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REVIEW

Optical Brain Imaging: A Powerful Tool for Neuroscience

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Abstract As the control center of organisms, the brain remains little understood due to its complexity. Taking advantage of imaging methods, scientists have found an accessible approach to unraveling the mystery of neuroscience. Among these methods, optical imaging techniques are widely used due to their high molecular specificity and single-molecule sensitivity. Here, we overview several optical imaging techniques in neuroscience of recent years, including brain clearing, the micro-optical sectioning tomography system, and deep tissue imaging.

Keywords Brain imaging · Tissue clearing · MOST · Optical microscopy · Deep tissue imaging

Introduction

The brain is a complex organ composed of neurons, glia, microglia, and vascular tissues. By exploring brain structure and function, scientists can not only understand the mechanisms of emotion, sleep, and cognition, but also bring relief to patients who suffer from nervous system diseases, such as depression, autism, Alzheimer's disease, and Parkinson's disease. Observation is the most straightforward way to understanding. Thus, brain imaging has turned out to be an effective technique to carve a path for neuroscience. Compared with general methods such as the molecular biological techniques, brain imaging has the advantage that it presents the real physical features and functions (*in vivo* imaging) of neurons.

Brain imaging has developed over centuries. At the turn of the last century, Camillo Golgi and Santiago Ramón y Cajal did the pioneering work of neuronal tracing using light microscopy [1, 2]. Subsequently, scientists opened the gate of this newborn field [3]. In the last decades, many techniques have been developed, including patch-clamp recording [4], electroencephalography [5], magnetic resonance imaging [6], positron emission tomography [7], and optical imaging, the latter being the main concern of this review.

Optical methods such as confocal [8] and two-photon microscopy [9, 10] have been widely used in brain imaging for a long time. Svoboda *et al*. [11] achieved *in vivo* brain imaging with two-photon excitation microscopy in the open-skull mouse [12–17]. However, the surgery probably induced inflammatory reactions [16, 18], resulting in impaired image quality. What is more, the axons and dendrites of neurons extend in many directions, some interneurons crossing several millimeters through a large volume of brain tissue. However, brain tissue is a strong scattering medium, which makes it difficult to focus the excitation light on a small target point and detect the emitted signal. Therefore, to reconstruct the three-dimensional (3D) morphology of neurons, deep tissue imaging techniques are needed.

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Here, we review the state-of-the-art optical techniques used to achieve deep brain imaging. First, brain-clearing treatments such as CLARITY [19] and CUBIC are presented [20]. Then, we introduce micro-optical sectioning tomography (MOST) [21], which can achieve whole-brain imaging at submicron resolution. Finally, we discuss the *in vivo* deep tissue optical imaging methods developed recently.

Brain Clearing

Brain-clearing techniques make the whole brain transparent by dramatically reducing light scattering and improving imaging depth.

From the perspective of optics, the brain remains opaque because of the mismatching of the refractive index. The lipids widely distributed throughout the brain cause the inhomogeneity of scattering. Therefore, removing the lipids, or replacing them with other molecules can effectively rematch the refractive index. Based on this, there are several promising methods, including Scale [22], BABB [23], SeeDB [24], Clear (T) or Clear (T2) [25], and 3DISCO [26–28], which are compared in Table 1.

