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► **To cite this version:**

Thomas Deffieux, Charlie Demené, Mickael Tanter. Functional Ultrasound Imaging: A New Imaging Modality for Neuroscience. *Neuroscience*, 2021, 474, pp.110 - 121. 10.1016/j.neuroscience.2021.03.005 . hal-04061406

HAL Id: hal-04061406

<https://hal.science/hal-04061406>

Submitted on 6 Apr 2023

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Functional Ultrasound Imaging: A New Imaging Modality for Neuroscience

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Abstract—Ultrasound sensitivity to slow blood flow motion gained two orders of magnitude in the last decade thanks to the advent of ultrafast ultrasound imaging at thousands of frames per second. In neuroscience, this access to small cerebral vessels flow led to the introduction of ultrasound as a new and full-fledged neuroimaging modality. Much as functional MRI or functional optical imaging, functional Ultrasound (fUS) takes benefit of the neurovascular coupling. Its ease of use, portability, spatial and temporal resolution makes it an attractive tool for functional imaging of brain activity in preclinical imaging. A large and fast-growing number of studies in a wide variety of small to large animal models have demonstrated its potential for neuroscience research. Beyond preclinical imaging, first proof of concept applications in humans are promising and proved a clear clinical interest in particular in human neonates, per-operative surgery, or even for the development of non-invasive brain machine interfaces.

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Key words: ultrasound imaging, ultrafast imaging, functional ultrasound imaging, Doppler, ultrasound localization microscopy, functional brain connectivity, neurovascular coupling, neuroimaging, stroke.

INTRODUCTION

Ultrasound imaging is one of the most used imaging modalities in clinics, praised for its low cost, portability, and real-time capability. However traditionally, ultrasound imaging has never played a major role in clinical neuroimaging and was limited to a few structural and anatomical characterizations (Bmode images) and blood flow measurements in major cerebral arteries (Transcranial Doppler). Niche applications like neonate brain imaging and per-operative brain imaging stand as notable exceptions, as the absence of the skull in those case enable further ultrasound quantifications. Even so, ultrasound imaging lacked any functional contrast regarding brain activity beyond large scale modifications of flow. The recent advent of ultrafast imaging (Tanter and Fink, 2014) vastly enhanced blood flow sensitivity (Bercoff et al., 2011) and led eventually to ultrasound-based brain functional imaging, using the detection of small blood volume changes as a surrogate for brain activity. It precipitated ultrasound into neuroimaging and recently, researchers pointed out that ultrasound could

become a major player in the field of neuroimaging and neuroscience, filling a gap between existing techniques in terms of portability, spatial and temporal resolution (Deffieux et al., 2018). The recent introduction of Ultrasound Localization Microscopy (Couture et al., 2011, 2018) based on ultrafast tracking of contrast agents further enabled to break the spatial resolution of brain vasculature down to microscopic resolutions non-invasively (Christensen-Jeffries et al., 2015; Errico et al., 2015). Those three ruptures, temporal resolution, sensitivity and spatial resolution, allowed ultrasound imaging to become a full-fledged neuroimaging modality

FUNCTIONAL ULTRASOUND IMAGING

History

The use of Doppler ultrasound to assess blood flow in the brain is not new, Transcranial Color Doppler Ultrasound (TCCD) has been routinely used to monitor blood flow in clinics in the large basal intracerebral arteries (Aaslid et al., 1982) for the last decades. The technique uses low frequency ultrasound (typically 1–3 MHz) transmitted by a phased array positioned over the temporal bone window to acquire a temporal profile of blood velocity using a conventional pulsed Doppler mode at a single location. TCCD was also used in functional lateralization studies by comparing rest and task conditions between right and left hemispheres. However, its limited sensitivity does neither allow to detect the slow blood flow in smaller ves-

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Abbreviations: CBV, cerebral blood volume; CPCA, class-wise principal component analysis; fUS, functional ultrasound imaging; ICA, independent component analysis; LDA, linear discriminant analysis; PET, positron emission tomography; PPC, posterior parietal cortex; rtPA, recombinant tissue plasminogen activator; SPECT, single photon emission computed tomography; ULM, Ultrasound Localization Microscopy.

sels nor to build detailed maps of the brain vasculature and was limited to very large arteries.

Interestingly, it became possible to detect such slow blood flow only by increasing the frame rate of ultrasound scanner up to thousands of frames per second compared to the typical 50 frames per second of conventional US scanners. This ultrafast imaging approach was made possible by the parallel acquisition of hundreds of channels simultaneously in combination to the transmit of a small set of unfocused waves (plane waves or diverging waves) that can be synthetically focused everywhere afterwards in software processing (Tanter and Fink, 2014). The clinical interest of ultrafast ultrasound was initially demonstrated in the field of elastography and cancer diagnosis (Tanter et al., 2014). Notably, the technique was used to track the propagation of shear waves at several thousand frames per second to estimate local tissue stiffness with a millimetric resolution. Based on ultrafast imaging, ultrafast Doppler was proposed as a mean to capture the complete hemodynamic information in both space and time in a way that was not yet possible, allowing for example to generate maps of resistivity index in neonates (Demené et al., 2014) or to compute vector flow maps (Hansen et al., 2017). But the main advantage of ultrafast imaging came from the large increase of the number of temporal samples for every pixel which allows to detect very slow blood flow and gain a 50-fold factor in sensitivity compared to conventional power Doppler imaging (Mace et al., 2013).

Applied to the imaging of cerebral blood volume variations during whisker stimulation in a rat model, ultrafast Doppler was shown to detect the subtle blood flow increase induced by the neurovascular coupling (See Fig. 1) leading to the concept of functional ultrasound imaging (fUS) (Macé et al., 2011).

Physical and physiological principles

The fUS signal is proportional to the local hematocrit ** (Rubin et al., 1995; 1997; Shung et al., 1976) and thus to the local cerebral blood volume (CBV), and as such increases when vasodilatation happens. We recall here the founding of this idea. Briefly, fUS is based on a power Doppler estimator which estimates the energy of the signals coming from moving scatterers (Mace et al., 2013). Let us begin to write, after the transmission of an ultrasonic wave, the signal p , measured in one pixel at a distance z from the transducer, at the slow time T corresponding to the successive transmissions of ultrasonic waves ($T = p/\Delta F$ where p is the frame number and ΔF is the frame rate of the imaging system) and backscattered from a single red blood cell i being in the exact location of the pixel center. If we put aside focusing considerations, this signal is built from the raw measurement r_i of the piezoelectric sensors at a fast time $t = \frac{2z}{c}$, and we measure at that moment a pulse similar to the emission s but multiplied by a backscattered amplitude coefficient a_i .

