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Advanced optical imaging techniques for neurodevelopment

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Over the past decade, developmental neuroscience has been transformed by the widespread application of confocal and two-photon fluorescence microscopy. Even greater progress is imminent, as recent innovations in microscopy now enable imaging with increased depth, speed, and spatial resolution; reduced phototoxicity; and in some cases without external fluorescent probes. We discuss these new techniques and emphasize their dramatic impact on neurobiology, including the ability to image neurons at depths exceeding 1 mm, to observe neurodevelopment noninvasively throughout embryogenesis, and to visualize neuronal processes or structures that were previously too small or too difficult to target with conventional microscopy.

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Introduction

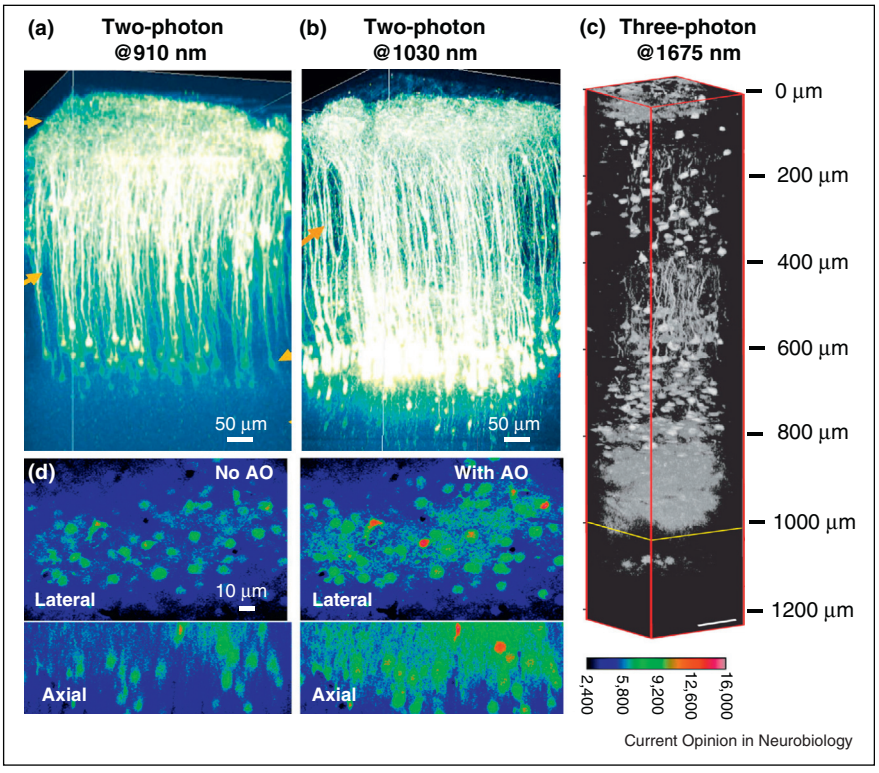
The components of the developing nervous system span a wide range of spatial scales — from synaptic vesicles 40 nm in diameter to axons several hundred micrometers long, and temporal scales — from processes lasting fractions of a second, to processes which might take years to complete. No single microscope is omniscient, so examining neurodevelopment requires a range of techniques with similar breadth in spatiotemporal resolution, while also allowing imaging to be performed noninvasively, at depth, and *in vivo*. Confocal and two-photon microscopies are established workhorses that partially satisfy these criteria, but many aspects of neurodevelopment still remain off-limits. We describe here new techniques, many of which have only recently been applied to neuroscience, that will greatly enhance the accessibility of the nervous system to researchers.

Imaging deeper

In the rodent brain, structures like the hippocampus and other deep brain areas are covered by a millimeter or more of tissue that scatter or absorb light, rendering them relatively inaccessible to conventional optical imaging. Scattering of the visible excitation wavelengths used in confocal microscopy limits the penetration depth to less than 100 μm. In two-photon microscopy, two longer-wavelength (usually near-infrared) excitation photons are absorbed instead of a single photon. Since this process depends on the near-simultaneous absorption of two photons, it is strongly enhanced when the excitation is concentrated in time (achieved with a pulsed excitation source) and in space (at the excitation focus). The resulting fluorescence is tightly confined to the focal region, and out-of-focus background is drastically reduced relative to single-photon microscopy. In addition, near-infrared excitation reduces scattering. These advantages improve depth penetration, enabling the study of neuronal activity and anatomy in the cortex (Figure 1a), sometimes at depths exceeding 800 μm (Figure 1b) [1].

