

# Principles of Two-Photon Excitation Microscopy and Its Applications to Neuroscience

## Primer

Karel Svoboda<sup>1,\*</sup> and Ryohei Yasuda<sup>2,\*</sup>

<sup>1</sup>HHMI

Cold Spring Harbor Laboratory  
Cold Spring Harbor, New York 11724

<sup>2</sup>Department of Neurobiology  
Duke University Medical Center  
Durham, North Carolina 27710

The brain is complex and dynamic. The spatial scales of interest to the neurobiologist range from individual synapses (~1 μm) to neural circuits (centimeters); the timescales range from the flickering of channels (less than a millisecond) to long-term memory (years). Remarkably, fluorescence microscopy has the potential to revolutionize research on all of these spatial and temporal scales. Two-photon excitation (2PE) laser scanning microscopy allows high-resolution and high-sensitivity fluorescence microscopy in intact neural tissue, which is hostile to traditional forms of microscopy. Over the last 10 years, applications of 2PE, including microscopy and photostimulation, have contributed to our understanding of a broad array of neurobiological phenomena, including the dynamics of single channels in individual synapses and the functional organization of cortical maps. Here we review the principles of 2PE microscopy, highlight recent applications, discuss its limitations, and point to areas for future research and development.

Fluorescence microscopy occupies a unique niche in biological microscopy. Fluorescent objects can be selectively excited and visualized, even in living systems (Lichtman and Conchello, 2005). The sensitivity of fluorescence detection is sufficiently high so that single fluorescent molecules can be detected in the presence of 10<sup>11</sup> nonfluorescent molecules (e.g., water, amino acids, lipids) (Eigen and Rigler, 1994). Chemists have developed myriads of synthetic fluorophores that allow the labeling of cellular structures of interest (Haugland, 1996). Other fluorophores report aspects of cellular function, such as Ca<sup>2+</sup> and Na<sup>+</sup> concentration and membrane potential. The advent of genetically encoded fluorescent proteins (XFPs) allows the tagging of most cellular proteins of interest to measure their spatial distributions and dynamics (Tsien, 1998). XFP-based probes can report a vast range of phenomena that are critical for intracellular signal flow, such as protein-protein interactions and the activation of kinases (Miyawaki, 2005). XFPs also allow the labeling of specific and sparse subpopulations of cells (Feng et al., 2000; Gong et al., 2003; Lendvai et al., 2000). Finally, new modes of microscopy are rapidly expanding the application of fluorescence microscopy to intact tissues (Conchello and Lichtman, 2005; Denk et al., 1990, 1994; Denk and Svoboda, 1997; Helmchen and Denk, 2002, 2005; Lichtman and Conchello, 2005; Mertz,

2004; Zipfel et al., 2003; Jung et al., 2004; Levene et al., 2004).

Fluorescence techniques are well matched to problems in neurobiology. Optical microscopy can resolve single synapses (Cajal, 1995; Muller and Connor, 1991) and track spatially complex dynamics over centimeters of cortical tissue (Grinvald and Hildesheim, 2004). Whenever possible, neurons need to be studied in their natural habitat, and intact brain tissue is an especially challenging place for light microscopy. In wide-field fluorescence microscopy, contrast and resolution are degraded by strong scattering (Denk and Svoboda, 1997). Confocal microscopy can overcome some of the effects of scattering, since the detector pinhole rejects fluorescence from off-focus locations (Conchello and Lichtman, 2005; Denk and Svoboda, 1997). However, scanning a single section excites, and thereby damages, the entire specimen. Furthermore, the pinhole also rejects signal photons emanating from the focus that are scattered on their way out of the tissue. Deep in tissue, confocal microscopy becomes unacceptably wasteful in terms of signal photons (Centonze and White, 1998; Conchello and Lichtman, 2005). Compensating for signal-loss with increased fluorescence excitation leads to phototoxicity and photobleaching. Wide-field and confocal microscopy are thus techniques that are best applied to thin specimens, such as cultured preparations or the most superficial cell layer in a tissue (<20 μm) (Lichtman et al., 1987). Experiments deeper in tissue benefit from two-photon excitation (2PE; also referred to as two-photon, or multiphoton) microscopy, which allows high-resolution and high-contrast fluorescence microscopy deep in the brain (Denk et al., 1994).

2PE microscopy was invented about 15 years ago (Denk et al., 1990). More than one thousand publications have employed, developed, or reviewed 2PE microscopy (Denk and Svoboda, 1997; So et al., 2000; Zipfel et al., 2003; Helmchen and Denk, 2002, 2005). In this Primer, we will follow a brief introduction of the principles of 2PE with a discussion of technical advances and applications to neurobiology. Throughout we will point out the limitations of 2PE microscopy and suggest areas for future development.

### Principles of Two-Photon Excitation Microscopy

Photobleaching and phototoxicity, together referred to as photodamage, limit the application of fluorescence microscopy to living systems. Each excitation event carries the risk of photodamage. Optimizing fluorescence microscopy often means to minimize photodamage by maximizing the probability of detecting a signal photon per excitation event. Compared to other techniques, 2PE microscopy dramatically improves the detection of signal photons per excitation event, especially when imaging deep in highly scattering environments.