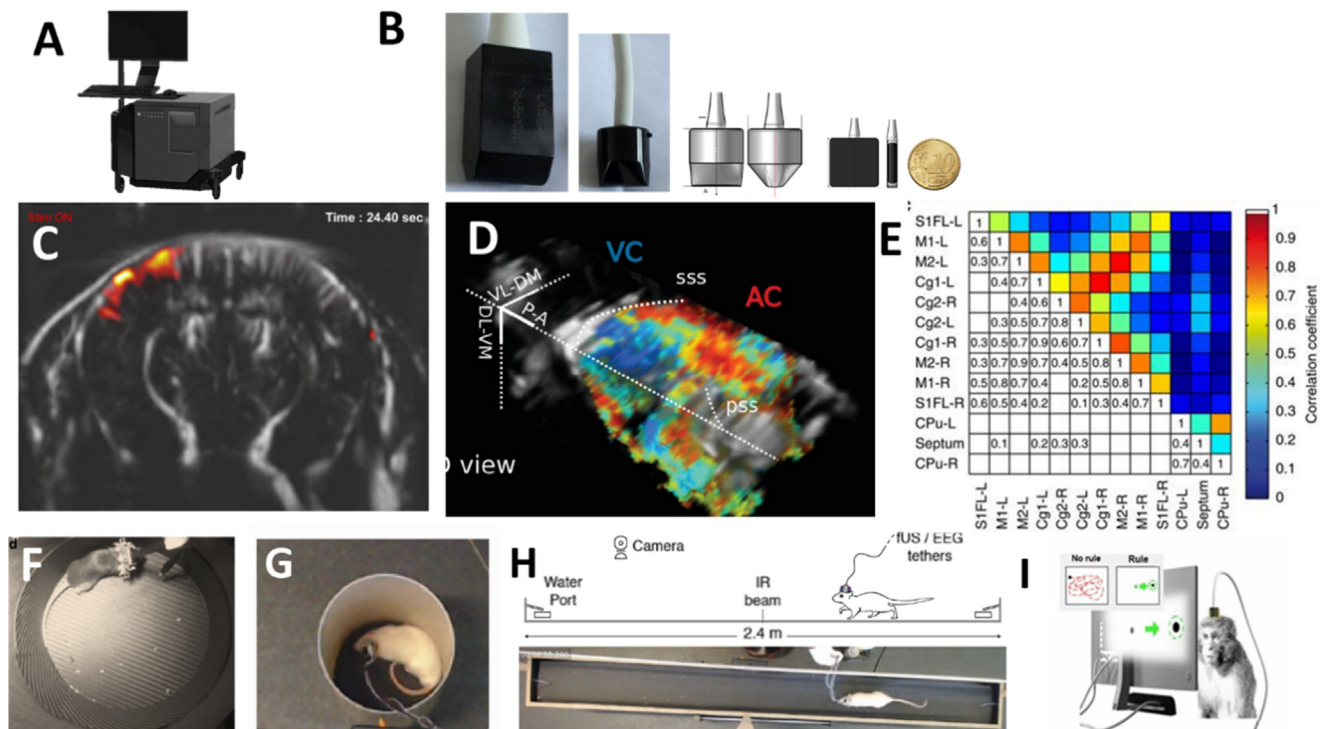


Fig. 1. (A) A ultrafast-capable ultrasonic neuroimaging scanner requires a special hardware to transmit plane waves and store in memory the backscattered echoes at an ultrafast framerate, before heavy computational image reconstruction. Still, it is available in a compact equipment. (B) Evolution of preclinical probe miniaturization with coin for scale. (C) Real-time relative increase in CBV during a whisker stimulation (orange) displayed over brain vessels in a transcranial mouse (gray). (D) Tonotopic map of the auditory cortex of awake ferrets (Bimbarb et al., 2018). (E) Connectivity matrix obtained in rat brain. (F) Awake mouse in a freely-moving setup (Rabut et al., 2020). (G) Sleeping rat in a cage with EEG and fUS (Bergel et al., 2018). (H) Rat running in a locomotion experiment in a corridor with EEG and fUS (Bergel et al., 2020). (I) Awake imaging of complex visual task in behaving non-human primates (Dizeux et al., 2019).

$$\rho_i(T) = r_i \left(\frac{2z}{c} \right) = a s_i \left(t - \frac{2z}{c} \right) \quad (1)$$

Then if this red blood cell is not in the exact center of the pixel:

$$\rho_i(T) = r_i \left(\frac{2z}{c} \right) = a s_i \left(t - \frac{2z}{c} - t_i \right) \quad (2)$$

where t_i is here to account for the path difference between the 2 situations and has a random value. Then if the scatterer starts to move at a speed v (the projection of \vec{V} on the axis z), the pixel signal at the time T becomes:

$$\begin{aligned} \rho_i(T) &= r_i \left(\frac{2z}{c}, T \right) = a s_i \left(t - \frac{2z}{c} - t_i - 2v \frac{T}{c} \right) \\ &= a_i s_{D_i} \left(\left[t - \frac{2z}{c} \right] \frac{c}{2v} - t_i \frac{c}{2v} - T \right) = a_i s_{D_i}(-\tau_i - T) \end{aligned} \quad (3)$$

where s_{D_i} is equivalent to s , but first translated by $(t - \frac{2z}{c})$ and then undergoing a dilation of time by a factor $\frac{2v}{c}$.

