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Deep brain imaging on the move

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New three-photon miniature microscopes open the study of neuronal networks to those deep in the brains of behaving animals.

In this issue, Zhao et al.[1] and Klioutchnikov et al.[2] introduce new types of miniaturized microscopes that are capable of three-photon imaging deep in the brains of freely moving mice. Their work builds upon recent developments in multiphoton in vivo calcium imaging that demonstrated the capability to record large populations of neurons (from thousands to hundreds of thousands) deep in the brains of head-fixed mice[3,4]. These developments revealed the tremendous potential of optical methods to enable large-scale, dense recordings of deep brain activity in longitudinal studies of complex neural dynamics across learning. To study neuronal correlates during natural behaviors in freely moving animals, miniaturized versions of single-photon[5] (1P, Fig. 1a,b), two-photon[6–8] (2P, Fig. 1c,d) and three-photon[9] (3P, Fig. 1e) microscopes have been developed. One of the major advantages of such miniaturization is that large-scale optical recordings can be integrated in a straightforward manner into well-established rodent behavior paradigms. These include pavlovian[10], instrumental[11] and avoidance conditioning[12] as well as anxiety[13], social[14], maternal[15] and spatial learning[16] paradigms.

For imaging deep brain regions, miniaturized multiphoton microscopes offer several advantages over their 1P counterparts, such as an improved optical sectioning and reduced background contamination. The resilience to light scattering at higher imaging depth can be explained by vascular and neuronal brain tissues having vastly different light absorption and scattering properties in the visible, far-red, and infrared wavelengths[17]. Second, in contrast to 1P imaging, which is dominated by background fluorescence excitation, the nonlinear absorption properties of multiphoton microscopy substantially reduce out-of-plane excitation. Two-photon microscopy suffers from this contamination at higher imaging depths and laser intensities, whereas 3P imaging greatly eliminates this residual out-of-plane excitation[18].

However, it is substantially more complex to design miniaturized multiphoton microscopes than 1P microscopes as the former require laser scanners and highly sensitive fluorescence detectors. In addition, multiphoton laser sources are too large to be mounted on a mouse or rat head, so optical fibers must be used to guide the femtosecond laser pulses to the microscope. Typically, single-mode hollow-core fibers are used to minimize pulse broadening and maintain transmission efficiency. However, such optical fibers potentially further limit the freedom of movement of the animal, a characteristic that is shared by all miniaturized multiphoton microscopes. Last, in multiphoton microscopy the light collected from the excited fluorophore needs to be acquired at sufficiently high speeds to associate the signal from each pixel with the raster scan location. When designing multiphoton microscopes, this speed requirement typically excludes traditional camera sensors, instead requiring the integration of fast single-pixel photomultipliers. For mice and rats, several 2P microscope prototypes have been successfully demonstrated[6,7,19,20] (Fig. 1c,d). Although an approximately 5 g 3P microscope has been developed for rats (Fig. 1e), designing lightweight 3P miniaturized microscopes that are suitable for mice has proven extremely difficult, as it requires an even slimmer optical design and assembly.

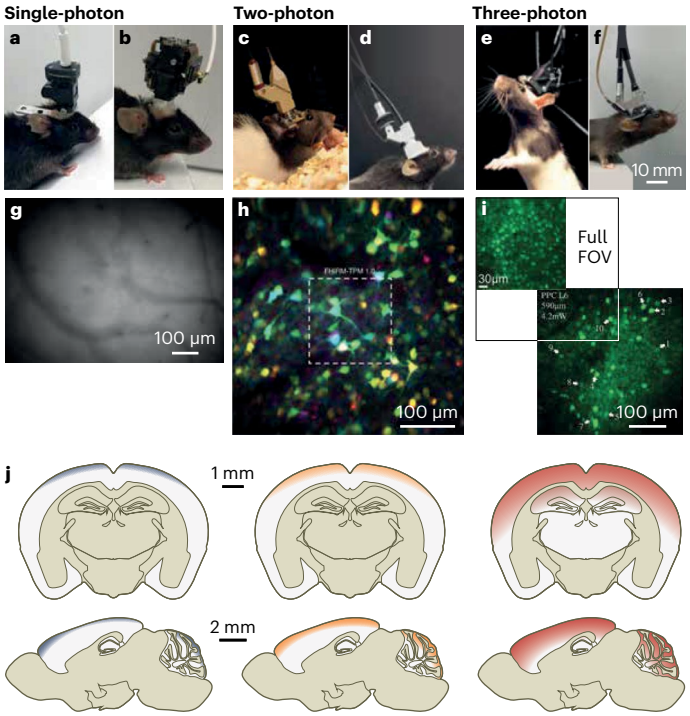

Fig. 1 | Single-photon and multiphoton miniature microscopy in freely behaving animals. a, Miniaturized 1P microscope based on the original design by the Schnitzer lab. **b**, Mouse with the open source 1P miniaturized microscope from UCLA (http://miniscope.org). **c**,**d**, Two-photon miniaturized microscopes designed for rats[6] (left) and mice[22] (right). **e**,**f**, Three-photon miniaturized microscopes suitable for rats[9] (left) and mice[1] (right). **g**, Example FOV of a 1P microscope (Inscopix) with a size of 650 × 900 µm. **h**, FOVs of a standard 2P (dashed square) and an extended-FOV 2P microscope (full image) that is capable of multiplane imaging. Colors indicate neurons at different depths. **i**, Cortical layer 6 FOVs from the two 3P prototypes developed by Klioutchnikov et al.[2] (upper left) and Zhao, Chen, Zhang et al.[1] (lower right). **j**, Coronal (top row) and sagittal (bottom row) sections of a mouse brain indicating the imaging depths of 1P, 2P and 3P miniaturized microscopy. Panel **c** reproduced with permission from ref. [6], National Academy of Sciences. Panels **d** and **h** adapted from ref. [22], Springer Nature. Panels **f** and **i** (lower right) reproduced with permission from ref. [1], Springer Nature. Panel **i** (upper left) reproduced with permission from ref. [2], Springer Nature.