CLARITY, first reported in 2013 by Chung *et al*. [19], was a revolutionary method to transform the brain into a transparent tissue. It removes lipids by electrophoresis, and is suitable for light-sheet fluorescence microscopy [19, 20] to achieve whole-brain imaging [34]. CLARITY first uses a hydrogel as a protein preserver by infusing the brain with acrylamide and bisacrylamide together with formaldehyde and thermally triggers initiators at 4 °C [19, 37]. The formaldehyde in the mixed reagent helps to crosslink the brain constituents as well as establishing covalent bonds between the hydrogel monomers and biomolecules [19]. After infusion, the treated brain is incubated at 37 °C for 3 h to make a hybrid construct of gel and brain [19]. Then, aiming at efficient clearing, Chung and colleagues chose an ionic extraction technique (electrophoretic tissue clearing) instead of hydrophobic organic solubilization [19]. However, this improvement resulted in other problems, such as the difficulty of selecting the optimal parameters for different samples [20], and the electrophoretic tissue clearing procedure may induce tissue degradation when heated [36]. The principle of CLARITY is illustrated in Fig. 1A. Using CLARITY, the imaging depth with traditional optical microscopes reaches the micron level [19]. Nonetheless, there is no doubt that CLARITY challenges common views. Using CLARITY, labeling specific cells in the brain reversibly is never a problem [37], and researchers can view to depths that are only limited by the working distance of the microscope objective [34]. What is more, there is less loss of proteins, fluorescence signal, and structure of axons and dendrites when using CLARITY compared with conventional methods [19, 34]. Besides, Chung *et al*., using postmortem human brains as samples, demonstrated that CLARITY is also suitable for the human brain [19].

Since CLARITY was published, a growing number of researchers have devoted themselves to improving this method [32, 33, 35]. In April 2014, Tomer *et al*. described an advanced protocol based on CLARITY to achieve simpler lipid removal [35]. The revised protocol allows deep imaging of clarified brains, taking advantage of advanced confocal microscopes and light-sheet microscopes (CLARITY optimized light-sheet microscopy) [35]. Besides, the time required is reduced and the imaging depth is extended to millimeters (a maximum of 5.78 mm) [35]. Poguzhelskaya *et al*. have also slightly amended the original CLARITY protocol, naming it CLARITY2 [33]. They inserted a cutting step after hydrogel fixation to improve the clearing efficiency [33]. However, the depth of imaging was not increased. What is worse, the cutting procedure may damage samples and thereby influence the 3D reconstruction. At the end of 2014, Lee *et al*. revised this protocol, making it suitable for many organs [32]. Although CLARITY has been improved, many deficiencies still need to be addressed.

Apart from CLARITY, Susaki *et al*. invented a protocol of whole-brain imaging using chemical cocktails named CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) [20] and its principle is shown in Fig. 1B. This protocol can be combined with immunofluorescence, and its scale ranges from the whole brain to subcellular structures. The most groundbreaking feature is that CUBIC also allows profiling of the time-course of expression [20]. Researchers first comprehensively screened the CUBIC reagents, resulting in the optimal reagents and procedure. In this procedure, it was confirmed that CUBIC could be completed within 2 weeks and with the preservation of fluorescence [20]. The depth of the immunostained signal that can be detected is > 750 μm [20]. Later, they extended CUBIC to whole-body imaging in both infant and adult mice. As a result, CUBIC is suitable for whole-organ imaging and the resolution can reach the single-cell level. More interestingly, taking diabetic pancreas samples, they found that CUBIC is able to reconstruct the 3D pathology of islets of Langerhans [31].

Other brain-clearing protocols include the passive clarity technique (PACT), refractive index matching solution (RIMS), and perfusion-assisted agent release *in situ* (PARS) [36]. The PACT reagents are applicable to transgenic tissue with fluorescent protein, and enhance the signal-to-noise ratio. RIMS is used to match a suitable imaging refractive index, while keeping the cleared tissues for a much longer time. PARS has been applied to whole organs, and the imaging depth can be extended to

Table 1 Pros and cons of major brain clearing methods.