The signal $p_i(T)$ in the pixel, generated from the backscattering of N scatterers randomly distributed in the pixel and moving at a speed v therefore be written as:

$$\begin{aligned} p(T) &= \sum_{i=1}^N \rho_i(T) = \sum_{i=1}^N a_i s_{D_i}(-\tau_i - T) \\ &= \sum_{i=1}^N a_i \delta(-\tau_i - T) * s_{D_i}(T) \end{aligned} \quad (4)$$

In the Fourier domain ($T \rightarrow f$), Eq. (1) writes:

$$P(f) = \sum_{i=1}^N a_i e^{-j2\pi f \tau_i} \cdot S_{D_i}(f) = S_D(f) \cdot \sum_{i=1}^N a_i e^{-j2\pi f \tau_i} \quad (5)$$

The Fourier transform $S_{D_i}(f)$ of the backscattered signal coming from each scatterer i does not depend on i but only the axial speed v and the Doppler spectrum of the transmitted signal. Due to the dilation factor of the time, $S_{D_i}(f)$ is similar to the spectrum of the emitted pulse, but with a contraction of the bandwidth and central frequency by a factor $\frac{2v}{c}$, giving a central frequency $f_c = \frac{2v}{c} f_{US}$, a.k.a the Doppler frequency.

Eq. (5) describes the fact that the total Doppler spectrum is the product of a white noise (τ_i being random) by the Doppler Spectrum $S_D(f)$ generated by a single scatterer of axial speed V . As the number N of scatterers within the focal spot is very large and the signal corresponds to a random variable, its ensemble average $\langle |R(f)|^2 \rangle$ can be written:

$$\langle |R(f)|^2 \rangle = N \sigma^2 \cdot \langle |S_D(f)|^2 \rangle \quad (6)$$

where σ^2 is the variance of the white noise. Eq. (4) shows that the power spectrum of the Doppler signal $\langle |R(f)|^2 \rangle$ is centered on the Doppler frequency corresponding to the axial speed V of the scatterers and proportional to the total number N of scatterers in the focal spot. Thus, the fUS signal which corresponds (according to Parseval's identity) to the integral of the power spectrum of the Doppler signal $\langle |R(f)|^2 \rangle$ is given by :

$$fus = \int_f \langle |R(f)|^2 \rangle df = N \sigma^2 \cdot \int_f \langle |S_D(f)|^2 \rangle df \quad (7)$$

The fUS signal is proportional to N and therefore to the local cerebral blood volume. Note also that in the case of a distribution of different speeds v , one could demonstrate

that $\int_f \langle |S_D(f)|^2 \rangle df$ is constant. Thus, the integral of the power spectrum over frequency would give a similar weight to the contribution of scatterers with different speeds.

In a more intuitive manner, one can see the blood ultrasound signal in one pixel as the summation of the contributions of N individual scatterer randomly distributed within the resolution cell, i.e. as the summation of N complex numbers with similar modulus but random phases. Therefore, this summation can be identified as a N -step random walk in the complex plane, the value in the pixel being one realization of this random walk. The expectation of the distance travelled with a N -step random walk is textbook and known to be proportional to \sqrt{N} . Calculating the power Doppler in that pixel (time average of the squared value in the pixel), under an ergodic assumption, boils down to estimate the expectation of the distance travelled squared, i.e. $(\sqrt{N})^2 = N$. We indeed find that the PW Doppler signal is proportional to the number of scatterers in the resolution cell.

In order to efficiently separate signals originating from moving blood scatterers from static tissue scatterers, a clutter-filter is also necessary. Since brain tissue is not completely static, fUS relies on advanced spatio-temporal filters to efficiently separate signals from blood and tissue based on the incoherent properties of blood scatterers. Scatterers from blood vessels have a low covariance whereas signals from the slowly moving tissue have a high covariance since the tissue motion is similar in many pixels (Demené et al., 2015).

fUS response amplitudes were shown experimentally to be proportional to local dendritic calcium concentration and optically measured CBV signals in the mouse olfactory bulb (Aydin et al., 2020) in a co-registered single voxel brain volume. There, the hemodynamic response function was found to have a typical delay of 2 s (Aydin et al., 2020) and could be modeled as a modified (accounting for time shift) Gamma function.

fUS was directly compared to the BOLD response in the case of olfactory stimulus and determined to have a better sensitivity in the very same experimental configuration (Boido et al., 2019). Typical cortical responses in mice and rats exhibit an increase of 20% relative to baseline of the blood volume, a much higher increase compared to what is usually obtained in fMRI with a typical increase of a few percents.

Regarding the spatial resolution, at 15 MHz, an ultrasound frequency suitable for most preclinical application, Doppler imaging has a typical 100 μm spatial resolution combined with a 10–100 ms temporal resolution and a 1 mm s^{-1} blood flow sensitivity. If conversely to the spatial resolution of power Doppler, the spatial resolution of functional activation was not measured directly, experimental results on different paradigms permits to obtain a rough estimate of fUS imaging resolution. Notably, in awake ferrets with multifrequency auditory stimulation (Bimbard et al., 2018), the use of a classifier allowed to discriminate functional responses of nearby pixels separated by only 300 micrometers. In other words, neighboring brain voxels

separated with less than 300 μm in the auditory cortex and also in deeper regions such as the inferior colliculus were shown to be specialized in the identification of tones with different frequencies although they were extremely nearby. In awake non human primates, ocular dominance columns, structures of 400–700 μm width, could be clearly resolved in the activation images by [Blaize et al. \(2020\)](#). Recent work have investigated the use of even higher frequency from 30 MHz to 40 MHz in mice and could provide to push a step further the fUS imaging resolution ([Koekkoek et al., 2018](#)).

In the first clinical applications, where a 6–8 MHz was preferred to image deeper regions (transfontanellar imaging and peroperative imaging), the Doppler spatial resolution is typically around 200 μm .

APPLICATIONS

Highly sensitive functional mapping

fUS imaging has been used in numerous animal models and clinics for brain activity mapping using different stimuli. The most common one has been whisker stimulation ([Fig. 1C](#)) but other sensory paradigms have also been used. fUS was applied to the mapping of cerebral responses to an olfactory stimulation ([Boido et al., 2019; Osmanski et al., 2014a, 2014b](#)) with excellent sensitivity compared to fMRI: it showed that different odors were encoded as different activation spatial patterns in the main olfactory bulb, but that they elicited similar diffuse activation patterns in the piriform cortex (a cerebral structure that is difficult to reach with other imaging modalities), suggesting the lack of spatial encoding of the stimulus in this cortex compared to other primary somatosensory cortices such as primary visual or auditory cortex.

Using different frequency tones in awake ferrets, [Bimbard et al. \(2018\)](#) demonstrated that fUS could be used to construct 3D tonotopic maps of the primary and secondary auditory cortices ([Fig. 1D](#)) but also in very small deep seated structures not reachable by optical and fMRI techniques such as the inferior colliculus, the medial geniculate body or the 1 mm-long dorsal nucleus of the lateral lemniscus for which no tonotopic organization had been described previously. Furthermore, those data suggest that the high resolution of fUS signal could capture the layer specific responses of the auditory cortex, paving the way for a wide range of applications.