In 2PE of fluorescence, two low-energy photons (typically from the same laser) cooperate to cause a higher-energy electronic transition in a fluorescent molecule (Figure 1A). 2PE is a nonlinear process in that the

\*Correspondence: svoboda@cshl.edu (K.S.); yasuda@neuro.duke.edu (R.Y.)

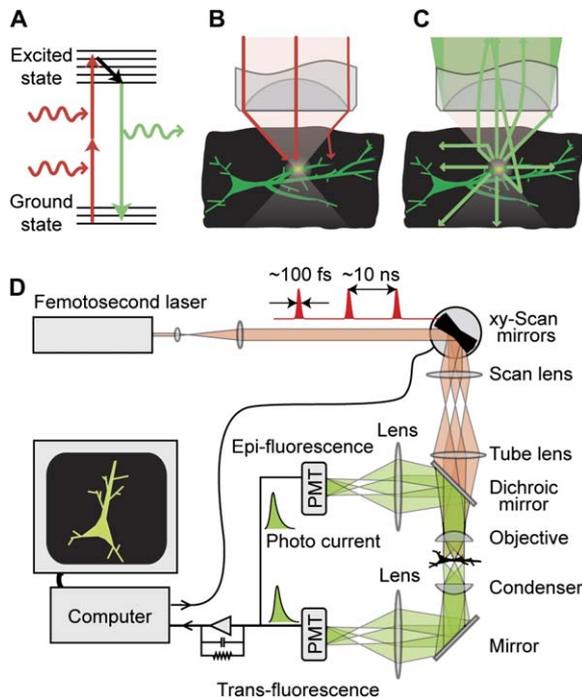


Figure 1. Two-Photon Excitation Microscopy

(A) Simplified Jablonski diagram of the 2PE process. (B) Localization of excitation in a scattering medium (black). The excitation beam (red) is focused to a diffraction-limited spot by an objective where it excites green fluorescence in a dendritic branch, but not in a nearby branch. The paths of two ballistic photons and one scattered photon are shown (red lines). Scattered photons are too dilute to cause off-focus excitation. The intensity of the beam decreases with depth as an increasing number of excitation photons are scattered. (C) Fluorescence collection in a scattering medium. Fluorescence photons are emitted isotropically from the excitation volume (red lines). Even scattered fluorescence photons contribute to the signal if they are collected by the objective. Since the field of view for detection is larger than for excitation, the fluorescence light exiting the objective back-aperture will diverge substantially (green). (D) Schematic of a 2PE microscope with epifluorescence and trans-fluorescence detection.

absorption rate depends on the second power of the light intensity. In a focused laser, the intensity is highest in the vicinity of the focus and drops off quadratically with distance above and below. As a result, fluorophores are excited almost exclusively in a tiny diffraction-limited focal volume (Figure 1B). If the beam is focused by a high numerical aperture (NA) objective, the vast majority of fluorescence excitation occurs in a focal volume that can be as small as  $\sim 0.1 \mu\text{m}^3$  (Zipfel et al., 2003).

The key consequence of localization of excitation is three-dimensional contrast and resolution (comparable to confocal microscopy) without the necessity for spatial filters in the detection path (e.g., the detector pinhole in the confocal microscope) (Wilson and Sheppard, 1984; Denk et al., 1990). To generate an image, the laser is scanned over the specimen. Since the excitation occurs only in the focal volume, all fluorescence photons captured by the microscope objective constitute useful signal. When imaging in thick specimens, the signal yield per excitation event is enhanced (Figure 1B).

The properties of 2PE discussed so far are independent of scattering (Denk et al., 1990). When excitation photons enter tissue, their paths are altered by inhomogeneities in the index of refraction (Denk et al., 1994; Denk and Svoboda, 1997; Helmchen and Denk, 2005). Depending on the type and age of the tissue and the wavelength of the light, about half of the incident photons are scattered every 50–200  $\mu\text{m}$  (Oheim et al., 2001; Yaroslavsky et al., 2002; Kleinfeld et al., 1998). The scattering of excitation light effectively reduces the light delivered to form the diffraction-limited focus (Figure 1B). Scattering also perturbs the trajectories of fluorescence photons on their way out of the tissue (Figure 1C).

Compared to one-photon techniques, 2PE provides three key advantages for microscopy in scattering specimens (Denk et al., 1994). First, the excitation wavelengths used in 2PE microscopy, deep red and near IR, penetrate tissue better than the visible wavelengths used in one-photon microscopy. This improved penetration is due to reduced scattering and reduced absorption by endogenous chromophores (Oheim et al., 2001; Svoboda and Block, 1994; Yaroslavsky et al., 2002). Second, because of the nature of nonlinear excitation, scattered excitation photons are too dilute to cause appreciable fluorescence (Figure 1B). Even deep in tissue, under conditions where most of the incidence photons are scattered, excitation is therefore still mostly limited to a small focal volume (but see the section on depth limitations). Third, because of localization of excitation, all fluorescence photons, ballistic and scattered, constitute useful signal if they are detected (Figure 1B). (In contrast, in wide-field and confocal microscopy, scattered fluorescence photons are either lost, or worse, contribute to background (Gentzone and White, 1998)). Since in typical experiments in tissue the majority of fluorescence photons are scattered, this advantage of two-photon microscopy can be huge.