By pushing the boundaries of miniaturized optical design, Zhao, Chen, Zhang et al.[1] and Klioutchnikov et al.[2] have now pioneered the development of two 3P miniaturized microscope prototypes that are suitable for mice. Interestingly, the two research groups chose slightly different technological approaches. Zhao, Chen, Zhang et al.

optimized their miniaturized light collection optical elements for the greatest collection efficiency at depth and coupled the light into a fiber connected to a standard bench-top photomultiplier tube (Fig. 1f). By contrast, Klioutchnikov et al. used an on-board dual-detector system made of silicon photomultipliers (SiPM), thereby eliminating the need for a larger collection fiber. Despite their different optical designs, the two 3P miniaturized microscopes are comparable in weight (2.1 g and 2 g). To achieve nonlinear excitation, the researchers use lasers at longer wavelengths (1,300 nm) with much lower repetition rates (typically 500 kHz to 1 MHz) than are typically used for standard 2P microscopy (80 MHz). The pixel-wise acquisition of fluorescence signals is synchronized to each laser pulse to maximize throughput and maintain a constant illumination intensity per pixel. Because of the low laser pulse repetition rate, fewer pixels and smaller fields of view (FOVs) than for typical 1P (Fig. 1g, up to 1000 × 1,000 µm) or 2P microscopes (Fig. 1h, up to 500 × 500 µm) can be recorded at frame rates between 5 and 10 Hz (Fig. 1i). Given the currently reported FOV sizes for 2P and 3P variants, we predict that local neuronal populations of $n \approx 200$ neurons and maximal $n \approx 100$ neurons, respectively, can be recorded in a single plane. If multiphoton excitation is combined with fast *z*-scanning, multiple 2D imaging planes and thus up to four times more neurons can be imaged simultaneously[19].

By contrast, 1P miniaturized microscopes usually feature larger FOVs (Fig. 1g) containing up to 400–1,000 neurons within a single plane[16]. However, 1P imaging of deeper brain areas critically depends on chronic optical access via implanted optical elements. During invasive brain surgery, gradient index lenses[16] or micro-prisms[21] are implanted. Invasive imaging preparations are particularly delicate when brain areas of interest, such as the cortex, have critical local connectivity. Moreover, the exact technical specifications of the imaging device, the quality of the tissue preparation and the calcium indicator labeling determine the imaging depth. A good rule of thumb is that 1P imaging can record neurons at a depth of about 100–200 µm, while 2P imaging starts to suffer from out-of-focus contamination at about 500–600 µm. Three-photon imaging greatly extends the reachable imaging depths to 1,500 µm (Fig. 1j). In mice, 3P miniscopes can therefore reach subcortical areas such as the hippocampus CA1 at a depth of 1,200 µm while preserving cortical projections. In fact, with the new 3P miniaturized microscopes, the study of several connected subcortical-cortical networks — for example, involving the striatum — is now within reach.

Miniaturized 1P, 2P and 3P microscopes not only are beneficial for recording from large populations of neurons in freely behaving animals, but also facilitate an economy of scale that is useful for manufacturing and dissemination of the technology. The full integration of all optical components and miniaturization makes 3D printing practical and even enables shipping of completely pre-aligned devices directly from the factory. This aspect was instrumental to the fast adoption and user-friendliness of 1P miniaturized miniscopes, and allowed researchers without expertise in optics to adopt the technology for neuroscience research. Three-photon imaging depends on higher-order photon interactions and, as such, the optical pathway is even more sensitive to aberration and misalignments. Depending on their ease of manufacturing, it is conceivable that 3P miniscopes could greatly facilitate a 'ready to image' factory deployment.

While the initial cost of 2P and 3P pulsed laser sources is still a barrier for large-scale adoption of multiphoton miniscopes in neuroscience, single-wavelength Ti:sapphire lasers could offer robust and cost-effective alternatives. With 3P miniaturized microscopy in mice now being a reality, the next set of technological developments could focus on several main directions. The extension of 3P miniaturized microscopy to multi-plane imaging will increase the recording throughput and the number of neurons sampled at standard 5–10 Hz volume rates. In addition, the integration of multiphoton devices with optogenetics will enable patterned multiphoton stimulation to investigate causal relations of brain activity and behavior. Furthermore, combining miniaturized imaging with other imaging modalities such as functional MRI recordings might help to better characterize the intricate nature of the blood-oxygen level-dependent signals. Finally, the combination of multiphoton miniscopes with post hoc cellular genetic identification and tracing will allow the functional and morphological characterization of cell types.

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Competing interests

The authors declare no competing interests.