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Current Opinion in Neurobiology

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

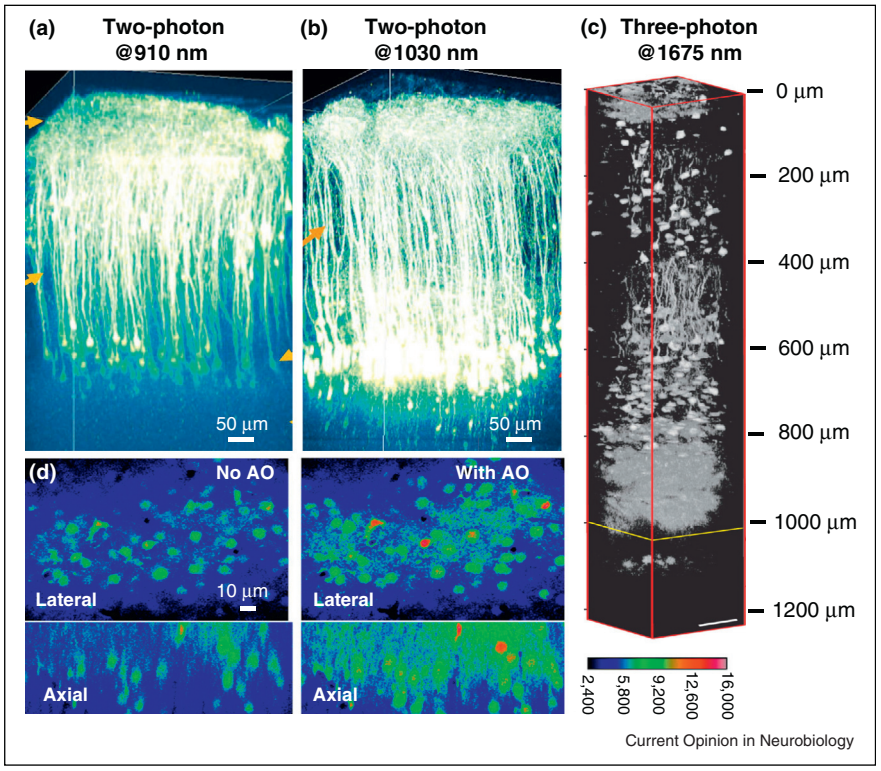
The components of the developing nervous system span a wide range of spatial scales — from synaptic vesicles 40 nm in diameter to axons several hundred micrometers long, and temporal scales — from processes lasting fractions of a second, to processes which might take years to complete. No single microscope 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••].

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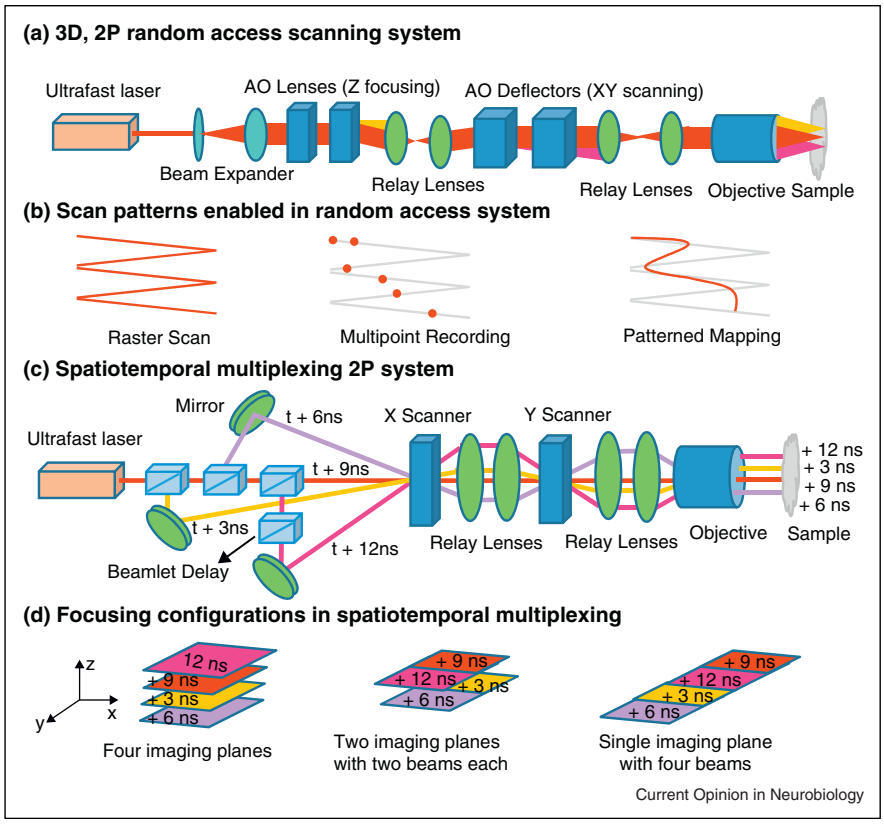
Figure 2