Techniques	Brief	Clearing Time	PROS	CONS	References
BABB	Clearing reagent containing several organic solvents	Days	First tissue clearing method	Severe fluorescence quenching; tissue shrinkage; toxicity	Becker *et al.* [23]
3DISCO	BABB-based reagent improvement	Days	Strong clearing capability	Fluorescence quenching; tissue shrinkage	Erturk *et al.* [26–28]
SeeDB	Simple immersion method to rematch RI with fructose	Days	Fluorescence and neuron morphology preserved	Incomplete clearing; no immunostaining	Ke *et al.* [24, 29]
FRUIT	SeeDB method improved by adding urea	Days	Better clearing than SeeDB	Minimal tissue expansion	Hou *et al.* [30]
Clear (T)/ Clear (T2)	SeeDB method improved by replacing fructose with formamide	Hours-days	Less time-consuming than SeeDB	Incomplete clearing; no immunostaining	Kuwajima *et al.* [25]
Scale	Hyperhydration method by denaturing protein with urea	Weeks	Strong clearing; fluorescence preserved	Severe tissue swelling; no immunostaining	Hama *et al.* [22]
CUBIC	Scale method improved with additional RI rematch	Weeks	Less expansion than Scale; immunostaining available	Protein loss during clearing	Susaki *et al.* [20], Tainaka *et al.* [31]
CLARITY [19, 32–35]	Hydrogel embedding method with SDS clearing	One week	Strong clearing; neuron morphology preserved	Electrophoresis equipment required	Chung *et al.* [19], Lee *et al.* [32], Poguzhelskaya *et al.* [33], Pointer *et al.* [34], Tomer *et al.* [35]
PACT, RIMS, and PARS	Improved CLARITY methods	Days	Less time-consuming than CLARITY	Tissue structure damage; partial fluorescence quenching	Yang *et al.* [36]

BABB, SeeDB, Clear (T), Clear (T2), Scale, 3DISCO, and CLARITY, names of the methods.

CUBIC clear, unobstructed brain imaging cocktails and computational analysis, *PACT* passive clarity technique, *PARS* perfusion-assisted agent release *in situ*, *RIMS* refractive index matching solution.

the millimeter level (maximum depth up to 6 mm in whole-brain imaging). In conclusion, using PACT, RIMS, and PARS, the whole brain can be imaged with single-cell resolution [36].

Brain-clearing techniques have flourished in recent years but still leave much room for improvement, such as the tissue deformation, long specimen treatment times, and the reagent toxicity. But these methods represent a milestone in neuroimaging and provide an effective method for the diagnostic analysis of several certain intractable brain diseases such as the 3D visualization of Aβ plaques and microglia in AD human clinic samples [38].

Micro-Optical Sectioning Tomography

Using conventional optical microscopy to examine a whole brain is challenging. Inspired by brain clearing and tissue sectioning, the micro-optical sectioning tomography (MOST) system was developed to achieve whole mouse-brain imaging at the micron level [21]. With a microtome, optical microscope, and data-processing unit, MOST can simultaneously section and image specimens, collecting ribbons from the microtome, and ultimately reconstructing 3D images of the whole brain. A schematic of this system is shown in Fig. 2 [21, 39, 40]. The resulting images reach mesoscopic resolution, and it is even able to resolve the neurites of neurons [21]. However, the raw images from this system are 8-bit grayscale and need subsequent pseudo-color to help visualization, which limits its visual effectiveness. What is more, it takes a long time (>10 days) to image a centimeter-sized whole mouse brain, collecting more than 15,000 coronal sections to generate a file of 8 terabytes [21]. These workers also indicated that a specific sectioning mode would be helpful for maintaining the accuracy and integrity of brain atlases [41]. Advanced specimen preparation processes, such as fluorescence MOST [21] using transgenic EGFP [42, 43] and EYFP [44, 45] would help in the labeling of specimens. The introduction of confocal techniques and acoustical optical

