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Advances in the speed and resolution of light microscopy

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Neurobiological processes occur on spatiotemporal scales spanning many orders of magnitude. Greater understanding of these processes therefore demands improvements in the tools used in their study. Here we review recent efforts to enhance the speed and resolution of one such tool, fluorescence microscopy, with an eye toward its application to neurobiological problems. On the speed front, improvements in beam scanning technology, signal generation rates, and photodamage mediation are bringing us closer to the goal of real-time functional imaging of extended neural networks. With regard to resolution, emerging methods of adaptive optics may lead to diffraction-limited imaging or much deeper imaging in optically inhomogeneous tissues, and super-resolution techniques may prove a powerful adjunct to electron microscopic methods for nanometric neural circuit reconstruction.

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Introduction

Fluorescence microscopy offers numerous advantages for the study of neurobiological systems: cell specific labeling; protein specific contrast; and functional imaging of neural activity. Two-photon excitation (2PE) of fluorescence in particular stands out in neuroscience for its ability to image deep into tissues, its intrinsic optical sectioning capability, and its negligible out-of-focus photobleaching and photodamage (for recent reviews, see [1,2]). However, as a scanning technique based on an intrinsically weak process, 2PE imaging speed is limited by the response of the beam scanning hardware and the magnitude of the fluorescence signal required to achieve an acceptable signal to noise ratio (SNR). Fast scanning was reviewed in this journal in 2006 [3], so here we survey only the progress over the past two years. We also consider techniques that reduce photobleaching and photodamage, for these effects crucially impact the imaging speed and ultimately determine the amount of information that can be extracted from the sample.

One underappreciated fact associated with optical imaging in biology is that the achieved resolution often does not reach the physical limit imposed by diffraction. This is particularly true when imaging *in vivo* or within acute brain slices: the optical inhomogeneity of the sample distorts the phase of the incoming beam, leading to a focal volume significantly larger than the diffraction-limited ideal. We therefore also review here recent technological advances in improving imaging resolution by correcting optical aberrations.

For more optically benign samples such as cultured neurons and ultrathin sections of resin embedded brain tissue, the diffraction limit can be not only achieved but also substantially surpassed, thanks to emerging super-resolution fluorescence techniques. These are summarized here as well, since they provide a unique means to determine the nanoscale distribution of neurologically significant proteins such as ion channels or at synapses.

Increasing the lateral beam scanning speed

In 2PE microscopy (see Glossary) (Box 1), the laser focus is scanned laterally by changing the angle of the laser beam at the objective back focal plane using galvanometer mirrors, polygonal mirrors, or acousto-optic deflectors (AOD). In raster scanning mode, frame rates can reach 30 Hz [1,3]. However, certain neurobiological questions such as rapid Ca^{2+} imaging from a population of neurons, require submillisecond random-access addressing of discrete user-selected positions, for which scanners involving moving mirrors are not suitable because of their inertia. AODs, however, deflect a beam via diffraction from an acoustic wave generated in a crystal. Since the acoustic frequency determining the degree of deflection can be modulated discontinuously and rapidly, AODs can reposition the laser focus to an arbitrary position much faster than inertia-limited scanners.

A random-access multiphoton (RAMP) microscope has been developed to achieve rapid random access in the focal plane using two AODs. It has an access time of 15 μs and has been applied to spatiotemporal mapping of back-propagating action potentials in rat hippocampal brain slices [4]. Because AODs are highly dispersive, however, extra optical components are needed to compensate for the spatial and temporal dispersion that occurs when ~100 fs pulses (typical of most commercial Ti:Sapphire lasers) are used. Alternatively, longer laser pulses that are

Box 1 Glossary

Two photon excitation (2PE) microscopy It uses near-simultaneous absorption of two photons to generate fluorescence, with the advantages of deeper tissue penetration and reduced out-of-plane photobleaching compared with its more common one photon counterpart.

Light sheet excitation It uses external illumination to selectively excite fluorescence only near the focal plane of an objective, combining the speed of widefield microscopy with reduced out-of-plane background and photobleaching.

A wavefront It is a surface of constant phase. A perfectly converging wave, as produced by an ideal microscope objective, has a spherical wavefront, and an aberrated wave has an irregularly shaped wavefront.

Optical aberrations These represent deviations of the wavefront from its ideal form. They lead to geometric distortions; decrease signal, contrast, and resolution and limit the effective imaging depth.

The point spread function (PSF) It is the image of a point source. Diffraction imparts a minimum size to the PSF comparable to the wavelength; and aberrations degrade the PSF, increasing its size.

Adaptive optics (AO) It improves imaging performance by controlling the wavefront of light.

The classical diffraction limit It dictates that the smallest resolvable feature size in a conventional microscope is approximately half the wavelength of light.

Super-resolution techniques These resolve features on a length scale finer than the diffraction limit.

Near-field microscopy It utilizes a subwavelength light source near the surface of an object to construct a super-resolution image.

Stimulated emission depletion (STED) microscopy It utilizes the stimulated emission of fluorophores to deplete and shrink the effective excitation volume to subwavelength dimensions.

Structured illumination microscopy (SIM) It uses a structured excitation pattern to detect otherwise inaccessible high-resolution information.