Higher-speed two-photon microscopy. Optical schematic **(a)** and scanning patterns **(b)** for 3D random-access scanning. The system allows conventional raster scanning for structural imaging, discrete point sampling for multiunit recording, and pattern mapping for functional imaging. In the high-speed 3D random-access mode, 2000 points can be scanned in 40 ms, enabling volumetric calcium imaging in hundreds of neurons *in vivo*. Compensating elements must be used because acousto-optical devices generate high spatial and temporal dispersion. Optical schematic **(c)** and focusing configurations **(d)** possible in a spatially and temporally multiplexed two-photon system. Ultrafast laser pulses are emitted every 12 ns and divided into four beams with 3 ns relative delay that are simultaneously focused at different positions within a sample. Different imaging configurations include a scan of four imaging planes, a single plane scan with four beams, or a scan of two imaging planes with two beams each. When used with a resonant scanning mirror, fast (250 Hz/plane at 500 × 500 pixels) calcium imaging in four axial planes is possible.

Two-photon microscopy also benefits when multiple excitation beams are used in parallel to increase scanning speed. When simultaneously focusing multiple excitation beams at different positions, a camera may be used to detect the resulting fluorescence, but this approach limits penetration depth relative to conventional two-photon microscopy due to increased sensitivity to scattering [10]. A better approach separates multiple excitation beams not only in space but also in time, using a fast point detector to temporally resolve the resulting fluorescence (Figure 2c,d) [11•]. Four beams with 3 ns relative delay are simultaneously focused at different positions within a sample, increasing speed fourfold and enabling simultaneous 3D calcium imaging at multiple axial planes to monitor network activity of ensembles of cortical neurons. This strategy only works when the fluorescence decay time is less than 3 ns; further increasing the speed by adding more beams ultimately depends on finding suitable fluorescent dyes.

Finally, axial refocusing may be eliminated entirely by using a diffraction grating to simultaneously record multiple focus-shifted images onto a single camera [12]. Increasing the number of simultaneously recorded planes per volume and improving the light efficiency of the grating are necessary before the technique is widely used in developmental neurobiology.

Imaging with higher resolution

Many structures within neurons, such as dendritic spines, are small enough that they lie close to or below the diffraction limit for optical microscopy. Given increasing interest in understanding the cellular compartmentalization of spines and synapses [13], imaging systems capable of sub-diffraction-limited resolution are required. Although the resolution of light microscopy was limited to ~250 nm for several hundred years, a variety of 'super-resolution' techniques have emerged in the last decade that are capable of visualizing structures as small as 20 nm

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[14]. The unifying ability in all these microscopes is the ability to isolate fluorescence from a subdiffractive region inside the sample. We review three of the most popular super-resolution implementations below.

Single-molecule imaging techniques (SMI) [15,16] repeatedly isolate and localize fluorescence emitted from sparse subsets of photoswitchable molecules, building up a super-resolution image from the centers of the fluorescence emitters recorded in thousands of raw images. These techniques provide images with 10–20 nm spatial resolution in ideal conditions [17] but several caveats apply. First, unless enough localizations are obtained, images possess low signal to noise ratio and appear artificially punctate. Second, the acquisition time for a single super-resolution image ranges from seconds to minutes. 3D imaging takes proportionately longer, and is best performed in combination with multiphoton imaging [18] or light sheet microscopy [19] to improve signal to background ratio. Finally, the $\sim$kW/cm^2 excitation intensities limit the duration of live applications due to phototoxicity and photobleaching. For these reasons, SMI has been most successfully applied to fixed preparations. Multicolor SMI has enabled $\sim$40 nm imaging of neurites in a hippocampal cell culture model, increasing neural tracing accuracy [20]. SMI also revealed that actin and adducin form periodic ring structures every $\sim$180 nm along axonal shafts in hippocampal cells, a discovery missed previously [21].