In the field of vision, [Gesnik et al](#) demonstrated the use of fUS to map the 3D main visual structures in the rat, a prelude to investigate visual restoration strategies such as retina implant ([Gesnik et al., 2017](#)). [Mace et al.](#) investigated the use of fUS in awake mice ([Macé et al., 2018](#)). [Rau et al](#) studied the response to visual stimulation in anesthetized pigeons ([Rau et al., 2018](#)), a proof of concept that could be used to further study the bird sensitivity to magnetic field which cannot be easily studied using fMRI. [Blaize et al. \(2020\)](#) demonstrated the use of fUS for retinotopic mapping and ocular dominance columns mapping in the visual cortex V1, V2 and V3 of awake primate using an imaging chamber.

[Song et al](#) used fUS to image spinal cord responses in rats to different electrical response patterns ([Song et al., 2019; Tang et al., 2020a, 2020b](#)). [Claron et al](#) further used fUS to study nociception in a rat pain model using fUS on the spinal cord in both normal and inflammatory scales, studying the response of different categories of afferent fibers (A β -, A δ -, C-fibers) to electrical stimulation and measuring their associated hemodynamic response function ([Claron et al, 2020](#)). Back to whisker stimulation, the functional response has been studied to investigate the role of circulating tPA in the neurovascular coupling using tPA-knocked out mice ([Anfray et al, 2020](#)).

Recently, acoustic biomolecules were also proposed for transcranial imaging and could pave the way for genetically targeted biomolecular imaging enabling exciting new molecular contrasts ([Shapiro et al., 2014; Bourdeau et al., 2018; Farhadi et al., 2019](#)).

A compatible technique for multimodal imaging

One exciting feature of fUS imaging is the high compatibility with other modalities commonly used by neuroscientists or neurologists. fUS imaging was used successfully in combination with electrophysiology in the rat using implanted electrodes during locomotion, natural seizures or even sleep phases ([Sieu et al., 2015; Bergel et al., 2018, 2020](#)). This combination enabled to map the correlation of the local field potential LFP envelopes with CBV in different bands in space and time, providing new information on the active neuron's population ([Sieu et al 2015](#)). fUS was also combined with an electroencephalography headset for neonates ([Demene et al., 2017](#)), enabling to discriminate sleep phases or to study epileptic seizures.

Other complimentary imaging techniques have been coupled with fUS such as PET and CT imaging ([Provost et al., 2018](#)), two-photon microscopy ([Rungta et al., 2017; Boido et al., 2019](#)) and optogenetic stimulation ([Rungta et al., 2017; Brunner et al., 2020](#)) or electrical stimulation ([Nayak et al., 2021](#)). In the latter study, the authors explored the feasibility of using functional ultrasound to visualize cerebral activation associated with thalamic electrical deep brain stimulation in the rat. The activation was found primarily in the primary motor cortex and varied greatly with the stimulus frequency. In those cases, the geometrical constraints of fitting both modalities together on the head is generally the only true limitation.

Studying fast and transient events

Functional ultrasound imaging has a combination of resolution and sensitivity that can also be helpful to seize information on fast or spontaneous phenomenon. One such example was illustrated in non-human awake and behaving primates in the context of complex visual tasks. During saccades and anti-saccades tasks, fUS synchronized with an eye tracker was able to detect robust activation in the supplementary eye field and anterior cingulate cortex ([Dizeux et al., 2019](#)) ([Fig. 1I](#)). Moreover, fUS sensitivity allowed to perform single-trial imaging, i.e., to detect the task right after the first trial.

This could thus drastically reduce the number of trials required to obtain a robust response compared to electrophysiology and fMRI. This could also allow to better study fatigue, motivation, volition, learning or other complex effects by assessing each trial response individually. On the same data and taking advantage of the high temporal sampling of fUS, it was also possible to measure sub-second delays between different activated structures using lag-correlation possibly further revealing valuable information on the sequence of activations.

Such study of lag-correlations could further be improved by using robust deconvolution approaches as proposed by Aydin et al who establishes experimental transfer functions between fUS and the underlying neural response in mice. These transfer functions approaches would need to be further studied in different physiological context and brain structures.

In another similar study on non-human primates, fUS provided insightful information about the planning of movement and its control (Norman et al., 2020) which allowed researchers to produce a first offline proof of concept of a brain-computer interface using functional ultrasound. The classifier, based on class-wise principal component analysis (CPCA) and linear discriminant analysis (LDA), used fUS data acquired on the posterior parietal cortex (PPC) through a minimally invasive cranial window and allowed offline decoding of the intended movement direction of the animals on a single-trial basis. The work could pave the way to future implanted brain-computer interface embedded units using for instance capacitive micromachined ultrasound sensors.

In several studies, fUS was also used to image spontaneous epileptiform crisis or induced spreading depression waves (Macé et al., 2011; Sieu et al., 2015; Demene et al., 2017; Rabut et al., 2019), which allowed to measure the spreading depression waves propagation speed and pinpoint its source location.

Stroke imaging

Being a blood flow imaging technique, fUS is extremely well suited to study cerebral perfusion and flow modification following an ischemic stroke or occlusion.

Martin et al. (2012) used fUS in a rat intraluminal middle cerebral artery occlusion model to detect longitudinal changes in tissue perfusion and elasticity in parallel with positron emission tomography (PET) and single photon emission computed tomography (SPECT). Using the same model, Brunner et al. (2018) mapped the reperfusion and investigated the slopes of the fUS signal time courses for the occluded-side and non-occluded side in somatosensory and motor structures.

It also has been demonstrated recently in a mouse model of thromboembolic stroke with an intact skull, that fUS was able to monitor the reperfusion following thrombolytic within the first 2 h post stroke onset (Hingot et al, 2020). The obtained data matches ischemic lesions measured 24 h after using MRI. In that way, fUS can be used to monitor in real-time the extent of a stroke and the effect of pharmacological stroke therapy such as recombinant tissue plasminogen activator (rtPA) at different time points. By imaging lesions early on, fUS allows to

understand better the dynamic of those therapeutic agents and to better understand the diversity of lesions that can be observed in MRI for the same model.