Photoactivated localization microscopy (PALM) This and related techniques obtain super-resolution images by the serial photoactivation and nanometric localization of fluorophores.

less sensitive to dispersion can be used, but these lower the 2PE efficiency and require either a different laser or realignment of the laser cavity.

Increasing the axial beam scanning speed

Focal scanning along the axial direction is commonly achieved by translating the microscope objective or changing the divergence of the beam entering the back aperture of the objective. This divergence may be controlled by various means, but the fastest thus far also relies on AOD elements [5,6]. Using two AODs with counter-propagating acoustic waves whose frequency continuously changes linearly (chirping), one can vary the divergence of incoming light, which leads to different axial focal positions. Combined with two other AODs for lateral scanning, 3D RAMP microscopy with an access time of 15 μs has recently been demonstrated, and used to study dendritic calcium dynamics in rat hippocampal slices [7•]. However, the axial scanning range was limited to 50 μm partly by the decreased diffraction efficiency of the AODs for diverging or converging beams.

Because microscope objectives are designed for illumination light of specific divergence (e.g. a collimated beam for an infinity-corrected objective), axial scanning by changing the beam divergence introduces spherical aberration, which leads to a degradation of the image quality [5,7•,8••,9]. Consequently, in commercial microscopes, axial scanning is usually achieved by moving the microscope objective relative to the sample. Gobel *et al.* induced 10 Hz sinusoidal motion of an objective in the axial direction by mounting it on a piezoelectric element and synchronized this motion with lateral beam scanning to generate 3D line scanning patterns that were able to sample more than 90% of cell somata across a 250 μm thick region of the rat somatosensory cortex [10•]. Furthermore, several 3D line scanning modes were designed and applied to dendritic excitation *in vivo* [11]. Typically, the axial scanning speed over such distances is limited to ~20 Hz by the response time of piezoelectric element and the weight of the objective. Furthermore, for certain samples (such as *C. elegans*) the objective movement may introduce unacceptable disturbance.

An alternative focusing method that does not require objective translation or beam divergence variation was demonstrated recently [8••,9]. A second microscope objective was introduced to form an intermediate image plane, aberration-free with respect to the focal plane of the first objective (i.e. the objective facing the sample). A focal spot created at the intermediate image plane could then be reflected by a small mirror to form a focus within the sample. Furthermore, by axially translating the mirror about the intermediate image plane, the focal spot imaged within the sample could be axially scanned without significant aberration over large distances (>100 μm)—potentially at very high speeds (>1 kHz), and without the danger of vibration-induced perturbations to the sample.

Increasing signal to enable faster imaging: pulse splitting and focal multiplexing

Fast raster scanning utilizing resonant galvanometers or AODs often reduces the pixel dwell time so much that repeated scans are needed to collect enough photons for an adequate SNR. Compensation with higher excitation intensity comes at a cost, since photobleaching and photodamage increase faster with excitation intensity than does the 2PE fluorescence signal [12••]. Thus, although the lasers most commonly used for 2PE microscopy can produce pulse energies of tens of nJ, photodamage limits the applicable pulse energy at the focus to ~0.125 nJ. A better approach that more effectively

utilizes the available laser power is to increase the pulse repetition rate while keeping the pulse energy constant. Recently, a pulse splitter was demonstrated that accomplishes this by dividing each pulse output by the laser into a train of temporally delayed, co-propagating subpulses of equal energy [12••]. When the energy of each subpulse was adjusted to equal the original pulse energy applied without the splitter, 128× pulse splitting was shown to decrease the dwell time by 128 times in 2PE microscopy imaging of GFP-labeled mouse brain slices. Significantly, the pulse splitter can be inserted between any pulsed laser and microscope to increase the pulse repetition rate and potential imaging speed, including those based on other nonlinear processes such as second harmonic generation microscopy.

In addition to temporally multiplexing the excitation, imaging speed may also be increased by spatially multiplexing the excitation, as in multifocal 2PE microscopy [3]. However, because a widefield detector must be used to discriminate the signals arising from different foci, this technique is most useful for intrinsically transparent samples, such as zebrafish larvae, or samples which can be made more transparent by the use of clearing agents. In strongly scattering samples such as brain tissue, it is limited to a much shallower imaging depth (~2× greater than the scattering mean free path length) than the single focal scheme [13,14].

Maximizing the photon budget

An unavoidable consequence of fluorescence excitation is photobleaching and photodamage. Mitigating these effects is important as the amount of information one can retrieve from optical imaging is ultimately determined by the number of detected photons, and meaningful results can be obtained only to the extent that the sample approximates its physiological state when imaged. The pulse splitting scheme described above can also help in this regard [12••]. For 2PE microscopy, a constant signal rate can be achieved as the pulse repetition rate is increased N-fold by reducing the energy of each subpulse by a factor of $\sqrt{N}$. However, achieving the desired signal rate in this manner reduces photobleaching and photodamage, as these effects increase even faster with excitation intensity than does the signal. For example, a 64× pulse splitter was shown to be able to decrease both GFP bleaching in live *C. elegans* and photodamage during calcium imaging of acute rat hippocampal slices by an order of magnitude (Figure 1a and b). The molecular mechanism responsible is speculated to be related to the reduced probability, with weaker, temporally separated pulses, of absorbing extra photodamage-inducing photons while in an excited vibrational level of an electronic excited state.