Stimulated emission depletion microscopy (STED) superimposes a donut-shaped 'depletion' beam on a focused excitation beam, forcing fluorophores at the perimeter of the excitation beam to preferentially undergo stimulated emission so that fluorescence is emitted only from a subdiffractive region in the center of the focus. Scanning the resulting focus through the sample while collecting the fluorescence in a confocal arrangement enables 'all-optical' super-resolution without the need for post-processing required by SMI. STED has been used to image dendritic spines in live nematodes [22] and changes in spine morphology 10–15 μm beneath the surface of the somatosensory cortex in anesthetized mice, at $\sim$70 nm resolution [23•]. The resolution in STED scales with the depletion intensity, requiring peak intensities that can lead to phototoxicity over prolonged imaging. If reversible photoactivation is used as the contrast mechanism, similar resolution can be achieved at much lower excitation intensity [24]. Such approaches have been used to study morphological changes in dendritic spines 10–50 μm inside live organotypic brain slices at $\sim$10-second intervals, offering a threefold improvement in resolution compared to confocal microscopy [25]. Continued development of multicolor, photostable, faster-switching fluorescent proteins [26] is necessary before these methods are routinely applied in neurobiology.

Linear structured illumination microscopy (SIM) uses spatially patterned excitation light rather than uniform illumination to selectively excite a subset of fluorophores. By shifting the illumination pattern through the sample and mathematically processing the resulting series of diffraction-limited images, it is possible to obtain higher resolution (up to twice the diffraction limit) than can normally be observed in conventional microscopy. While offering a more modest improvement than STED or SMI, SIM is much faster (10 Hz in 2D, $\sim$0.2 Hz in 3D [27]) and uses significantly lower intensities, enabling volumetric imaging over tens of timepoints. As it is not necessary to use photoswitchable dyes, multicolor imaging [28] is much easier in SIM than in SMI. The recent combination of SIM with confocal pinholing improves background rejection, permitting volumetric imaging of samples $\sim$10$\times$ thicker than previously possible [29•], and allowing neurodevelopment to be studied *in vivo*.

Imaging less invasively

Many neurodevelopmental processes are sensitive to the photobleaching and photodamage that occur during imaging, and that limit the duration over which a given process can be interrogated. Light sheet-based fluorescence microscopes (LSFM) [30–32] minimize photodamage/photobleaching by only illuminating the focal plane (Figure 3a), and enable 3D acquisition by sweeping the excitation and focal plane through the sample. Since fluorescence is read out with a widefield detector (camera), imaging rates are 10–1000$\times$ faster than point-scanning techniques such as confocal or two-photon microscopy.

Photobleaching reduction is advantageous in combination with chemical clearing agents that reduce scattering in fixed samples. LSFM has been used in conjunction with clearing agents to investigate development of whole mouse ears over embryonic and postnatal periods [33] and to study monosynaptic connectivity of different neuronal populations in whole adult mouse brains [34]. LSFM can image whole live embryos with spectacular success, as its high speed and low illumination dose enable continuous tracking of neuronal migration and neurite outgrowth in *Caenorhabditis elegans* [35••] (Figure 3b–d), lineaging of hair cell progenitors in the zebrafish primordium over tens of hours [36], and neuroblast lineaging and observation of subcellular neuronal dynamics in *Drosophila* [37].