Since localization of excitation is a direct consequence of the nonlinear nature of 2PE, other nonlinear contrast mechanisms (Mertz, 2004; Wilson and Sheppard, 1984), including 3PE of fluorescence (Maiti et al., 1997) and second harmonic generation (Campagnola et al., 1999; Gannaway and Sheppard, 1978), can be used for optical sectioning microscopy. 3PE may be useful to study endogenous tissue chromophores with one-photon spectra in the UV (Maiti et al., 1997; Zipfel et al., 2003). Second harmonic generation can selectively excite probes that are aligned at interfaces and may therefore be useful to study the structure and function of membranes (Moreaux et al., 2000; Dombeck et al., 2004).

#### Hardware and Software

2PE microscopy is typically implemented in a simple laser scanning microscope (Figure 1D). A laser is focused to a tight spot in the specimen plane and scanned in a raster over the sample. When the laser focus overlaps with fluorescent molecules in the sample, fluorescence photons are generated selectively in the tiny focal volume and detected by photodetectors. The signals are summed over pixel times (microseconds) and mapped to individual pixels of an image by the data acquisition computer. The simplicity of 2PE microscopes has

allowed numerous labs to adapt confocal microscopes for 2PE microscopy (Majewska et al., 2000b; Nikolenko et al., 2003; Ridsdale et al., 2004). Other labs have opted for complete custom design (Mainen et al., 1999a; Polgruto et al., 2003; Tsai et al., 2002).

The principle differences between confocal and 2PE microscopes are the laser (see below) and the fluorescence detection path. In confocal microscopy, the epifluorescence light passes back through the scan mirrors and through a pinhole before detection (Conchello and Lichtman, 2005). In 2PE microscopy, all fluorescence photons collected by the objective constitute useful signal, and the detector pinhole is not required. The optimal solution for the detection path is to project the objective back-aperture onto the photosensitive area of the photodetector (whole-field detection; Denk et al., 1995a; Mainen et al., 1999a; Tsai et al., 2002) (Figure 1D). Since fluorescence photons may be scattered multiple times before exiting the tissue, they can appear to originate from a large effective field of view (Oheim et al., 2001). Objectives with low magnifications (but maintaining high NA, e.g., 20× 0.9NA) are best for imaging in scattering tissue. However, the back-apertures of these objectives are large, and the scattered fluorescence light exiting the objective diverges rapidly (Oheim et al., 2001). To collect as many fluorescence photons as possible, it is therefore necessary to bring large focusing elements close to the objective back-aperture, which is difficult to implement in commercial microscope stands. Similar considerations apply for transfluorescence collected through the condenser (Mainen et al., 1999a) (Figure 1D).

#### **Lasers**

Because two-photon cross-sections are small, very high instantaneous intensities of excitation light are required to generate sufficient signal levels. 2PE microscopy was made practical (Denk et al., 1990) by the advent of lasers producing rapid trains of short pulses (Gosnell and Taylor, 1991). With the average power and pulse repetition rates constant, the 2PE efficiency increases as the inverse of the pulse duration. Mode-locked Ti:sapphire lasers have nearly ideal properties for 2PE microscopy. They produce a stream of pulses with repetition rates of ~100 MHz; more than 100 pulses impinge on the sample within a typical pixel time, implying that variations in the number of pulses per pixel are small (<1%). The pulse duration produced by turnkey systems is on the order of 100 fs, which may be optimal; shorter pulses would be severely distorted by dispersion in the microscope optics (Guild et al., 1997). Finally, the excitation wavelength is tunable from below 700 nm to above 1000 nm, allowing excitation of most useful fluorophores.

Ti:sapphire lasers perform relatively poorly at wavelengths that are optimal for the excitation of red fluorescent proteins (>1000 nm) (Zipfel et al., 2003). In this wavelength regime, other powerful light sources are available, for example mode-locked Ytterbium-doped lasers (Deguil et al., 2004; Honninger et al., 1998) (fixed wavelength: ranging from 1030 nm to 1060 nm) or Cr:forsterite lasers (Chen et al., 2002a) (tunable, 1200–1300 nm).

#### **Scanners**

The vast majority of laser scanning microscopes use galvanometer mirrors. They have excellent optical properties and allow zooming and image rotation. Their ma-

ior drawback is their relatively slow speed (>1 ms per line); a typical image requires ~1 s. Since many neurophysiological processes occur over milliseconds, faster scanning methods are needed.

In multifocal scanning, the beam is divided into beamlets that are simultaneously scanned across the sample (Andresen et al., 2001; Bewersdorf et al., 1998; Kurtz et al., 2006). This approach demands imaging detectors (i.e., CCDs) since the fluorescence excited by different beamlets needs to be distinguished. In scattering samples, the image will be blurred. In addition, dividing the beam into beamlets with lower power reduces nonlinear excitation.

In highly scattering tissue, point scanning with whole-field detection is preferable. Rapid raster scanning can be performed with rotating polygonal mirrors (Kim et al., 1999) or resonant galvanometers (Fan et al., 1999; Nguyen et al., 2001). These methods have excellent optical properties. However, they have the disadvantage that they are limited to raster scanning with fixed scanning speeds and do not allow zooming or image rotation.

