requires commercial software (Bertolo et al., 2021). To remedy this fact, two groups (including ours) have collaboratively published a complete fUS protocol that includes free software tools to acquire and analyze fUS data (Brunner et al., 2021). We anticipate that data and software sharing will grow in the future, to guide non-experts, increase reproducibility, and foster a collaborative fUS imaging community.

Focusing on functional connectivity experiments, the characteristics of fUS has several advantages. First, the high temporal resolution of compound imaging (~500 Hz) allows for the detection of fast physiological oscillations, such as the respiratory and cardiac cycles, that otherwise would appear as confounders of the low-frequency neural signals, i.e., aliased, thus introducing unwanted correlations (Gorges et al., 2017; Pan et al., 2015). This feature has been exploited to design filters that remove those oscillations directly from the compound images without external measurements (Demené et al., 2015). Second, the fast acquisition rate of fUS also makes it a good option for tracking transient changes in functional connectivity, namely dynamic functional connectivity (Deco et al., 2011; Hutchison et al., 2013; Preti et al., 2017), as discussed in Section 2.3. This subfield may therefore benefit the most from fUS imaging. Third, it has been demonstrated that fUS signals, although relying on changes in blood volume, provide a faithful and linear readout of neural spiking rate up to ~0.3 Hz (Nunez-Elizalde et al., 2021), coinciding with the frequency band that underlies resting-state activity fluctuations (Helps et al., 2007; Hiltunen et al., 2014; Mantini et al., 2007) and thus giving confidence in the interpretation of fUS studies. Finally, most data-analysis strategies that are available for other neuroimaging approaches can be applied to fUS output as well.

## 6. Discussion

In this review, we have examined the fUS literature with a functional connectomics angle. By "functional connectomics", we mean experiments that aim to expand our knowledge of the networks and pathways that underlie various brain functions at the whole-brain level. In that context, fUS imaging is a relatively young technique. From its conception, a myriad of proof-of-concept experiments have validated its capabilities by replicating results obtained by other methods. This tool-developing phase reached an inflexion point last year, when the most recent papers started to apply fUS to obtain new insights on brain function. Functional ultrasound has now been successively used to generate finer-grained maps of sensory systems, uncover unexpected regions involved in genetically defined pathways, unveil dynamic patterns associated with behavioral states, and detect disease-associated brain states.

The possibility of evoking brain activity with circuit manipulation tools (optogenetics, chemogenetics, and electrical stimulation) during high-resolution brain-wide recordings in behaving animals is in our opinion the most important asset of fUS imaging, inasmuch as it enables large-scale network functions to be investigated at a level otherwise innacessible. Direct manipulation of neural circuits creates highly-controllable experimental conditions that enable accurate fUS mapping of brain circuits (Bimbard et al., 2018; Brunner et al., 2020a; Sans-Dublanc et al., 2021), even with higher sensitivity than state-of-the-art opto-fMRI (Edelman et al., 2021). Importantly, whole-brain circuit studies can be performed during complex behavioral tasks (Brunner et al., 2020a) or in freely-moving animals (Bergel et al., 2018), which so far is out of reach for rodent fMRI. We believe that this approach is, to date, the most promising research path where fUS can make innovative contributions to our understanding of brain function at the network level.

Nonetheless, we call for caution before fUS studies proliferate. In that regard, the study of "evoked" activity patterns with fUS (addressed in Section 3) should be treated differently from "functional connectivity" studies (described in Section 2), as the interpretation and statistical analyzes are more straightforward in the first case. Indeed, the biggest challenge in resting-state fMRI functional connectivity studies, both in humans and small animal models, is the difficulty to access the ground truth, to verify findings with other methods, and to fit their observations within a generalized theoretical framework (Reid et al., 2019). The goal of fUS imaging should therefore not be to merely repeat this literature with yet another technique, but to complement it. For example, since fUS is a portable method that may find a place in many neuroscience labs, it offers the opportunity to design studies that include cross-validation of the results (for example, using electrophysiology or calcium imaging). However, in most of the fUS functional connectivity proof-of-concept studies that we have presented here, distinct connectivity patterns are found to correlate with pathophysiological or behavioral states, but independent validations of those patterns are absent. If that is addressed, we believe strong findings could emerge.

Along the same lines, fUS imaging, although having been applied to several animal models, is most advanced in rodents. What fraction of the functional connectivity findings made in rodents can be extrapolated to human brain function is unknown. First, because large-scale network architecture and function may differ drastically across species (Gozzi and Schwarz, 2016), especially those involving the areas that share the least homology, such as the frontal cortices (Carlen, 2017; Laubach et al., 2018). Second, because the behavioral context in which resting-state data are acquired is different, with humans lying still and awake during the whole recording while rodents are either anesthetized or allowed to

switch between different levels of arousal (Pan et al., 2015). Tangentially to the rodent studies that suffer from these intrinsic limitations, the possibility to non-invasively image functional connectivity patterns in human newborns with fUS at the bedside (Baranger et al., 2021) is a promising avenue for fUS to enrich our understanding of normal and abnormal development of human large-scale networks.

Given the technique's potential, we expect in the next few years to witness an upsurge of publications that apply fUS to the study of brain-wide networks. We envisage the biggest discoveries coming from combining fUS with other circuit interrogation tools, such as optogenetics, and from applying it to the study of behavior and brain states less accessible by other methods.

## Declaration of Competing Interest

The authors declare no conflicts of interest.

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## Credits

Both J.M.M.d.P and E.M. conceptualized, wrote and edited this review article.

## Data and code availability statements

(1) All papers must include a statement regarding the availability of all data used in the study.

This is a review article that does not include data.

(2) All papers must also include a statement regarding the availability of software and code used in the study.

This is a review article that does not include code.

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