Early LSFM implementations embedded the sample in an agarose gel, rotating and translating the gel as required for 3D acquisition. Although appropriate for samples that do not change shape significantly during development, embedding in agarose can cause severe developmental defects in zebrafish. Systematic evaluation of different mounting agents may help address this problem [38]. Alternatively, mounts such as glass coverslips may be

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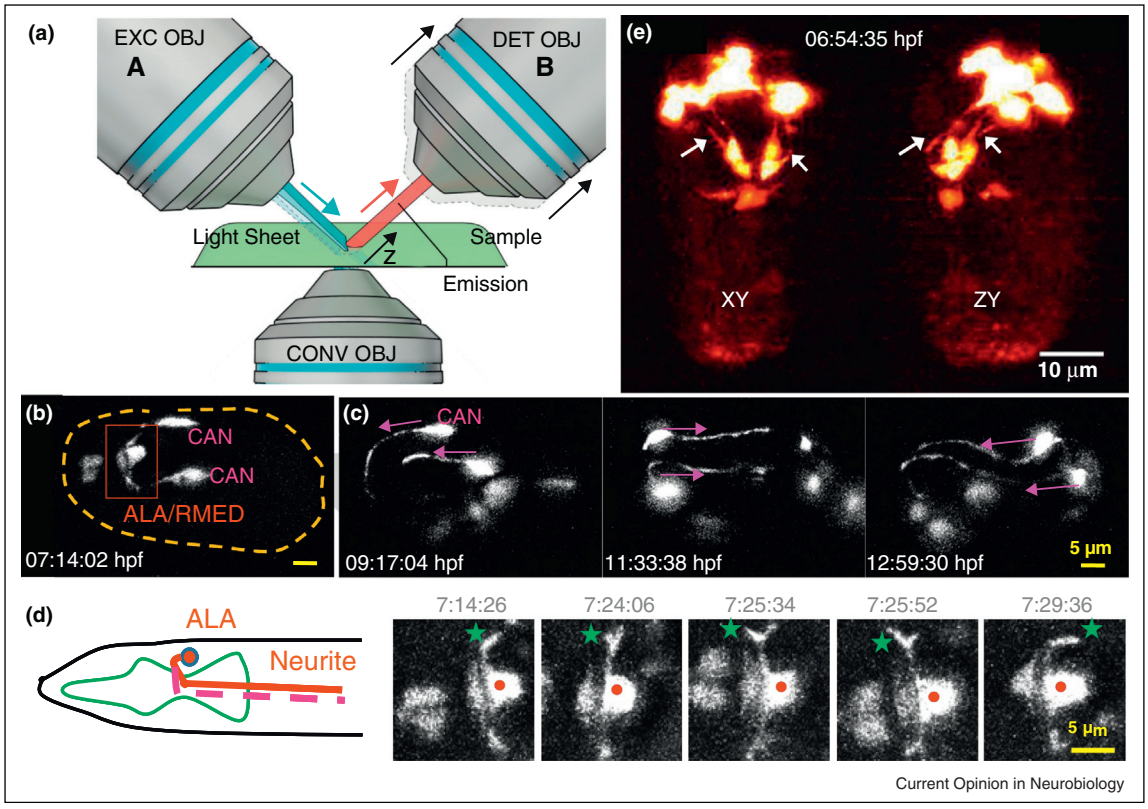
Figure 3



Light sheet fluorescence microscopy enables high-speed, long-term neurodevelopmental imaging during embryogenesis. **(a)** Inverted selective plane illumination microcopy (iSPIM) schematic. Two long working distance, water immersion objective lenses enable orthogonal SPIM excitation and detection. The excitation objective (EXC OBJ) introduces a light sheet at the sample, and the resulting fluorescence emission is collected by the detection objective (DET OBJ). High-speed volumetric imaging is achieved by sweeping the light sheet and focal plane through the sample, along the detection axis. The bottom objective provides an additional view of the sample and allows other optical modalities. *i*SPIM enables conventional mounting of specimens. **(b–d)** Visualization of neuron migration and neurite outgrowths in *C. elegans* embryos with *i*SPIM: (b) maximum-intensity projections of *ceh-10p*:GFP, highlighting ALA/RMED and CAN neurons before twitching. Scale bar, 5 μm. (c) Time series of the CAN neurite outgrowth through the entire twitching period. (d) The cartoon shows the ALA neuron in the adult worm. Both neurites of ALA project toward the posterior end of the animal. The time-series images display a higher magnification view of the red box in (b). Red dot: ALA soma, green star: left neurite outgrowth. The images show the neuronal outgrowth of ALA through twitching and reveal when the bilateral neurites project toward the posterior end of the embryo. **(e)** Dual-view iSPIM (diSPIM) imaging highlighting GFP-tagged AIY neurons. Arrows indicate AIY neurites, clearly visible in both lateral projection (left image) and axial projection (right image).
Panels (b, c and d) are adapted from Wu *et al.* [35••] with permission from *PNAS*.

used by placing objectives in an inverted geometry over a microscope stage [35••].