Functional connectivity

Imaging intrinsic brain connectivity can be a powerful non-invasive approach to map the dynamic networks in the brain. This can be done by studying correlations of activity between all brain structures during rest periods. This resting-state connectivity can serve as a robust readout of the brain state. Moreover, there exists a vast literature on the alteration of resting-state functional connectivity based on fMRI studies linked to several brain diseases in patients, including but not limited to Alzheimer disease, Autism, Depression, Bipolar disorder, Parkinson or Pain.

Functional ultrasound was first applied with success to functional connectivity mapping in the rat brain (Osmanski et al., 2014a, 2014b) allowing the creation of connectivity matrices by estimating the CBV correlation between different pairs of regions of interest (Fig. 1E). It is also possible to generate seed-based maps by correlating the CBV of a structure to other pixels. Finally, one can also use unsupervised approaches such as global spatial modes or independent component analysis (ICA) to untangle the different brain resting state networks with high spatial resolution.

Recently, fUS provided the first evidence for task-induced deactivation and disconnection of what appears to be a default mode network (DMN) in the awake mouse (Ferrier et al., 2020).

Functional ultrasound has been used to study brain connectome alterations in many configurations for example it showed in rat pups that a fetal growth restriction model presented a general decrease of connectivity compared to controls and that early injection of oxytocin could mitigate this effect (Novais et al., 2016; Mairesse et al., 2019) or that the presence of chronic pain could be identified based on the brain networks identified in fUS in an arthritic rat model compared to controls (Rahal et al., 2020).

Functional connectivity mapping using functional Ultrasound is a particularly well-suited readout for pharmacological studies and drug discovery (Rabut et al., 2020; Vidal et al., 2020a, 2020b) including in awake mice without anesthesia bias. Together with the use of unsupervised classifiers for fingerprinting of connectivity matrices, it was demonstrated to be able to robustly quantify the effect of scopolamine, an anticholinergic agent that bounds to muscarinic receptor and previously used as Alzheimer model, on the brain networks and its reversal through its antagonist milameline.

Anesthetized or awake imaging

Another feature of fUS compared to fMRI is its ability to perform deep brain imaging in behaving subjects (Sieu et al., 2015). This is possible thanks to the miniaturization of ultrasonic probes which can now be mounted on the animal head using an implanted frame (Fig. 1F). fUS could provide whole brain imaging in behaving rodents

during absence seizures (Sieu et al., 2015), during locomotion (Fig. 1G) or operant tasks (Sieu et al., 2015; Urban et al., 2015; Bergel et al., 2020) and even during different sleep phases (Fig. 1H) (Bergel et al., 2018). In this last study by Bergel et al., fUS has shown that REMs phases were characterized by global hyperemic events that were previously unknown and are not yet understood.

The technique can be used either in freely moving specimens such rats or mice (Sieu et al., 2015; Urban et al., 2015, Bergel et al. 2018, Rabut et al 2020) or in head-fixed setups use for example for mice, ferret and even primate (Macé et al., 2018, Bimbard et al., 2018, Ferrier et al., 2020, Dizeux et al. 2019, Blaize et al. 2020, Brunner et al. 2020) where it is possible to use a motorized stage or array for 3D imaging.

Awake imaging is important to study behaving animals during their interactions with their environment or with other animals but also simply to avoid the confounds of anesthesia, for instance for pharmacological studies. In that regards, fUS can be used to assess functional connectivity in awake mice (Ferrier et al., 2020; Rabut et al., 2020) and to study the effects of different compounds on the brain, creating a platform for unbiased drug screening in different mice models. We expect that such platform for assessing functional connectivity in awake mice will become a standard tool in the coming years for research on new compounds in addition to the creation of large online database.

Another aspect of active research is the use of functional ultrasound imaging for behavioral studies which are more complex to perform due to the tradeoffs between experimental setup constraints and natural behavior of the animal. In that regard, by allowing hours of unsupervised acquisitions, functional ultrasound imaging might help to gather enough data to robustly match behavior traits to functional activation. Such analysis will require to monitor every aspects of the experiment and retrospectively classify and associate events to functional responses.

In humans, the portability of fUS makes it a well-suited modality for monitoring neonates in a bedside configuration or for the development of minimally invasive Brain Machine Interfaces if the ultrasonic transducer is implanted within the skull bone to image through the intact dura as recently demonstrated for decoding of movement intentions in the posterior parietal cortex of macaques (Norman et al., 2020). Obviously, the development of real brain-computer interfaces will require much more miniaturization effort to become truly portable but this represents an exciting proof of concept.

fUS clinical imaging in humans

In humans, fUS has been applied in awake patients in scenarios where the skull is absent, including functional cortical mapping during intra-operative craniotomy procedures (Fig. 2A) (Imbault et al., 2017; Soloukey et al., 2020). Obtaining functional measurements during the surgical procedure is of highest interest since any functional mapping (e.g. via fMRI) done prior to the surgery will be distorted by the brain shift at skull opening.

Classically those measurements are obtained via electrical surface measurements, and in those two papers, mapping precisely in depth sensitive or motor brain areas helped for the resection of the deep parts of the tumor. In Soloukey et al., fUS was also used to detect brain areas involved in lip pouting, finger tapping (Fig. 2A) and language processing allowing to map selective areas for spoken and unspoken words.

Finally, fUS was demonstrated in neonates through the anterior fontanelle window. The technique was able to discriminate between sleep phases (using EEG measurements as a control) and to locate ictal brain activity during seizures in an epileptic neonate (Demene et al., 2017), revealing previously undescribed slow waves of activity in the cortex during post-ictal bursts of electrical activity. Recently, the first demonstration of fUS resting state connectivity evaluation in human was performed in preterm babies (Baranger et al., 2021) (Fig. 2B). It showed that the peculiar spatio-temporal resolution of fUS enabled new connectivity assessments such as homotopic connectivity at a 200 μm resolution (Fig. 2C) or dynamic connectivity evaluation showing switching between brain states at a few seconds resolution. Occurrence of brain states of the preterm neonates were found significantly different from the ones of term babies. In particular, thalamocortical connectivity was found to be increased in term babies. Beyond this proof of concept, the approach could be a powerful tool for bedside assessment of brain networks maturity and possibly as a biomarker for neurodevelopmental disorders and autism spectrum disorders.