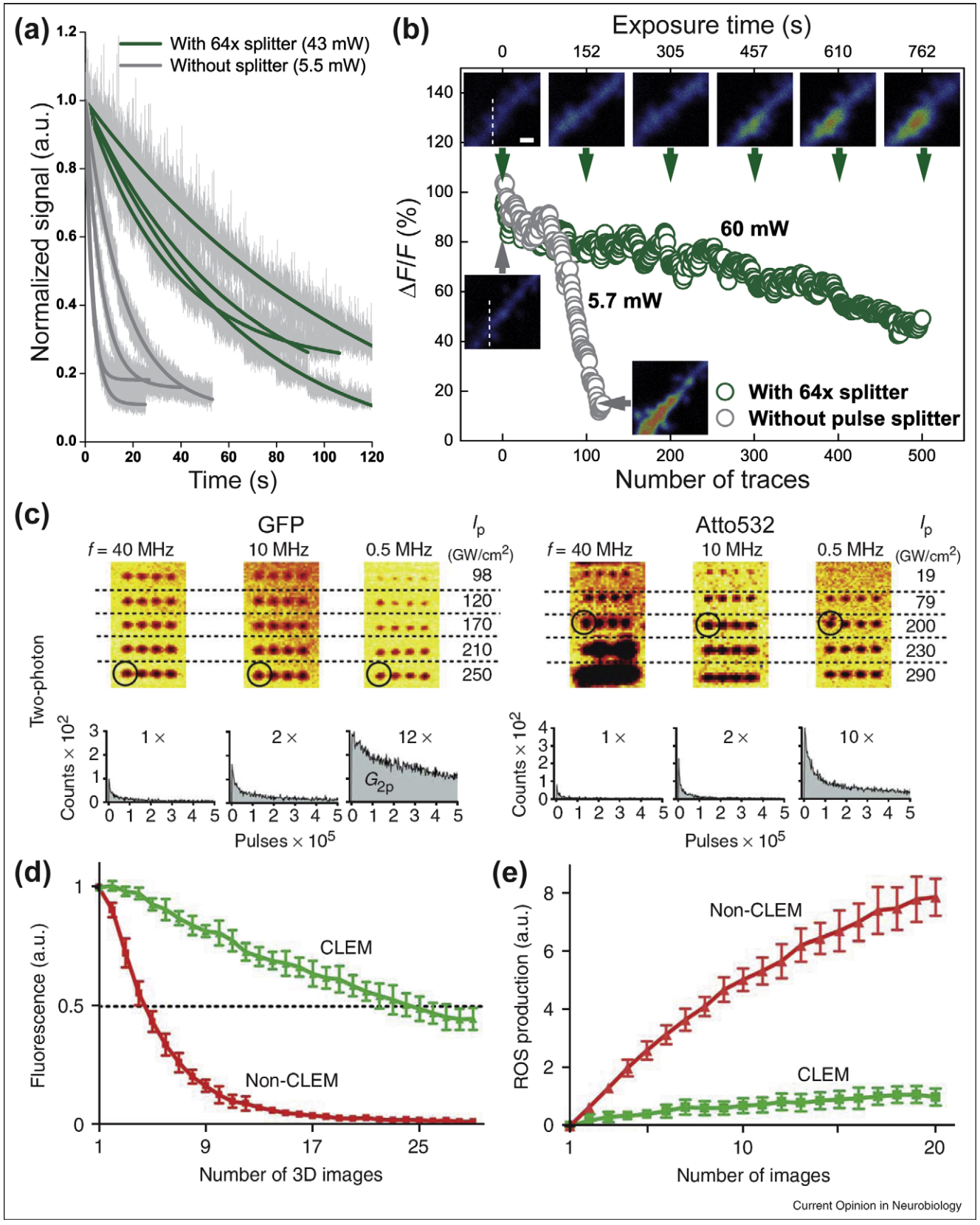
Photobleaching reduction has also been achieved by *decreasing* the pulse repetition rate to allow previously excited molecules trapped in metastable dark states to relax back to ground state before the next pulse arrives, since such molecules are susceptible to bleaching upon the absorption of an additional photon [15]. Decreasing the repetition rate from 40 MHz to 0.5 MHz, Donnert *et al.* used this approach to reduce GFP photobleaching during 2PE by 12-fold (Figure 1c). Decreasing and increasing the pulse repetition rate therefore may both reduce photobleaching by acting on different photochemical pathways. Of course, low repetition rates lead to low signal rates and consequently slow imaging speeds, but for some samples, this is not a concern.

Another class of methods optimizes the photon budget across multiple frames and reduce phototoxicity by controlling the excitation intensity to ensure that only enough signal is collected at each point to achieve an adequate SNR. In controlled light-exposure microscopy, an acoustic-optical modulator was used to block the excitation once a preset number of photons was collected at each pixel. In this manner, regions with high fluorophore concentration were not unnecessarily illuminated, thereby reducing both photobleaching and phototoxicity to the sample (Figure 1d and e) [16]. Similarly, in adaptive illumination, an electro-optic modulator was used to deliver higher power illumination to weakly fluorescent regions [17]. Both methods increase the imaging dynamic range and may be combined with pulse splitting or dark-state relaxation to further minimize photobleaching and photodamage.