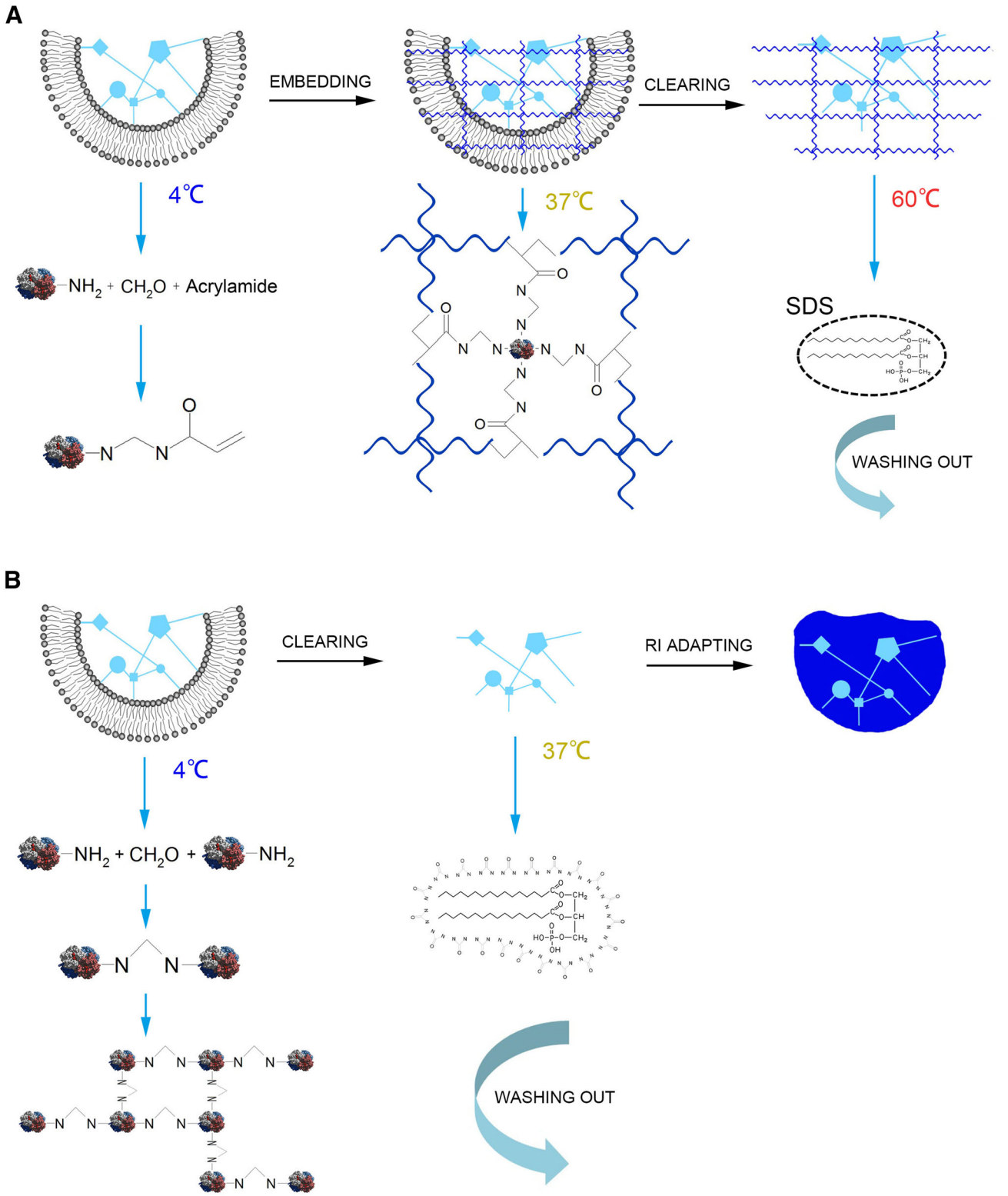

Fig. 1 Principles of CLARITY and CUBIC. **A** Principle of CLARITY. The amino groups from brain tissue protein covalently combine with formaldehyde and acrylamides at 4 °C. Then, the whole brain tissue is embedded in acrylamide gel so the residual acrylamide-connected proteins bind tightly to the framework of the gel at 37 °C. Finally, lipids are completely washed out with sodium dodecyl sulfate (SDS) at 60 °C. **B** Principle of CUBIC. The amino groups in brain tissue protein covalently combine with each other via PFA. Then the lipids are completely washed out with CUBIC Reagent 1.