Many technical improvements that improve LSFM speed and spatial resolution have occurred recently. Using near-infrared excitation for two-photon LSFM enables imaging 2× deeper than single-photon LSFM and allows imaging rates ~10× faster than conventional two-photon microscopy [39]. Placing two detection objectives at opposite ends of the sample increases penetration depth and light collection, permitting live *Drosophila* embryos to be imaged every 30 seconds throughout embryonic development [37]. Combining LSFM with confocal slit detection increases image contrast in thick samples [40], as does combining LSFM with structured illumination and post-processing [8]. Finally, improved spatial resolution is possible if ultrathin light sheets are created from Bessel beams [41] and combined with SIM [42], although such methods significantly increase illumination dose and phototoxicity and are ~5–10× slower than conventional LSFM. A simpler method that provides isotropic 300 nm resolution without these drawbacks relies on acquiring and appropriately combining perpendicular LSFM views (Figure 3e).

Imaging with intrinsic contrast

Extraneous fluorescent probes may interfere with neuronal function, eventually bleach, and sometimes are impossible to target specifically, limiting their utility in examining neurodevelopment. Intrinsic, label-free

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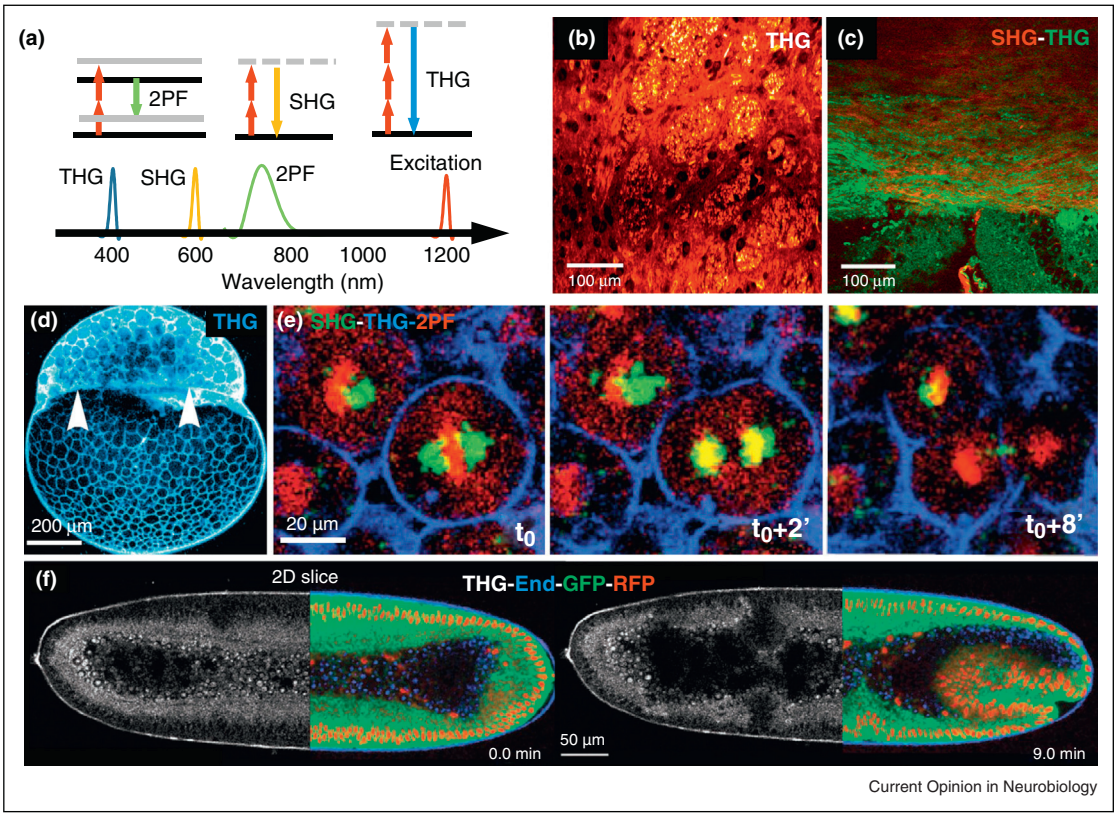
image contrast mechanisms such as autofluorescence imaging [43] (fluorophores include NADH, FAD, collagen and elastin); optical coherence tomography (OCT) [44,45,46•], optical projection tomography [47], optoacoustic tomography [48] (using tissue scattering and absorption for contrast); and second and third harmonic generation [49,50••] (SHG, using noncentrosymmetric structures such as collagen; THG, using lipids) microscopy enable the direct visualization of neurodevelopment without extraneous fluorescent probes.