It is expected that those two clinical applications (peroperative brain imaging and neonate brain imaging) will see the most straightforward clinical investigations while the technology improves toward transcranial applications.

CURRENT CHALLENGES AND PERSPECTIVES

Functional ultrasound imaging was introduced ten years ago and has progressed at a steady pace. In those years, the technique has gained in maturity and was demonstrated in many experimental paradigms. Commercial fUS systems are now on the market to improve the technique dissemination towards neuroscientists.

Despite this, several inherent limitations are still being worked on by researchers and will remain important challenges for years to come. The first limitation is the difficulty to produce volumetric functional imaging with good quality and sensitivity due to technological limitations of the current state of the art of matrix arrays. The reduction in size of the piezoelectric elements when moving from a 1D array to a 2D array imposes an important reduction of the sensitivity of the imaging. Another challenge is raised by the skull which drastically limit the applicability of functional ultrasound. It imposes distortion to the ultrasound wavefronts, but also very important absorption, especially at mid to high frequencies. Other applications might require to improve either the spatial resolution or the temporal resolution,

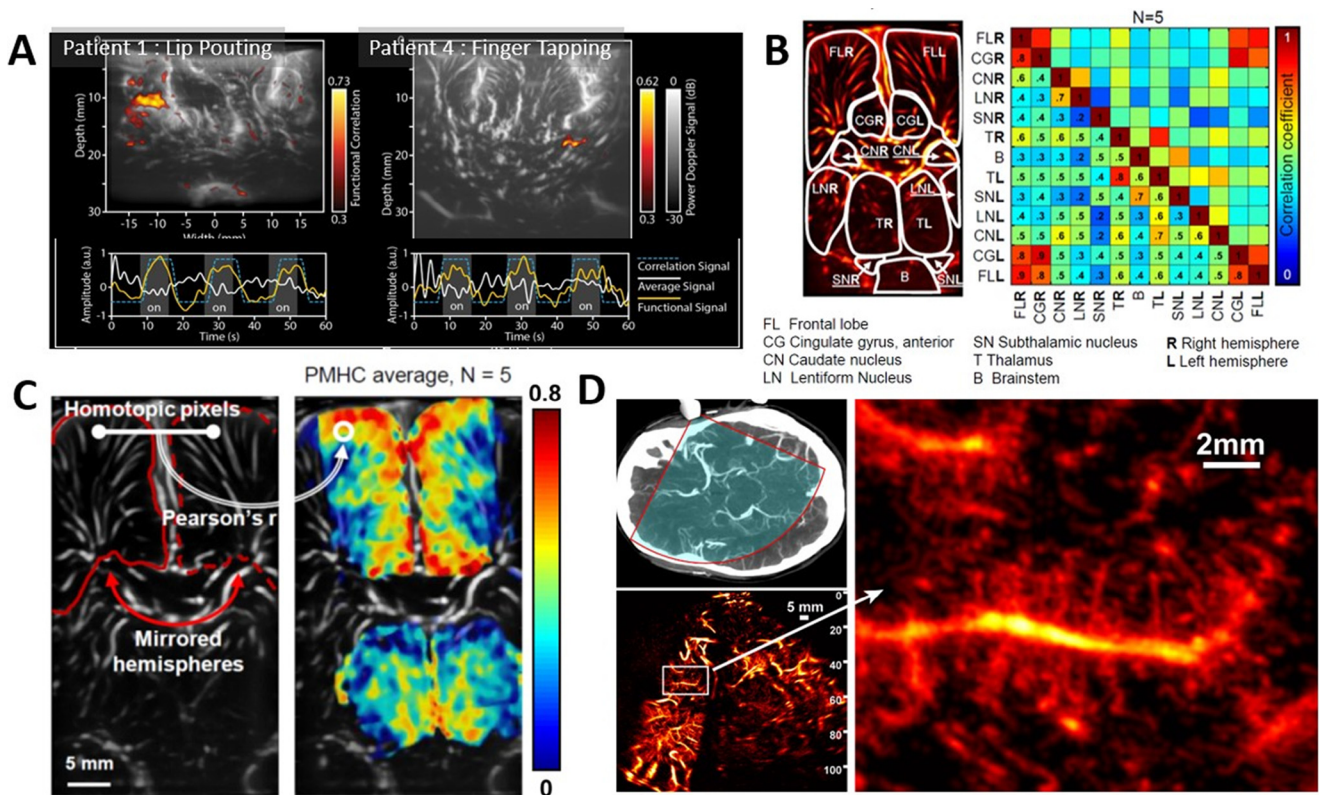


Fig. 2. (A) Functional ultrasound imaging in patient during peroperative imaging before tumor resection (with craniotomy). Activation maps and CBV response corresponding to lip pouting and finger tapping (Soloukey et al., 2020). (B) Noninvasive brain connectivity matrix in a neonate using the transformantellar window showing high intra-cortical and thalamo-cortical connectivity (Baranger et al., 2021). (C) Homotopic connectivity: fUS signal in one voxel is correlated to the fUS signal in the mirror pixel in contralateral, and the value of correlation is mapped on the whole image. It detects a higher connectivity in the grey matter compared to the white matter in the cortex, and interesting heterogeneities in the thalamus. (D) Transtemporal ultrasound localization microscopy (ULM) in human adults showing micro vessels down to the $< 100 \mu\text{m}$ scale (left top: CT imaging, left bottom, ULM image, right: ULM detail). (from Demene et al., 2021).

as for instance for research on low-latency brain-computer interface or cortical layers imaging. The next part addresses some of these challenges and on ongoing research.

Initially limited to the acquisition of a single plane, fUS was extended to 3D imaging (Demene et al., 2016; Gesnik et al., 2017; Bimbard et al., 2018; Macé et al., 2018; Rau et al., 2018). By mechanically translating the transducer, fUS can provide whole-brain functional activity mapping at high sensitivity and resolution albeit increasing significantly the acquisition time. This simple but efficient approach was further optimized using fast scans approach to allow simultaneous slices acquisitions for both functional imaging and functional connectivity. Fast scans allow to monitor hundreds of regions of interests within up to four distinct brain areas and planes.