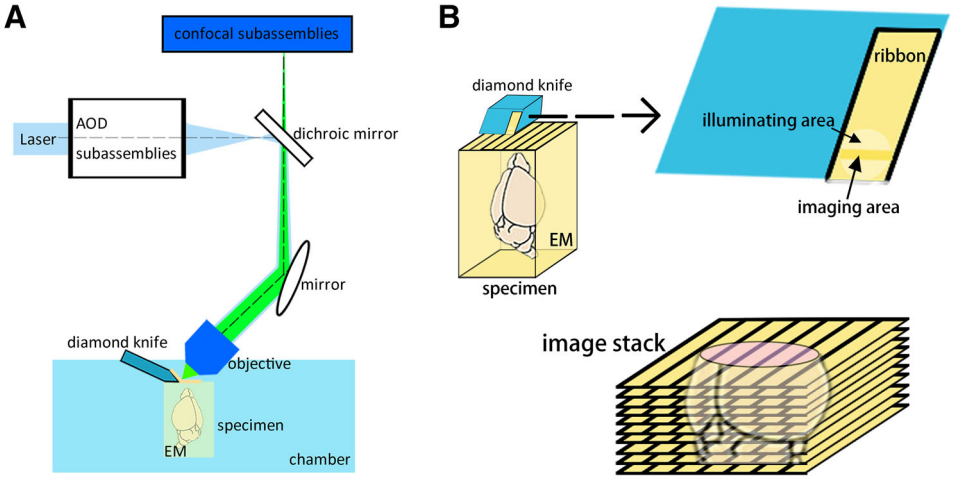

Fig. 2 Schematic of the fluorescence MOST system [21, 39, 40]. **A** The system usually consists of a confocal microscope, an acoustical optical deflector scanner, and a microtome with a diamond knife and a moveable chamber. The motion of the chamber matches spatial coordinate axes. The specimen is embedded in embedding materials (EM) and mounted in the chambe. **B** Using the diamond knife, the system generates a series of coronal sample ribbons, and images each small horizontal stripe simultaneously. Finally, an image stack is collected, and maintains almost all important regions and fine structures of the brain. 3D reconstruction can then be carried out.

deflectors (Fig. 2) are able to rescue some of the information loss during sectioning and imaging [46], and help to achieve a higher resolution as well as a faster scan imaging [39, 45]. Furthermore, to maintain the fluorescence signals, different embedding approaches during specimen preparation also have diverse effects on both the processes and results of the MOST system; these include glycol methacrylate, Unicryl, and LR White, among which modified glycol methacrylate is relatively more suitable [44]. In addition, the MOST system can provide direct 3D brain reconstruction, showing both individual cells and vessels including capillaries, at a resolution of 1-μm voxels [47–49].

In conclusion, the MOST system provides a new path to imaging the whole brain with high resolution and accuracy. However, its long imaging acquisition time, requirement for large storage space, and sectioning methods still leave many challenges.

Deep Tissue Imaging

Imaging deep inside tissue has been a major challenge with the high scattering properties of brain. Both brain clearing and the MOST system aim to bypass the problem of light scattering by using sample treatments of transparent process and muti-sections. So they cannot be applied *in vivo* and are thus unable to exploit neuronal function in the brain. Furthermore, the sample processing also generates artifacts after slicing or chemical washing. Therefore, scientists have endeavored to develop optical microscopy to break through the depth limit for *in vivo* imaging [50]. These efforts can be broadly categorized into two groups: the first reduces the light scattering, whereas the second makes use of the scattered light.

Since ultrasonic scattering is 2–3 orders weaker than optical scattering in the brain, ultrasonic imaging can greatly reduce the scattered light and thus provide better penetration depth. Wang *et al*. developed functional photoacoustic microscopy, which provides multi-wavelength imaging of optical absorption. The imaging depth is >1 mm below the sample surface and the ratio of maximum imaging depth to depth resolution exceeds 100 [51].

Our group reported one-photon optical microscopy for high-resolution molecular imaging in thick biological tissue, called focal modulation microscopy. It uses a focal modulation technique to suppress the out-of-focus fluorescence signal excited by scattered light. We obtained *in vivo* one-photon fluorescence imaging with diffraction-limited resolution up to 600 μm deep inside highly-scattering media [52, 53]. When this technique is combined with two-photon microscopy, the imaging depth can be further improved [54].