When regions of interest are dispersed sparsely across the sample, raster scanning is slow and wasteful in terms of excitation. In random-access scanning, the beam is directed to predetermined targets in the sample. Galvanometer-mirrors support random-access scanning, but their inertia limits scan speeds to ~1 ms per spot. Acousto-optic deflectors (AOD) offer an attractive alternative. AODs use soundwaves to deflect laser beams, and they can be orders of magnitude faster than galvanometer-mirrors. However, their highly dispersive properties make compromises between excitation efficiency and image quality necessary (Iyer et al., 2003). While picosecond pulses can pass through AODs more or less unaffected, shorter pulses will be temporally and spatially distorted, leading to degradation of the excitation efficiency and the point spread function (Iyer et al., 2006). Temporal dispersion (group velocity dispersion) can be compensated with prism pairs (Iyer et al., 2003; Lechleiter et al., 2002; Roorda et al., 2004), and angular dispersion can be compensated by a prism (Lechleiter et al., 2002; Roorda et al., 2004), grating (Iyer et al., 2003), or another AOD (Ngoi et al., 2001). AODs can also be used to implement lenses for rapid scanning along the optical axis (Kaplan et al., 2001; Reddy and Saggau, 2005). Miniaturization of mechanical scanning systems using microelectromechanical systems (MEMS) technology is promising to provide other versatile approaches to fast scanning in the near future (Flusberg et al., 2005; Wang et al., 2004a).

#### **Objectives**

Objectives are critical for 2PE because they generate the tight laser focus required for localization of excitation. All major microscope manufacturers now produce objectives suitable for 2PE microscopy. Important factors include the NA, which determines resolution and the collection angle for fluorescence; the magnification, which determines the field of view; the working distance; and the efficiency of transmission of the near-IR excitation light.

#### **Detectors**

The optical demands of the detection path limit the choice of photodetectors that are suitable for 2PE

Table 1. Fluorophores and Chromophores for Two-Photon Excitation

| Fluorophores/Chromophores  | $\Phi^a$ (GM) | 2PE <sup>b</sup> (nm)                    | Em. (nm)  | Note   | References  |
|--|---------------|--|-----------|--|---|
| <b>Calcium indicators</b>  |               |  |           |  |   |
| Fluo -3, -4, -5F, 4FF et al.   |               | 810 <sup>c</sup>                         | 520–530   |  | (Yasuda et al., 2004)   |
| Oregon Green BAPTA -1, -2 et al.   |               | 810 <sup>c</sup>                         | 520       |  | (Yasuda et al., 2004)   |
| Calcium green-1 + Ca <sup>2+</sup> ; Calcium green-1 – Ca <sup>2+</sup>      | 30, 2         | 820                                      | 530       |  | (Xu and Webb, 1996; Xu et al., 1996)                              |
| Fura-2 + Ca <sup>2+</sup> ; Fura-2 – Ca <sup>2+</sup>                        | 6, 0.2        | 800                                      | 505       |  | (Wokosin et al., 2004)  |
| Indo-1 + Ca <sup>2+</sup> ; Indo-1 – Ca <sup>2+</sup>                        | 3.5, 1.5      | 700                                      | 400       |  | (Xu and Webb, 1996; Xu et al., 1996)                              |
| <b>Quantum dots</b>  |               |  |           |  |   |
| Quantum dots   | up to 47,000  | broad                                    | variable  |  | (Larson et al., 2003)   |
| <b>Fluorescent proteins</b>  |               |  |           |  |   |
| eCFP   | 100–200       | 800–900                                  | 505       |  | (Zipfel et al., 2003)   |
| eGFP   | 100–200       | 900–1000                                 | 510       |  | (Zipfel et al., 2003)   |
| eYFP   | 100–200       | 930–1000                                 | 530       |  | (Zipfel et al., 2003)   |
| mRFP, mCherry  |               | 1030 <sup>c</sup>                        | 610       | Ytterbium-doped laser                              | (Campbell et al., 2002; Shaner et al., 2004)                      |
| <b>Photoswitchable fluorescent proteins (see also Lukyanov et al., 2005)</b> |               |  |           |  |   |
| paGFP  |               | 750 <sup>g</sup>                         | 515       |  | (Patterson and Lippincott-Schwartz, 2002; Schneider et al., 2005) |
| Kaede  |               | 730 <sup>d</sup>                         | 520 → 580 | green to red; tetramer                             | (Ando et al., 2002)   |
| KFP1   |               | 1120 <sup>d</sup>                        | 600       | tetramer   | (Chudakov et al., 2003)   |
| Dronpa   |               | 780 <sup>d,e</sup> , 1010 <sup>d,f</sup> | 520       | reversible   | (Ando et al., 2004; Habuchi et al., 2005)                         |
| psCFP  |               | 800 <sup>d</sup>                         | 470 → 510 | cyan to green                                      | (Chudakov et al., 2004)   |
| PA-mRFP  |               | 760 <sup>d</sup>                         | 605       |  | (Verkhusha and Sorkin, 2005)                                      |
| KikGR  |               | 760 <sup>c</sup>                         | 520 → 590 | green to red; tetramer                             | (Tsutsui et al., 2005)  |
| Dendra   |               | 960 <sup>d</sup>                         | 505 → 575 | green to red                                       | (Gurskaya et al., 2006)   |
| mEosFP   |               | 780 <sup>d</sup>                         | 520 → 580 | green to red                                       | (Wiedenmann et al., 2004)   |
| <b>Caged glutamate</b>   |               |  |           |  |   |
| MNI-glutamate  | 0.06          | 730                                      |           |  | (Matsuzaki et al., 2001)  |
| <b>Caged calcium</b>   |               |  |           |  |   |
| DM-nitrophen   | 0.013         | 730                                      |           | $K_d$ : 2 nM <sup>h</sup> , 1.5 mM <sup>i</sup>    | (Brown et al., 1999; Momotake et al., 2006)                       |
| Azid-1   | 1.4           | 700                                      |           | $K_d$ : 230 nM <sup>h</sup> , 0.12 mM <sup>i</sup> | (Brown et al., 1999; Momotake et al., 2006)                       |
| NDBF-EGTA  | 0.6           | 710                                      |           | $K_d$ : 14 nM <sup>h</sup> , 1 mM <sup>i</sup>     | (Momotake et al., 2006)   |