High speed and reduced photobleaching using light sheet excitation

Recently, microscopy utilizing light sheet illumination (selective plane illumination microscopy [18], ultramicroscopy [19•], or planar illumination microscopy [20]) was revived as a technique with the potential for high speed volumetric imaging and minimal deleterious photo-induced effects. Here the sample is illuminated by a sheet of light introduced externally to the fluorescence collection objective yet coincident with its focal plane. Because the image at any given plane can be quickly captured with a widefield detector such as a CCD camera, an entire 3D volume can be rapidly mapped by sweeping the focal plane and excitation together through the specimen. Furthermore, since the excitation is largely confined to the focal plane, out-of-focus photobleaching or photodamage is greatly reduced compared to confocal microscopy. For intrinsically transparent samples, such as live embryos of Medaka and fruit fly [18], as well as chemically cleared samples such as mouse brain, mouse embryos, and the entire body of a fruit fly [19•], single cell resolution has been achieved in 3D. Limitations of the method include a diffraction dictated tradeoff necessitating thicker light sheets to cover larger fields of view (thereby degrading axial resolution), and restriction to samples and imaging depths where optical aberrations

Figure 1


(a)
With 64x splitter (43 mW)
Without splitter (5.5 mW)
Normalized signal (a.u.)
Time (s)
(b)
Exposure time (s)
0
152
305
457
610
762
ΔF/F (%)
60 mW
5.7 mW
With 64x splitter
Without pulse splitter
Number of traces
(c)
GFP
Atto532
f = 40 MHz
10 MHz
0.5 MHz
Ip (GW/cm2)
Two-photon
Counts × 10^2
Pulses × 10^5
(d)
Fluorescence (a.u.)
CLEM
Non-CLEM
Number of 3D images
(e)
ROS production (a.u.)
Non-CLEM
CLEM
Number of images
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and scattering are sufficiently benign to yield images from the focal plane of acceptable quality. Possible applications include functional Ca^{2+} imaging of neural populations in 3D [20], and high throughput mapping of neural anatomy.

Recovering optimal spatial resolution with adaptive optics

A microscope objective can only create an ideal, diffraction-limited focus when the excitation light travels to the focus through the immersion media for which it was designed. Thus, the spatial variation in refractive index typical of biological samples creates distortions in the converging spherical excitation wavefront, known as aberrations, that yield a focus that is no longer diffraction-limited. Aberrations lead to decreased signal, contrast, and resolution, distort the image geometrically, and limit the imaging depth in thick biological samples such as brain [13,14,21,22]. One remedy is to use corrective optical elements, such as a deformable mirror, to introduce wavefront distortions that cancel out the sample-induced aberrations. This approach, called adaptive optics (AO), was originally developed in astronomy to obtain diffraction-limited images of stars even when viewed through the optically inhomogeneous atmosphere.

A central question in applying AO to microscopy is how to determine the aberrations that exist in order to know what corrective measures to apply. A recent review can be found in [23]. Here we highlight a few approaches that may prove relevant to neurobiology. Rueckel *et al.* determined the aberrations by measuring the wavefront of the backscattered light [24•], and demonstrated that AO correction could improve both signal and resolution when imaging the olfactory bulb of transgenic zebrafish. However, this approach is probably best suited to weakly scattering samples where the assumption that the measured backscattered light comes mostly from laser focus is probably justified. A different approach proposed by Booth [25•] applies a series of known aberrations to the imaging system and measures their effect on the fluorescence signal to determine the sample-induced aberrations present. Because the measurement does not directly involve the excitation light, it is less sensitive to scattering effects and therefore may be more useful when imaging deep into scattering tissues. However, this method has not yet been demonstrated on a biological sample.

Another technique [26] utilizing corrective optics was used not to improve resolution, but to remove the fluorescence background caused by out-of-focus 2PE that arises when laser light is focused deep into brain tissue [27]: by *adding* aberrations to the imaging system, the signal from the focus was eliminated, so that only the background remained. This background was then subtracted from the images obtained without the induced aberrations, in order to increase the image contrast. Examples from the mouse olfactory bulb and CA1 pyramidal cells were given. Of course, this approach is only applicable when the in-focus signal is greater than the shot noise associated with background photons.