Longer wavelengths undergo less scattering, so they can penetrate deeper inside the brain. By using a fluorescent probe in the 1.3–1.4 μm near-infrared window, Hong *et al*. reported *in vivo* imaging to a depth of >2 mm in mouse brain with sub-10-μm resolution [55]. Xu *et al*. have made an exceptional contribution to multi-photon microscopy. They first improved two-photon microscopy and achieved a 1-mm imaging depth in the adult mouse brain *in vivo* with

an excitation wavelength of 1280 nm [56]. Later, they further extended the excitation wavelength to 1700 nm with three-photon microscopy to image fluorescence-labeled vascular structures and neurons within the hippocampus *in vivo* [57].

Instead of reducing the light scattering, scientists have also made efforts to use the scattered light. Optical phase conjugation can focus light through millimeter-thick strongly-scattering media by modifying and optimizing the wave-front of the input light field [58]. Our group combined digital optical phase conjugation with ultrasound, and achieved 3D fluorescence imaging with ~38 μm spatial resolution up to 2 mm deep inside brain slices [59]. Later, the resolution was further improved to 10 μm [60].

Another effective method of using scattered light is to measure and correct wave-front aberrations through adaptive optics. Ji *et al*. proposed a method of adaptive optics based on pupil segmentation and achieved high-resolution two-photon images at 400 μm deep inside mouse brain [61, 62]. Wang *et al*. demonstrated the adaptive correction of complex optical aberrations at a high numerical aperture. They compensated the rapid spatial aberrations and recovered diffraction-limited images over large volumes up to 240 μm per side [63, 64]. Wang *et al*. modulated the intensity or phase of light rays with pupil segmentation based on parallel adaptive optics. As a result, they improved the structural and functional imaging of fine neuronal processes over a large imaging volume [65].

There is no doubt that deep tissue imaging is extremely important for brain imaging *in vivo*. However, the strong scattering properties of the brain pose a major challenge for deep imaging at high resolution. Besides, many deep tissue imaging techniques, such as adaptive optics, need additional processing time for aberration compensation. Therefore, future work may focus on the improvement of imaging depth, spatial resolution, and image acquisition time.

Discussion and Outlook

Optical brain imaging allows an understanding of the structure and function of neurons and this will further help to explain the mechanisms underlying such processes as decision-making, emotion, and memory. Here, we briefly reviewed the current techniques from three points of view: brain clearing, micro-optical sectioning tomography, and deep tissue imaging.

Brain clearing techniques transform the brain from a strong scattering medium to a homogeneous medium. After that, the excitation light can penetrate much deeper so that a larger volume sample can be scanned with a traditional optical microscope. However, brain clearing still faces many challenges. For example, sample deformation is obvious in many tissue-clearing methods. BABB and 3DISCO shrink the brain, while Scale, CUBIC, and CLARITY expand it. Moreover, how the tissue deformation changes the structure of neurons is still unknown. In another aspect, the clearing procedure is inefficient. Most clearing methods are time-consuming. No method can complete clearing of the whole mouse brain within 3 days and most require more than a week. The greatest drawback is that after chemical reagent treatment, imaging information can only be obtained from a dead brain. For *in vivo* imaging, other means have to be found.

MOST is unique and special in achieving whole-brain imaging. It is widely acceptable for its thorough reconstruction of a whole-brain model. It has become an effective approach to investigating the fundamental structure, spread, and connections of neurons. However, similar to tissue clearing, *in vivo* signals cannot be obtained and a large data-processing capacity is also required.

We understand that one of the challenges of optical brain imaging is to obtain *in vivo* signals. The current optical imaging technique for mouse brain requires open-skull or thinned-skull operations [12–18, 66, 67]. The surgery is either severely or mildly traumatic. Light-scattering in brain tissue is the toughest problem. While making use of longer excitation wavelengths can increase imaging depth, special corresponding fluorophores are required. Adaptive optics can significantly increase the imaging depth by compensating wave-front aberration. However, this sacrifices imaging speed.

The development of *in vivo* brain imaging in the future must be noninvasive, with high resolution, rapid imaging speed, and deep penetration, so it still has a long way to go.

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