<sup>a</sup> Two-photon cross-section, if known.

<sup>b</sup> Wavelength corresponding to the measured two-photon cross-section, unless indicated otherwise.

<sup>c</sup> Wavelength typically used for two-photon excitation.

<sup>d</sup> Wavelength corresponding to twice the peak of one-photon absorption. For fluorescent proteins, this is typically a good estimate of the location of the peak of the two-photon cross-section (Zipfel et al., 2003).

<sup>e</sup> Photoactivation wavelength.

<sup>f</sup> Photoquenching wavelength.

<sup>g</sup> Although its absorption maximum is at 750 nm, we recommend longer wavelength to photo-activate paGFP (e.g., 810 nm). To image paGFP without photoactivating, we use 990 nm (Ti:Sapphire) or 1030 nm (Ytterbium-doped laser).

<sup>h</sup> Affinity for calcium before photolysis.

<sup>i</sup> Affinity for calcium after photolysis.

microscopy. 2PE microscopy requires large photosensitive areas (millimeters) (Oheim et al., 2001), which precludes the use of avalanche photodiodes. Other important factors include the quantum efficiency (QE), gain, absorption spectra, dark noise, and the acceptance angle of the detector. Photomultiplier tubes (PMTs) are best for most applications. The recently developed GaAsP photocathode PMTs (Hamamatsu, H7422P) have improved QE, and short transit time spreads (jitters) which make them useful general purpose detectors, including for fluorescence lifetime imaging (see below). However, their small acceptance angles may lead to signal losses when combined with high-NA/low-magnification objectives.

#### Software

2PE microscope hardware can be assembled mostly with off-the-shelf components. An important component is suitable software to control the microscope and acquire data. Flexible and user-friendly open-source

custom software tools are freely available (Pologru et al., 2003; Tsai et al., 2002; Nguyen et al., 2006).

#### Fluorophores and Chromophores

The photophysical properties of fluorescent probes are critical for experimental design (Table 1). It is difficult to predict 2PE spectra from the one-photon spectra, because different quantum mechanical selection rules apply. The 2PE spectra of several useful fluorescent molecules have been measured (Albota et al., 1998a, 1998b; Xu and Webb, 1996; Xu et al., 1996; Bestvater et al., 2002; Spiess et al., 2005; Fisher et al., 2005; Shear et al., 1997; Wokosin et al., 2004). 2PE cross-sections are expressed in units of Göppert-Mayer (GM  $\equiv 10^{-50}$  cm<sup>4</sup> s). Excellent fluorophores, such as rhodamine B, have cross-sections larger than 100 GM (Xu and Webb, 1996). Bright one-photon fluorophores typically make good two-photon fluorophores, but exceptions have been reported (Xu and Webb, 1996). Compared

to one-photon spectra, two-photon spectra tend to be broader and shifted toward the blue. EGFP, YFP, CFP, and red proteins all have good 2PE cross-sections (Zipfel et al., 2003) ( $\sim 100$  GM). Even some endogenous chromophores can produce usable fluorescence signals under 2PE (Kasischke et al., 2004).

One advantage of the broad 2PE spectra is that a single laser can simultaneously excite multiple fluorescent molecules. The different colors can be separated using dichroic mirrors on the detection side (Xu et al., 1996; Yasuda et al., 2004). This implies that multicolor imaging often requires only a single laser and that the different color images are perfectly aligned.

All fluorescent molecules used in 2PE microscopy so far have been designed with one-photon excitation in mind. Fluorophores optimized for 2PE with very large cross-sections ( $\sim 1000$  GM) have been synthesized (Albota et al., 1998a; Porres et al., 2004). Unfortunately these probes have not yet been successfully used in biological systems.

Semiconductor nanocrystals (quantum dots, QDots) are also promising fluorescent labels for 2PE microscopy. They have broad excitation spectra, narrow emission spectra, and excellent photostability (Fu et al., 2005). QDots are reported to have superb 2PE cross-sections, up to 47,000 GM (Larson et al., 2003). A major challenge in the use of these fluorophores is their large size (hydrodynamic diameter  $\sim 30$  nm; Larson et al., 2003) and their targeting within the specimen.