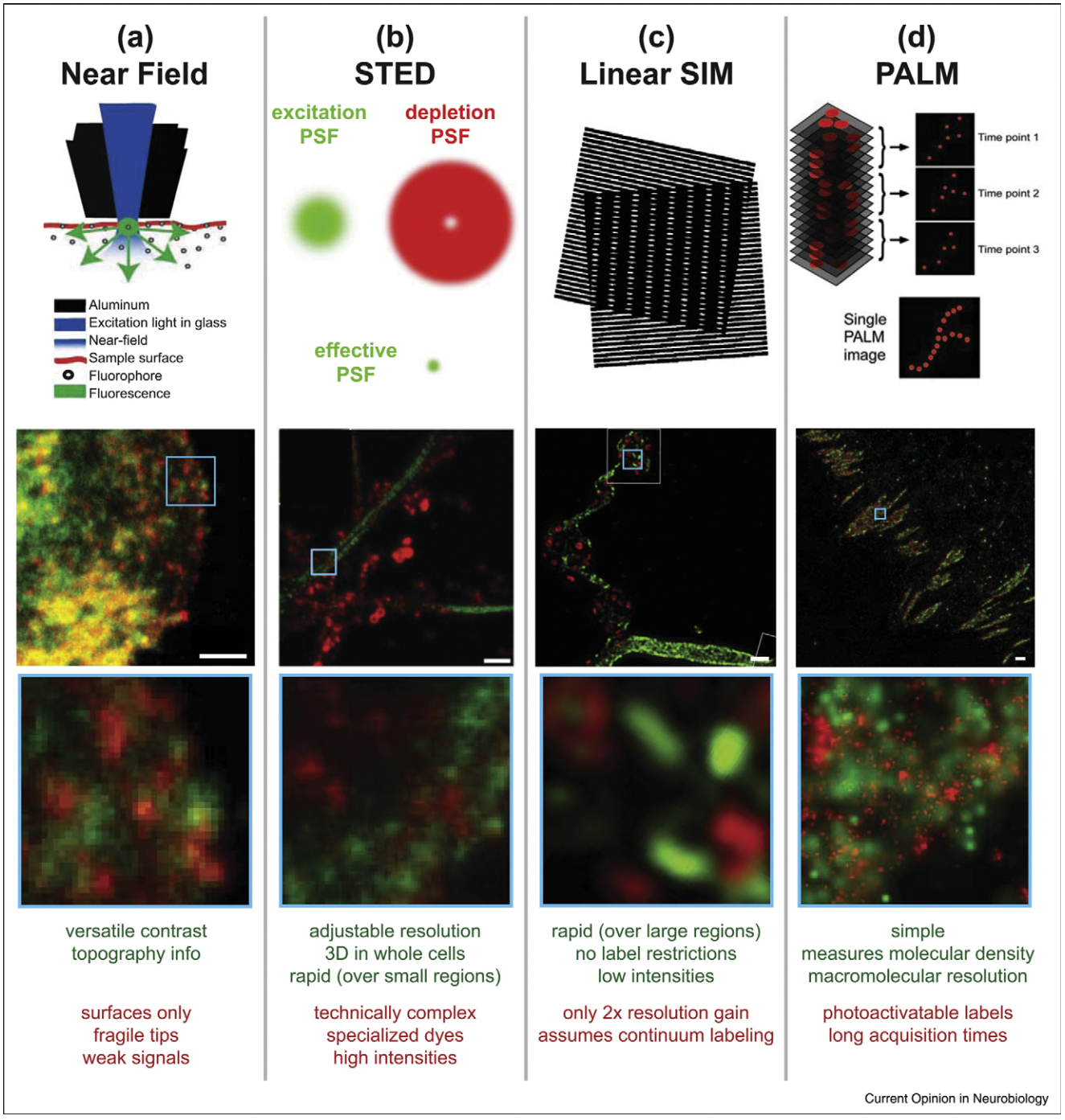
Spatial resolution beyond the diffraction limit

For certain samples where aberrations and scattering are negligible, imaging beyond the conventional diffraction limit (~200 nm) can be achieved. At least four unique super-resolution methods have been demonstrated. Near-field microscopy [28–32] scans a subwavelength sized light source and/or detector in close proximity (~10–20 nm) to the surface of a sample (Figure 2a) to generate, point-by-point, an image with resolution related to the size of the probe (typically, ~30–100 nm). Stimulated emission depletion (STED) microscopy [33,34•,35–42] creates a nanometric focal region (Figure 2b) by first exciting fluorophores to an excited state over a diffraction-limited region, and then forcing all of them, except those at the very center of the region, back to the ground state (before they can emit fluorescence photons), by using a second, doughnut-shaped light beam. Structured illumination microscopy (SIM) [43,44,45•,46–49] is a widefield technique wherein multiple images captured with a finely structured excitation pattern (Figure 2c) are used to demodulate high spatial frequencies that encode information about unresolvable, nanometric sample features down to lower frequencies that can be optically resolved. Finally, photoactivated localization microscopy (PALM) and related techniques [50••,51–58,59•] use the stochastic photoactivation of single molecules and their subsequent nanometric localization over thousands of widefield image frames to construct a super-resolution image (Figure 2d). Multi-color imaging, a tool essential to unravel the spatial relationship between different subcellular features or constituent proteins, has been demonstrated with all four methods [32,35,44,53,54,60], and 3D imaging has been demonstrated with STED, SIM, and PALM [36,45•,46,55,56,61,62,63•]. These latter far-field methods were also the focus of a recent review [64].

Each method has its own unique advantages and disadvantages. Near-field microscopy is limited to the surfaces of samples of slowly varying topography, and its sharp,

(**Figure 1 Legend Continued)** Methods for reducing photobleaching and photodamage: Pulse splitting to increase the repetition rate **(a)** reduces photobleaching of GFP in *C. elegans* and **(b)** mitigates photodamage during calcium imaging of CA1 pyramidal neurons of rat hippocampus (reproduced from [12••]); **(c)** *Decreasing* the repetition rate to allow dark-state relaxation reduces photobleaching of GFP and Atto532 (reproduced from [15]); Controlled light-exposure microscopy (CLEM) **(d)** reduces photobleaching of GFP and **(e)** inhibits the formation of reactive oxygen species (ROS) in tobacco BY-2 cells (reproduced from [16]).