Optical coherence tomography has been implemented at 200 Hz and up to depths of 500 μm, clearly discriminating dorsal white and gray matter in rodent spinal cord *in vivo*, as well as resolving vessels with ~10–20 μm diameters in the microvascular network [46•]. OCT may thus be an attractive alternative to fluorescence microscopy for studying neurovascular coupling in the developing spinal cord.

Second and third harmonic generation microscopy (Figure 4a) enables label-free live brain imaging using polarized microtubules inside axons and lipids in neuronal membranes and myelin sheaths surrounding axons (Figure 4b,c) [50••]. In zebrafish embryos, THG signals highlighted cell contours and revealed the yolk–blastoderm interface (Figure 4d) [51]. Combining THG/SHG with two-photon microscopy in transgenic embryos allows visualization of cytoplasmic streams and mitosis (Figure 4e) [51]. Also, multichannel imaging of THG with simultaneous three color fluorescence imaging in gastrulating *Drosophila* embryos revealed the rapid process of mesoderm invagination (Figure 4f) [52]. These methods may provide an alternate means for reconstruct-

Figure 4



Label free imaging. **(a)** Energy diagrams and wavelengths in two-photon fluorescence (2PM), second harmonic generation (SHG), and third harmonic generation (THG) imaging. **(b)** THG image of striatum in a mouse brain (coronal section), showing white-matter fibers and neurons. The bright grainy structures are axon bundles that run perpendicular to the image plane. **(c)** Merged THG (green) and SHG (red) signal of a mouse corpus callosum. White-matter structures are visible in both SHG and THG images, but gray matter is only visible in the THG image. THG signals originate mostly from the myelin sheaths surrounding axons, whereas SHG signals are produced by polarized microtubules inside the axons. **(d)** Sagittal THG image of a zebrafish embryo during the 512 cell stage. The THG signal, generated mostly from the lipids in the plasma membrane, highlights cell contours and reveals the yolk–blastoderm interface as indicated by the white arrowheads. **(e)** Temporal sequence (0, 2 and 8 min) of β-actin:H2B/mcherry transgenic zebrafish embryo, highlighting mitosis, simultaneously with SHG (green), 2PM (red) and THG (blue). **(f)** Gastrulating *Drosophila* embryos with contrast derived from THG (white) and 2PM (mRFP-red, EGFP-green, and autofluorescence-blue) imaging.Panels (b and c) are adapted from Witte *et al.* [50••] with permission from *PNAS*; panels (d and e) are adapted from Olivier *et al.* [51] with permission from *Science*; and panel (f) is adapted from Mahou *et al.* [52] with permission from *Nature*.

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ing neuronal connectivity and for tracking cell migrations during embryogenesis.

Summary

The technical advantages presented here enable researchers to directly visualize the expression patterns of genes involved in neurodevelopment, examine neuronal migration, neurite outgrowth, and synapse formation in living animals, and to examine plasticity in the nervous system throughout the lifespan of an organism. We anticipate that significant discoveries in neuroscience will ensue as these methods are applied to questions well-matched to their unique capabilities.

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