2PE allows photochemistry in femtoliter volumes (Denk et al., 1990). For example, in combination with suitable caged ligands, 2PE can be used to map the distribution of ligand-gated channels on membranes (photochemical microscopy) (Denk, 1994). However, 2PE uncaging has been limited by the small cross-sections of most available cages. The cross-sections are only appreciable at very short wavelengths ( $\sim 700$  nm), where absorption by endogenous chromophores can result in unacceptable phototoxicity. The time constant of uncaging after absorption is another important factor. Some cages have usable cross-sections, but release is slow after photostimulation so that diffusion destroys the spatial resolution of photochemical microscopy (Furuta et al., 1999). Recently, a flurry of activity in the development of caging chemistry has begun to ameliorate these problems (Brown et al., 1999; DelPrincipe et al., 1999; Kantevari et al., 2006; Kiskin et al., 2002; Klein and Yakel, 2005; Matsuzaki et al., 2001; Momotake et al., 2006; Nikolenko et al., 2005; Niu and Hess, 1993).

Developments of photo-activatable and switchable fluorescence proteins (Ando et al., 2002; Chudakov et al., 2004; Patterson and Lippincott-Schwartz, 2002; Ando et al., 2004; Chudakov et al., 2003; Habuchi et al., 2005; Verkhusha and Sorkin, 2005; Wiedenmann et al., 2004; Tsutsui et al., 2005; Pakhomov et al., 2004; Gurskaya et al., 2006; Lukyanov et al., 2005) allow measurements of protein transport and recycling. 2PE cross-sections of photo-activatable GFP (Patterson and Lippincott-Schwartz, 2002; Schneider et al., 2005) are sufficiently high to visualize proteins in individual spines (Bloodgood and Sabatini, 2005).

Because of the uncertainty principle, trains of short light pulses have broad spectra (Denk et al., 1995a; Gosnell and Taylor, 1991). The dynamic interactions of these

short light pulses with fluorescent molecules are complex and not well understood. Over the last 10 years it has become clear that pulse-shaping, the control of the phases of individual spectral components, can be used to tune a variety of nonlinear processes (Dudovich et al., 2001; Meshulach and Silberberg, 1998). Pulse-shaping can be used to increase the brightness of GFP by a factor of two and, more importantly, reduce photobleaching by a factor of four, while keeping average power constant (Kawano et al., 2003). Similarly, fluorescence excitation in scattering tissue can also be enhanced (Dela Cruz et al., 2004). More work needs to be done to evaluate the exciting opportunities for pulse-shaping in the context of biological 2PE microscopy.

### Photobleaching and Phototoxicity

Photobleaching and phototoxicity play a critical role in biological imaging. It is therefore important to understand photobleaching under conditions typical for 2PE microscopy. In one-photon excitation, the rate of photobleaching is proportional to the rate of fluorescence excitation. The situation is more complicated for 2PE. For several popular fluorophores, including fluorescein and GFP, the bleaching rate increases more rapidly with intensity than the fluorescence intensity (i.e., with an exponent larger than 2) (Patterson and Piston, 2000; Chen et al., 2002b). This indicates that highly labile excited states are accessible by multiphoton absorption (Eggeling et al., 2005).

Multiphoton bleaching has three implications for 2PE microscopy. First, because photobleaching in the focal plane is higher for 2PE than 1PE, 2PE may have little benefit and may even be detrimental for imaging thin samples (i.e., a few focal sections thick). Second, the maximal rate of fluorescence that can be emitted by individual fluorophores is lower with 2PE than with 1PE. Third, the number of fluorescence photons generated before photobleaching depends on the excitation level and the pulse shape. It is therefore not necessarily advantageous to minimize the pulse duration for 2PE microscopy (Koester et al., 1999).

Even less is known about phototoxicity than photobleaching, mainly because measurements of phototoxicity require laborious physiological assays. It is likely that different processes lead to phototoxicity when using different specimens, fluorophores, and excitation wavelengths. Phototoxicity can be mediated by 1PE (Neuman et al., 1999), 2PE (Koester et al., 1999; Konig et al., 1999), or higher-order processes (Hopt and Neher, 2001). In most cases phototoxicity is dominated by excitation of exogenous fluorophores (Koester et al., 1999), but in other situations endogenous chromophores can be more important (Hopt and Neher, 2001; Konig et al., 1999).

### Depth Limitations

Depending on the properties of the tissue (and the exact definition of imaging depth) 2PE microscopy can image up to 1 mm in tissue (Beaurepaire et al., 2001; Theer et al., 2003). Although this is impressive compared to other high-resolution techniques, this depth still covers only a small fraction of the mammalian brain. The imaging depth is determined by scattering: with increasing depth, a smaller fraction of the incidence photons are

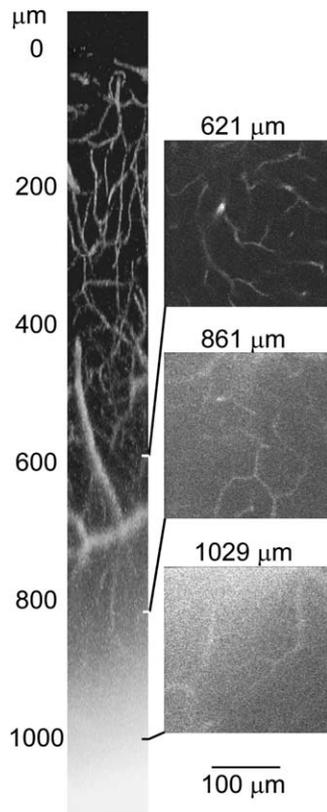


Figure 2. Cerebral Blood Vessels Imaged In Vivo

The vasculature was labeled by injecting fluorescein dextran into the circulatory stream. The light source was a regenerative amplifier. “0 μm” corresponds to the top of the brain. Left, XZ projection. Right, examples of XY projections. Note the increase in background fluorescence deeper than 600 μm in the brain due to out-of-focus 2PE. (Reprinted from Theer et al., 2003, copyright 2003, with permission from the Optical Society of America).

delivered to the focus. Since rays entering the brain at higher angles have longer paths to reach the focus they are more likely to be scattered, causing a loss of resolution with imaging depth. For example, in the mouse neocortex it is possible to image the basal dendrites of L5 pyramidal neurons, but their spines cannot be routinely resolved (unpublished data).