Figure 2



Super-resolution imaging techniques. *Top:* Schematic representations of **(a)** Near-field microscopy (reproduced from [28]); **(b)** Stimulated emission depletion (STED) microscopy; **(c)** Linear structured illumination microscopy (SIM, reproduced from [43]); and (**d**) Photoactivated localization microscopy (PALM, adapted from [72]). *Middle*: Dual color images and comparative 1 μm × 1 μm subregions, for each of the techniques shown at top: **(a)** Immunolabeled human T cell receptors (adapted from [32]); **(b)** Immunolabeled β-tubulin and syntaxin-I in rat hippocampal neurons (adapted from [60]); **(c)** Immunolabeled giant ankyrin and Fas II at the *Drosophila* neuromuscular junction (adapted from [47]); and **(d)** Fusion proteins paxillin and vinculin within adhesion complexes at the periphery of a human fibroblast (adapted from [53]). All scale bars = 1 μm. *Bottom*: Strengths (green) and weaknesses (red) of each technique.

nanometric probes are easily damaged, yet it can exploit many optical contrast mechanisms (e.g. absorption, polarization, and spectroscopy) in addition to fluorescence. STED requires precise control of the position, phase, and amplitude of two laser beams, and its best resolution is restricted to certain dyes able to withstand repeated cycles of excitation and de-excitation at high intensities. However, its final spot size can be tuned to balance resolution against signal and imaging speed, and, as a point scanning technique, it can cover small fields of view at high speed [37•]. SIM, in its linear form, can provide only a two-fold resolution increase beyond the diffraction limit, but it can be readily adapted to most widefield microscopes and is capable of high frame rates over wide fields of view. Lastly, PALM requires photoswitchable fluorophores and imaging conditions consistent with single molecule detection but can quantitatively map relative molecular densities at very high resolution, also over wide fields, even in living cells [59•].

All four methods have been applied to biological problems. Near-field microscopy has most recently been employed in several studies of immunoreceptors [30–32]. PALM has been used to track large populations of single protein molecules in the plasma membrane of living cells [57,58], and in studies of the spatial organization [53] and dynamics [59•] of cell/substrate adhesion points. SIM has elucidated the 3D structure of the nuclear periphery [44], as well as the organization of specific proteins at the neuromuscular junction in *Drosophila* [47]. However, for neurobiological questions, STED has been the technique most aggressively applied thus far [37•,38–42]: for example, in the study of syntaxin clusters and acetylcholine receptors on fixed cultured neurons, as well as to the dynamics of synaptic vesicles.

Caveats regarding the application of super-resolution microscopy

The rapid progress in super-resolution microscopy has raised hopes that these methods might soon make major contributions to biology. However, considerable obstacles must be overcome before they can rival the consistency and impact of electron microscopy (EM)—the current gold standard for biological imaging at the nanometric scale. Here we enumerate some of these challenges.

Labeling with fluorescent markers

One of the most fundamental, yet underappreciated, factors determining the ultimate resolution of fluorescence microscopy is the density of fluorescent markers within the specimen: in essence, it is impossible to image that which is not there. Thus, by the Nyquist–Shannon theorem, the mean distance between label molecules must be at least twice as fine as the desired resolution (Figure 3a, and Supplementary Table 1 in [59•]). Furthermore, this is a minimum requirement: for stochastic labeling, the number L of labels per pixel will vary approximately by $\sqrt{L}$, introducing considerable noise at lower labeling densities or higher spatial resolution (i.e. smaller pixel sizes). Dimensionality is also important: local densities of 10^4 molecules/μm^2 are required to image densely packed features at 20 nm resolution in 2D, but 10^6 molecules/μm^3 are needed to achieve the same resolution in 3D.

