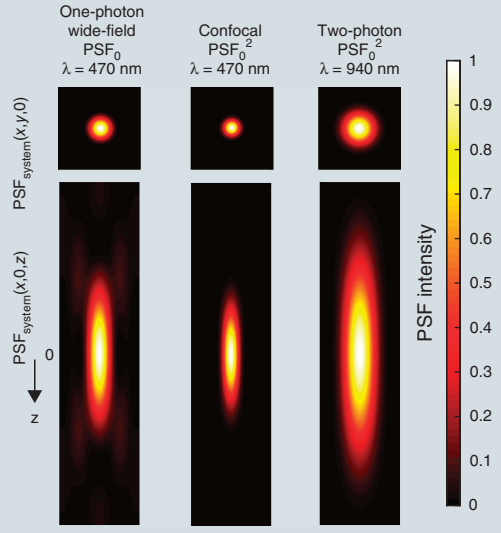
BOX 1 POINT-SPREAD FUNCTION, SPATIAL RESOLUTION AND OPTICAL SECTIONING

The point-spread function (PSF) characterizes how a point source appears upon detection in the microscope. The image of any object can be obtained by mathematically convoluting its intensity density with the microscope PSF. The smaller and sharper the PSF, the better the resolution of a microscope. For an aberration-free system, the PSF depends on wavelength (λ), refractive index (n) in the objective medium and the numerical aperture (NA), which is related to the microscope's aperture shape. For laser scanning microscopy, an illumination PSF can also be defined to characterize the spatial profile of the excitation spot. The overall PSF is the product between the illumination PSF and the detection PSF convoluted with the 2D pixel area (D) of the detector projected into the object space.



For a typical microscope with a circular aperture (assuming uniform illumination), the PSF is circular with a central bright disk and progressively weaker concentric dark and bright rings. The distance from its center to the first lateral minimum r_{xy} and axial minimum r_z is commonly quoted as the lateral and axial resolution (by Rayleigh criterion) of wide-field microscopes. The r_z is proportional to the depth of field.

$$r_{xy\_\text{wide-field}} = 0.61\lambda/\text{NA}$$

$$r_{z\_\text{wide-field}} = 2\lambda n/\text{NA}^2 \text{ (standard wide-field)}$$

For confocal microscopes, the pinhole restricts the collection at the focus center, leading to a squared PSF (ignoring wavelength difference between illumination and emission), which provides a tighter confinement. Thus[15]

$$r_{xy\_\text{confocal}} = 0.4\lambda/\text{NA}$$

$$r_{z\_\text{confocal}} = 1.4\lambda n/\text{NA}^2 \text{ (confocal)}$$

For standard two-photon microscopes, the excitation also scales as light intensity squared, so that resolution can also be expressed as above. Because the wavelength in two-photon microscopy is about twofold that used in confocal, its theoretical spatial resolution should be twofold as well. In practice they are similar, as the finite pinhole in confocal microscopy broadens its PSF.

A microscope capable of optical sectioning can discern the z location of a uniform thin fluorescence plane. The laterally integrated PSF describes this capability. In standard wide-field microscopy, the laterally integrated PSF is z independent and thus does not confer optical sectioning. But the squared PSF in confocal and two-photon renders a z dependency and thus optical sectioning.

Temporal focusing imposes additional nonlinearity at the focus and enables wide-field two-photon excitation. The calculation of its excitation PSF requires consideration of the spatial dependency of the laser pulse width[24–27].

strategy by simultaneously scanning multiple points. Although out-of-focus photons are not used for image formation in these approaches, they can nevertheless contribute to phototoxicity. Therefore, confocal microscopy is best suited for imaging fixed samples or those with low sensitivity to photodamage. For functional imaging *in vivo*, where phototoxicity must be minimized, methods using excitation photons more efficiently are preferred. This has led to methods that achieve optical sectioning by avoiding the generation of out-of-focus background in the first place.

One such technique is light-sheet microscopy, which uses a thin sheet of light projected into the sample from the side, exciting only a two-dimensional (2D) section of the sample. The emitted fluorescence signal is then imaged in an orthogonal direction to the excitation plane (**Fig. 2a**)[3,17–22]. Light-sheet microscopy is particularly applicable for functional mapping of circuits across large brain volumes in transparent or semi-transparent samples and for measuring spatiotemporal responses to sensory, pharmacological or optogenetic stimuli; volumetric recording can be implemented with speeds of >10 vol/s (refs. 19–22). More detail on light-sheet microscopy is given in another Review in this issue[23].

A different method for selective illumination of focal planes is two-photon excitation. Two-photon light sources are femtosecond lasers that emit periodic pulse trains with high peak power at infrared wavelengths. At these long wavelengths, a single photon's energy is not enough to excite the fluorophore; it has to absorb two photons to emit fluorescence. Thus, the two-photon absorption rate is proportional to the light intensity squared, so only fluorophores in the focal spot receive enough photon densities to emit fluorescence, suppressing out-of-focus excitation and background, enabling optical sensing (**Box 1**). Although typical two-photon microscopes scan a focal spot across the sample and are often used for scattering samples (see below), wide-field two-photon imaging is also possible. However, the axial extent of the excitation volume depends on its lateral extent[24]. This can be