Imaging deeper than 1 mm is difficult, as the fluorescence originating from the focal plane gets progressively scattered and attenuated at depth. Increasing the two-photon intensity or using even longer wavelengths to further reduce scattering helps [2], but only to a point. Beyond a certain depth, the out-of-focus fluorescence background generated at superficial layers overwhelms the increasingly faint signal at the focal plane. Near-simultaneous absorption of *three* photons results in fluorescence emission that is better confined to the focal plane and further reduces background, breaking the depth limit inherent to two-photon microscopy. Such three-photon microscopy requires pulsed lasers with higher energy and longer wavelengths for exciting the same fluorophores as in two-photon microscopy. The advent of high-pulse-energy lasers at ~1700 nm makes three-photon excitation of red fluorescent proteins practical, extending the imaging of mouse hippocampus from 1060 to 1120 μm (Figure 1c) and enabling vascular imaging to 1.4 mm depth within the brain [3••]. Even higher-order multiphoton microscopy is conceivable, although the risk of photodamage increases due to the increasingly intense excitation required. Finally, we note that cleverly combining ultrasound with visible excitation has enabled fluorescence imaging at an unprecedented depth of 2.5 mm within *ex vivo* tissue, albeit at a lateral resolution of tens of microns [4].

Figure 1



(a–c) Deep *in vivo* fluorescence imaging with NIR excitation. Two-photon fluorescence imaging of cortical pyramidal neurons with (a) 910-nm and (b) 1030-nm excitation in an adult eYFP-labeled mouse brain. (c) Three-photon fluorescence imaging with 1675-nm excitation of RFP-labeled pyramidal neurons in a mouse brain. **(d)** AO correction improves calcium imaging. Left: OGB-1 AM labeled neurons 155 μm below the brain surface without AO correction. Right: The same neurons with AO correction.
Panels (a and b) are reprinted from Kawakami *et al.* [1] with permission from *Science*; panel (c) is adapted from Horton *et al.* [3••] with permission from *Nature*; panel (d) is adapted from Ji *et al.* [6•] with permission from *PNAS*.

Besides scattering, optical aberrations resulting from imperfect optics and heterogeneity in sample refractive index prevent the formation of a diffraction-limited focus, also limiting imaging depth. Adaptive optics (AO) methods measure these aberrations and iteratively change the shape, phase, or intensity of the excitation in order to improve imaging. AO has been most useful in two-photon microscopy, where depth penetration, signal, and resolution are critically dependent on forming a high-quality excitation focus. AO has improved imaging at depth [5], provided near diffraction-limited imaging 450 μm inside tissue, enabled a fivefold signal enhancement for small neuronal structures, and increased axial resolution threefold when performing functional Ca^{2+} imaging in single neurons (Figure 1d) [6•]. More recently, AO was used to reduce excitation scattering in mouse brain, thereby improving signal strength 10–100 fold at a depth of 400 μm *in vivo*, and operating at speeds ~10× faster than previous efforts [7].

Imaging faster

Conventional technology is often too slow for capturing rapid neurodevelopmental dynamics. For example, the slow serial scanning employed in most two-photon microscopy systems restricts recording of neurophysiological signals to a single 2D plane. Observing neuronal activity (occurring on the millisecond timescale) through a population of neurons requires faster acquisition capable of interrogating neuronal activity in 3D. Advances in two-photon imaging and light-sheet microscopy now allow the interrogation of such processes in large tissue volumes [8,9••].

It is frequently desirable to rapidly image a series of discrete points (e.g. jumping from neuron to neuron) after assessing the entire imaging volume with conventional imaging. This mode of two-photon microscopy is called 'random-access', and is implemented using specialized hardware that switches between user-selected imaging points much faster than a conventional galvanometric mirror. For example, acousto-optic deflectors enable positioning of a laser's focus within a large volume (700 μm × 700 μm × 1400 μm) essentially instantaneously (Figure 2a,b) [9••]. Up to ~2000 points can be scanned in 40 ms, enabling volumetric calcium imaging of activity in hundreds of neurons in the mouse visual cortex at unprecedented speed [9••].