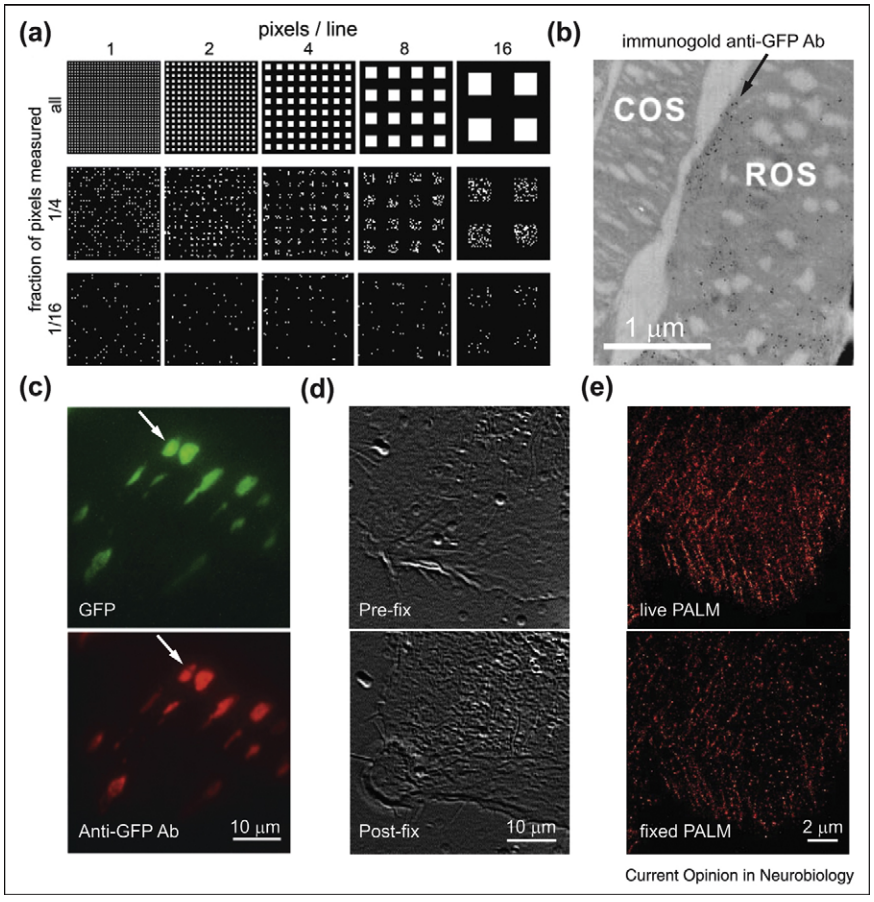
Such densities require very high expression of the target protein, or assembly of the protein in dense macromolecular structures. For example, cytosolic expression at 10 μM, a reasonably high concentration, corresponds to only 6×10^3 molecules/μm^3. Then there exists the challenge of labeling as many of these targets as possible (high binding affinity) while avoiding extraneous labeling elsewhere (high specificity). Labels can be introduced either exogenously (e.g. with antibodies, Abs) or endogenously (with fluorescent proteins, FPs). FPs can have perfect specificity, but label density can be reduced by competition with the wild-type (WT) protein, improper folding, or damage during sample processing. Abs exist for a great many targets, but their affinity and specificity at the levels required for super-resolution microscopy remain to be determined: label density has been an enduring concern in immunogold EM studies (Figure 3b), and a direct comparison of FP and Ab labeling on the same specimen [65] shows noticeable differences at the diffraction limit (Figure 3c), calling into question the labeling fidelity at finer length scales.

Sample perturbations

Another challenge is how to achieve the necessary labeling density and specificity while still preserving the sample in a physiologically relevant state. For endogenous labels, artifacts due to aggregation or overexpression of FP/target fusion proteins should be checked by diffraction-limited structural and functional comparison to WT or more weakly expressing transfected cells. For exogenous labels, metrics must be established to confirm that fluorescence originates predominately from the intended target. In addition, large labels such as Abs (~10 nm) require substantial cell permeablization via fixation or detergents to permit access to all but surface targets, as well as aggressive washing to remove excess label. Morphological changes due to these procedures need to be assessed. For example, cells fixed for 15 min in 2% paraformaldehyde show clear differences before and after fixation when imaged by differential interference contrast (DIC) microscopy (Figure 3d), and a similar live cell/fixed cell PALM comparison indicates substantial loss of cytosolic protein, the formation of some clusters, and slight nanoscale changes in pre-existing structures (Figure 3e).

Live cell imaging under physiological conditions is even more challenging. FPs have long been used in this context, but Abs can invoke atypical cellular responses due to the labeling process, non-specific protein aggregation, and dynamical perturbations caused by their

Figure 3



Challenges in super-resolution microscopy. **(a)** The importance of molecular label density (represented here as pixels in a test pattern): features are imaged at progressively worse SNR as fewer pixels are measured and become unresolvable when the mean pixel separation approaches the feature size (reproduced from [59•]). **(b)** Immunogold electron microscopy of GFP-expressing rod cells (reproduced from [65]), illustrating the poor labeling density that has historically plagued this field. Analagous problems may occur when immunofluorescence labeling is applied to super-resolution optics. For example, a direct comparison **(c)** between GFP and antiGFP-labeled structures in rod cells reveals differences (arrows) even when viewed with only diffraction-limited resolution (reproduced from [65]). Sample preparation protocols such as chemical fixation are also potentially quite perturbative, as attested to by **(d)** diffraction-limited DIC and **(e)** super-resolution PALM images of fibroblasts.

large size. However, even more crucial for super-resolution live cell imaging is the need to minimize potential artifacts introduced by the imaging process itself. Regardless of the method used, an R-fold increase in spatial resolution in D dimensions requires R^D times as much signal per frame to maintain a constant SNR per pixel, raising the specter of increased phototoxicity or even apoptosis unless the frame rate is reduced accordingly. Furthermore, for point scanning techniques such as near-field and STED, the excitation must increase by another factor of R^D at constant frame rate to compensate for the smaller volume interrogated at each point [59•]. Nevertheless, over small fields of view, point scanning can achieve high frame rates [37•], whereas over sufficiently large fields, widefield methods such as SIM and PALM will be faster, even though they must record an increasingly large number of images to construct a super-resolution frame of increasingly fine detail [49,50••].

In short, one of the frustrations of super-resolution microscopy is that it is easy to get images, yet extremely difficult to get biologically meaningful ones. As the novelty of super-resolution microscopy wears off, and the focus shifts to its biological application, it will become increasingly important to adopt careful controls such as correlative (Figure 3d and e) and/or simultaneous [59•] diffraction-limited imaging to insure that the results are physiologically relevant.