To acquire full 3D + t volumetric fUS, one must change the probe technology from linear to matrix arrays. The use of 2D matrix arrays (32×32 elements) for fUS was demonstrated for the first time using a custom 1024 electronic channels scanner (Rabut et al., 2019). The authors implemented multi-plane wave pulse sequences to balance the lower sensitivity of matrix arrays (Fig. 3B) and demonstrated brain activity mapping in response to visual and whisker stimulations, whole-brain functional connectivity (Fig. 3A) and epileptiform cri-

sis tracking on a rat model (Fig. 3C). This concept was also investigated for less-demanding research systems (using typically 256 channels) by proposing the use of sparse matrix technologies such as Row-Column arrays (RCA) (Sauvage et al., 2020) (Fig. 3B, D) or by using multiplexing systems with 2D matrix arrays (Brunner et al., 2020) which essentially acquire the volume subapertures by subapertures. Matrix or RCA probes are generally too large to be used on freely-moving animals but can be used in head-fixed configuration.

While there is little doubt that 3D + t volumetric fUS will be essential in the future, finding the best way to perform whole-brain imaging is still an active field of research and *in fine* highly dependent on the experimental paradigm and scientific questions asked. Acquisition time, volume rate, field of view, image resolution and most importantly sensitivity must be balanced to answer the scientific questions the most efficiently.

On a longer time scale, it is expected that disruptive technology such as on-chip transducers (CMUT) could pave the way to embedded fUS acquisition module which would be helpful for both preclinical applications in awake animals and for brain-computer interfaces.

Despite its rapid development in preclinical applications and some clinical applications such as

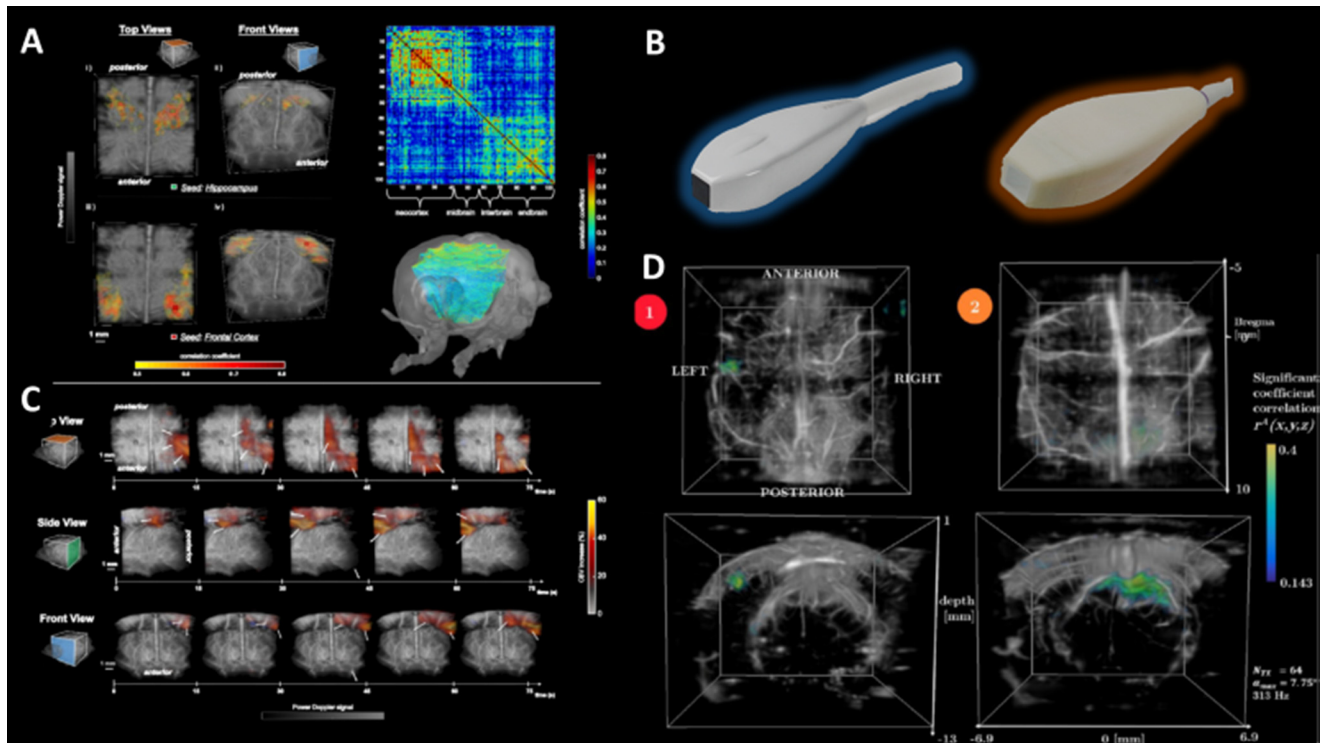


Fig. 3. (A) Matrix-array based seed-based functional connectivity imaging (seeds in the hippocampus and frontal cortex), associated connectivity matrix and representations of connections between brain structures (Rabut et al., 2019). (B) Matrix array (1024 channels) and Row-Column array (256 channels) probes. (C) Matrix-array based imaging of an epileptiform crisis in a rat model. (D) Row-Column array based volumetric imaging of functional brain activations linked to whisker (D.1) and visual (D.2) stimuli (Sauvage et al. 2020).

neonates and peroperative imaging, the human skull remains a major for its broader application in clinics. The strong attenuation and diffraction effects induced by the skull bone highly disturb the ultrasound propagation (Pinton et al., 2012).

In mice and young rats, the skull thickness allows transcranial fUS imaging through the intact skull (Tiran et al., 2017) and transcranial fUS imaging in mice is now widely used for pharmacological studies (Rabut et al., 2020). In chronic rat models or larger animals, one has to use thinned-skull procedures (Osmanski et al., 2014a, 2014b; Urban et al., 2014) or the implant an acoustically transparent cranial windows (Sieu et al., 2015; Urban et al., 2015; Bergel et al., 2018) to image the brain, this procedure can also be performed in chronic mice to further improve imaging quality (Rungta et al., 2017; Macé et al., 2018; Tang et al., 2020a, 2020b). In non-human primates the thickness of the skull imposes heavier surgical procedures, and fUS has been performed in awake animals by placing the ultrasonic probe within the electrophysiology chamber (Dizeux et al., 2019; Blaize et al., 2020; Norman et al., 2020).