To compensate for scattering, higher laser powers have to be delivered to the sample. Limitations of laser power can be overcome by using more intense light pulses at the expense of repetition rate (Beaurepaire et al., 2001; Theer et al., 2003). However, imaging depth is ultimately limited by fluorescence generated at the surface of the sample by out-of-focus light, which degrades localization of excitation and contrast (Theer et al., 2003) (Figure 2). After this point is reached, increasing laser power or pulse energy further would enhance background and signal equally.

For imaging depths beyond 1 mm, it may be necessary to remove the overlying structures (Mizrahi et al., 2004). Alternatively, fiber-like lenses can be inserted into the brain to image deep structures with excellent resolution (Levene et al., 2004; Flusberg et al., 2005; Jung et al., 2004). In both of these approaches, damage to the structures of interest is a concern.

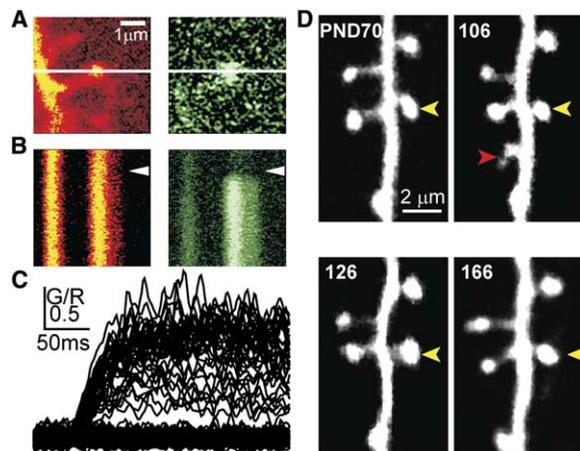


Figure 3. Imaging Small Compartments: Dendritic Spines

(A–C) 2PE microscopy calcium imaging in a neuron loaded with a  $[Ca^{2+}]$  indicator (Fluo-5F; green) and a  $Ca^{2+}$ -insensitive dye (Alexa 594, red). (A) Left, dendrite and spine (red fluorescence). The line indicates the position of the line scan used for (B) and (C). Right,  $[Ca^{2+}]$  transient after synaptic stimulation (green fluorescence,  $\Delta G$ ).

(B) Line scan images.

(C)  $[Ca^{2+}]$  changes after synaptic stimulation measured as the ratio of green/red. Note the clear separation of failures and responses, reflecting the stochastic nature of glutamate release. (Adapted from Oertner et al., 2002, with permission from Nature Publishing Group).

(D) Long-term in vivo imaging of dendrites and spines expressing GFP (A. Holtmaat, unpublished data).

### High-Resolution Imaging in Intact Tissue

The first applications of 2PE microscopy to neurobiology exploited its exquisite resolution in scattering tissue to image the structure and function of dendritic spines in brain slices. 2PE microscopy combined with  $[Ca^{2+}]$  imaging has revealed that spines function as isolated  $Ca^{2+}$  compartments.  $Ca^{2+}$  influx into spines can be dominated by different  $Ca^{2+}$  sources, depending on the physiological stimulus (Yuste and Denk, 1995; Raymond and Redman, 2006; Sabatini et al., 2002; Wang et al., 2000) (Figures 3A–3D). These measurements have further demonstrated that 2PE microscopy-based  $[Ca^{2+}]$  imaging can have sufficient sensitivity to detect the opening of a single  $Ca^{2+}$ -permeable channel (Nimchinsky et al., 2004; Sabatini and Svoboda, 2000; Yasuda et al., 2003b). Similar measurements have been used to analyze the dynamics of  $Ca^{2+}$  in dendrites (Egger et al., 2005; Goldberg et al., 2004; Nevian and Sakmann, 2004; Rozsa et al., 2004) and presynaptic boutons (Cox et al., 2000; Koester and Sakmann, 2000; Rusakov et al., 2004). 2PE microscopy-based  $[Ca^{2+}]$  imaging is an artifact by itself, and the technical details have been discussed elsewhere (Yasuda et al., 2004).

Electrophysiological analysis of synaptic currents has been an invaluable tool to study synaptic transmission (Hestrin et al., 1990), but it has been difficult to isolate the responses of individual synapses. Since there is a one-to-one correspondence between spines and synapses,  $[Ca^{2+}]$  imaging in single spines can be used to study excitatory synaptic transmission at single synapses (Yuste and Denk, 1995; Denk et al., 1995b). This approach has revealed that NMDA receptors are not saturated during low-frequency synaptic transmission (Mainen et al., 1999b) and that the release of glutamate

at single synapses can involve more than one vesicle of glutamate per action potential (Oertner et al., 2002). Measurements of  $\text{Ca}^{2+}$  accumulations mediated by  $\text{Ca}^{2+}$ -permeable AMPARs have been used to dissect the mechanisms underlying synapse-specific signaling in aspiny GABAergic neurons (Soler-Llavina and Sabatini, 2006; Goldberg et al., 2003).