Signal, background, and practical resolution limits

Every method of super-resolution microscopy faces the challenge of discriminating the signal over a nanometric region from the background over a much larger diffrac-

tion-limited region (DLR). Such background arises from sources unique to each method. In near-field, it can result either from light not fully confined to the probe dimension or from fluorescent bodies beyond the near-field regime. In STED, it can occur when molecules in the depletion region are not returned to the ground state, either by unfavorable dipole orientations, local excited states non-resonant with the depletion wavelength, shelving into intermediate states, or sheer chance. In PALM, it can be attributed to the weak emission from the many inactive molecules in close proximity to the single activated one. In SIM, the discrimination problem occurs in Fourier space: untangling different spatial frequencies mapped into the same spectral band is made more difficult in the presence of noise.

The chief effect of background is to set a practical limit to the resolution obtainable by each technique. Clearly, achieving 20 nm resolution in 3D (a ~1:2500 ratio of signal volume to DLR) is a far more daunting task than attaining 100 nm resolution in 2D. However, one condition that greatly simplifies the problem is when only a small fraction of the DLR is filled with fluorescent molecules—the contrast ratio of signal to background is then proportional to the relative number of molecules in the two regions, rather than their respective volumes. As a result, it can be possible to resolve a few punctate bodies at high resolution within a DLR (the so-called Rayleigh criterion), and still not be possible to resolve many such bodies at the same resolution within the same region. A more rigorous standard of resolution is the aforementioned Nyquist criterion, because it assumes no *a priori* information about the nanometric spatial organization of the sample or the density of the features therein. It does however demand the ability to label at increasingly high density at increasingly high resolution, and consequently the ability to discriminate the signal arising from a decreasingly small region over the background from an increasingly large pool of molecules elsewhere in a DLR. PALM is particularly noteworthy in that, when used in conjunction with the endogenous label EosFP, labeling densities and contrast ratios between the active and inactive forms of the label can be achieved that are sufficient for 10–20 nm 2D resolution by the Nyquist criterion on fixed cells [50••] and 60 nm resolution at 30 s frame rates on live cells [59•].

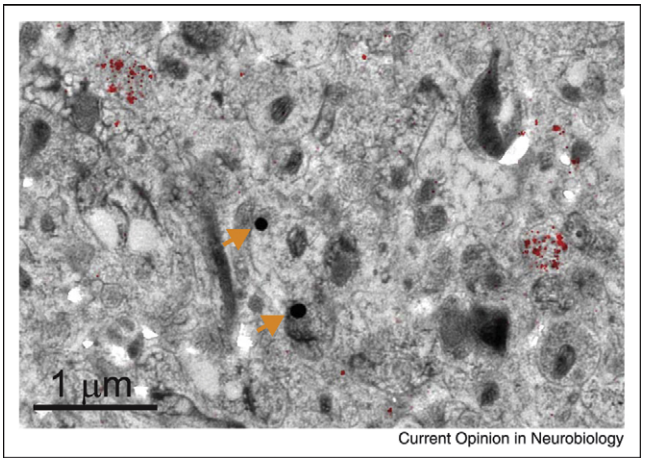
High resolution neuroanatomy by correlative light and electron microscopy

In addition to background, other factors such as aberrations, scattering, and autofluorescence contribute to the practical resolution limits of super-resolution microscopy. As these factors are of greater significance on thicker specimens, it is not surprising that super-resolution imaging has thus far been predominantly confined to thin samples, such as single-layer cultured cells. However, the structure within neurons and their interconnections that form neural circuits are best studied physiologically in the context of the tissues in which they reside. To reveal this structure on the nanometric scale, physical sectioning of the tissue is required.

Such sectioning has a long history in EM and forms the basis of one approach [66] toward volumetric reconstruction of complete neural circuits ('connectomics' [67]). Combining EM and fluorescence microscopy on thin (~50–70 nm) sections of resin-embedded tissue offers additional information by combining the global context typical of EM staining with the protein specific contrast of fluorescence labeling [65,68,69,70••]. For example, in array tomography [70••], serial sections imaged by conventional fluorescence microscopy after successive steps of antibody labeling and elution reveal the spatial relationship between multiple proteins that can then be compared to subsequent EM images.

Extending this approach with super-resolution microscopy may permit 3D nanometric imaging of an entire suite of proteins (e.g. synaptic proteins or ion channels) and their surrounding structure anywhere within any organism. Indeed, both PALM [50••] and STED [60] have been applied to fluorescence imaging on thin sections: in the former case, with correlative EM as well. Nevertheless, two challenges remain: labeling at sufficiently high density to achieve the necessary resolution and establishing a sample preparation protocol that preserves both the label density and the EM ultrastructure. We have recently established such a protocol and used it to identify specific dendritic processes in the mouse barrel cortex (Figure 4). With stochastic expression of multiple colors, such as in the

Figure 4



Correlative PALM/TEM in a high pressure frozen, LR-White embedded, 70 nm thick section. Cytosolic mEosA (S McKinney and L Looger, unpublished) was sparsely expressed in the mouse barrel cortex, and is present at ~25 μM concentration in dendritic structures (red). Orange arrowheads mark Au bead fiducials used in aligning the PALM and TEM images.

Brainbow method [71], and/or stochastic targeting of ubiquitous subcellular features, such identifications might be used to drastically reduce the error rate in neural tracing for connectomics below that which is possible with EM reconstruction alone.

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