The use of microbubble contrast agents and nanometric gas vesicles was also proposed to enhance the fUS signal and reach non-invasive fUS in rats (Errico et al., 2016; Maresca et al., 2020). In human adults, such a proof of concept of transcranial functional ultrasound has not been done yet, but ultrafast Imaging through the temporal window in the presence of contrast agents has been validated, showing the possibility to perform ultrasound localisation microscopy (ULM).

Ultrafast ultrasound combined with acoustic contrast agents intravenous injection can also be used to perform Ultrasound Localization Microscopy (ULM) using the same device. ULM is the analogue ultrasound of super-resolution imaging approach in optics such as fPALM. In ULM, the acoustic signature of individual microbubbles travelling within the cerebral vasculature are localized at ultrafast frame rates (Couture et al., 2011). ULM provides quantitative angiographic imaging of the deep cerebral vasculature down to microscopic resolution (Errico et al., 2015; Couture et al., 2018; Christensen-Jeffries et al., 2020). The technique can be used to compute quantitative velocity maps of the microvasculature tree in the brain and was recently extended to 3D in the rat brain. Non-invasive transcranial ULM was recently demonstrated clinically in human adults and led to human brain angiographic imaging with a very high sensitivity and a 25 μm -resolution (Fig. 2D), outperforming any clinically available angiographic modality (Demene et al., 2021). Beside vascular imaging, it is expected that ULM could allow one day to perform functional mapping of brain activity at high spatial resolution albeit with a much lower temporal resolution due to the need to accumulate enough microbubbles in each pixel.

New approaches are also being investigated to improve the temporal resolution and latency of functional ultrasound imaging. Researchers have used machine learning in a technique called deep-fUS to reduce the number of ultrafast frames necessary to produce a single Doppler image, allowing to reduce the needed images down to 5% of the usual number of

frames needed (Di Ianni and Airan, 2020). Further work is needed to better understand the tradeoffs and implicit assumptions of those approaches. Finally, other authors are also exploring the use of other imaging contrast than power Doppler such as flow velocity which could provide quantitative measurements (Tang et al., 2020a, 2020b).

Functional ultrasound still reads brain activity through the prism of the neurovascular coupling and as such remains limited by its space and time scale but reaching new resolutions might allow researchers to get more insight in brain mechanisms while better understanding the characteristics of the spatial and temporal transfer functions associated with the neurovascular coupling.

fUS imaging is facing today a wide range of perspectives in neuroscience. First, its ability to study brain connectomics in animal models during pharmacological studies with a much simpler approach than fMRI makes it a potential game changer for future drug discovery. The possibility to identify relevant pathology biomarkers from the functional connectivity matrix will both help to stage disease evolution but even more importantly to monitor the efficacy of drug treatment. This identification in animal models could be also used for clinical translation as fUS imaging is already ready for clinics in niche applications such as neonate imaging (Demené et al., 2019). The extension to adult human imaging using contrast agents makes it also an interesting modality for translational research. Secondly, fUS imaging in awake and freely moving animals is envisioned to provide a great tool connecting behavioral studies with neuroimaging. The identification and deciphering of brain networks interactions involved in a wide range of behaviors ranging from spatial navigation to social interaction becomes possible with a moderate cost technology and could fast spread in neuroscience research labs. It will certainly even be accelerated by the use of machine learning approaches applied to very long sessions of behavior monitoring. Thirdly, although fUS imaging is at a early stage of proof of concepts for brain machine interfaces, a domain hampered by requested number of implantable electrodes, its ability to image functional activations without penetrating the dura mater with a very large number of recording sites even in deep brain regions could pave the way to new generation fUS-guided brain machine interfaces. The development of ultrasonic matrix arrays implantable in the skull bone of patients could be used in the future to decode gesture early intention and trajectory, for wearable rehabilitation robots such as arm prosthetics. It could also detect adverse events such as seizures in epileptic patients. Finally, fUS imaging is particularly suited for whole brain neuroimaging during neurodevelopment and in the early months after birth. Such neuroimaging in term and pre-term neonates is today really hampered by the complexity of functional exams using fMRI. fUS could fill a gap in functional brain imaging at this stage of development which dramatically lack techniques for the prognosis and monitoring of abnormal neurodevelopmental trajectories.

Our understanding of the brain complex organization and interaction have been pushed forward by advances in neuroimaging. In the past decades, the advent of

ultrafast ultrasound has allowed ultrasound imaging to break key barriers in both sensitivity and spatiotemporal resolution, a breakthrough that has changed ultrasound into a full-fledge neuroimaging modality both for functional and vascular information. Functional ultrasound imaging, the ultrasound analogue of fMRI, enables to image whole-brain brain activity with high spatio-temporal resolution and high sensitivity. It has been used in numerous animal models from mice to primate under diverse experimental setups, and it can easily be combined with other neuroscience techniques such as EEG or optogenetics. Moreover, fUS can easily be adapted for awake head-fixed or freely-moving animals and is suitable for pharmacological studies using functional connectivity as a readout. Despite, the issue of the skull barrier which is not transparent to ultrasound of large animals, fUS has been applied in clinics in specific context: in adults during per-operative surgery for cortical mapping and in neonates, through the transfontanelle window, for seizure monitoring or brain functional connectivity assessment. On the technological side, fUS has gained in maturity and applicability in the past years but several limitations remain challenging such as volumetric imaging and transcranial imaging. Recently, new technological developments have allowed the first proof of concepts of 4D functional ultrasound imaging allowing the mapping of brain activity and functional connectivity at a whole-brain scale, although the sensitivity of such approach is still not as high as for 2D imaging. Using contrast agents may also help with imaging through the skull which would allow exciting new clinical applications in adults. Functional ultrasound is a portable and affordable technique that is uniquely positioned between fMRI and optical techniques to study the brain. On the application side, fUS imaging has already emerged as an interesting tool for a wide range of topics, including vision, pain, decision, volition, social interactions, sleep, spatial navigation and pathologies such as epilepsy, stroke, alzheimer's disease, multiple sclerosis, autism, neurodevelopmental diseases, and this application spectrum continues to grow rapidly.

ACKNOWLEDGMENTS

TD and MT are cofounders and shareholders of Iconeus Company which commercializes functional ultrasound systems for preclinical research. TD, CD and MT are co-inventors of several patents in the field of functional ultrasound imaging.

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(Received 23 January 2021, Accepted 2 March 2021)
(Available online 13 March 2021)