From an imaging perspective, little is different when imaging *in vivo* compared to brain slices. 2PE [ $\text{Ca}^{2+}$ ] imaging has been applied to study the excitation of dendrites in the intact neocortex (Svoboda et al., 1997; Waters and Helmchen, 2004) and the olfactory bulb (Delaney et al., 2001).

The introduction of fluorescent proteins has made new types of experiments possible. XFPs can be delivered to neurons in brain slices and *in vivo* using a variety of noninvasive techniques, including transfection, viral transduction, and transgenesis methods. GFP has been used to image the structure and structural dynamics of dendritic spines in brain slices (Maletic-Savatic et al., 1999; Lang et al., 2004) and *in vivo* (Lendvai et al., 2000). Similarly, the dynamics of GFP-tagged proteins can be imaged with high resolution (Shi et al., 1999; Zito et al., 2004). In transgenic animals expressing XFPs in a subpopulation of neurons, it has been possible to image dendrites and their spines over times of months (Grutzendler et al., 2002; Lee et al., 2005; Mizrahi and Katz, 2003; Trachtenberg et al., 2002; Holtmaat et al., 2004, 2005; Zuo et al., 2005) (Figure 3E). Even thin neocortical axons (diameter  $<100$  nm) can be imaged with high signal-to-noise ratio, not only in adult transgenic mice (De Paola et al., 2006) but even in macaque monkeys (Stettler et al., 2006). These and similar approaches promise to resolve long-standing controversies about structural plasticity in the adult brain and may reveal the cellular correlates of learning and memory.

2PE microscopy is especially well suited for imaging in the retina. Since the retina's photoreceptors are exquisitely sensitive to visible light, they are rapidly bleached by conventional fluorescence microscopy methods. In contrast, the IR light used for 2PE microscopy is not absorbed by photopigments and therefore allows simultaneous functional imaging of retinal neurons and visual stimulation of photoreceptors. 2PE microscopy has been exploited to dissect the mechanisms underlying direction-selectivity in the dendrites of retinal interneurons (Euler et al., 2002; Oesch et al., 2005).

2PE microscopy has also revealed rich dynamism of non-neuronal structures *in vivo*. GFP-labeled microglia, the main immune cells of the brain, show pronounced structural dynamics over tens of seconds and rapidly migrate to sites of brain injury (Davalos et al., 2005; Nimmerjahn et al., 2005). Networks of neocortical astrocytes, bulk-labeled with  $\text{Ca}^{2+}$  indicators, show pronounced [ $\text{Ca}^{2+}$ ] fluctuations that are correlated with the state of the neuronal network (Hirase et al., 2004) and are coupled to vasodilation (Takano et al., 2006). 2PE microscopy of blood flow (Chaigneau et al., 2003; Kleinfeld et al., 1998) has been combined with nonlinear techniques for tissue ablation to study the hemodynamics in response to vascular occlusion (Schaffer et al., 2006). 2PE microscopy can track the structure of thioflavine S-stained senile plaques over time in transgenic mouse models of Alzheimer's disease (Bacskaï et al.,

2001; Christie et al., 2001). In combination with transgenic mice expressing GFP, the effects of plaques on dendritic structure (Tsai et al., 2004) and dendritic spines (Spires et al., 2005) are beginning to be assessed.

#### FRET and Fluorescence Lifetime Measurements

Fluorescence resonance energy transfer (FRET) can be used to image protein-protein interactions in cells. FRET refers to the process of energy transfer from an excited donor fluorophore to an acceptor fluorophore, mediated by dipole-dipole interactions (Förster, 1948). FRET decreases the donor fluorescence and increases the acceptor fluorescence. The efficiency of FRET,  $Y_{\text{FRET}}$ , is the fraction of absorbed photons transferred to the acceptor.  $Y_{\text{FRET}}$  is a steep function of the distance between the fluorophores over distances of  $\sim 5$  nm (Patterson et al., 2000; Selvin, 2000; Yasuda et al., 2003a), and hence FRET reports interactions between proteins fused to fluorophores (Stryer, 1978; Stryer and Haugland, 1967; Uster and Pagano, 1986). FRET can also be used to detect intramolecular conformational changes of proteins tagged with pairs of fluorophores (Clegg et al., 1992; Selvin, 2000; Yasuda et al., 2003a). Numerous protein-protein interactions have been probed with FRET microscopy (Erickson et al., 2001; Miyawaki, 2003; Zacharias et al., 2002). Combining 2PE microscopy with FRET could allow the measurement of biochemical dynamics in neuronal microcompartments within intact neural networks. FRET has been measured using two types of approaches. Intensity measurements at two or more wavelengths can report the quenching of the donor fluorescence and the enhancement of acceptor fluorescence due to FRET (Figure 4). For example, a simple measure of FRET (often used for relative measurements, i.e., before and after a stimulus) is the ratio of donor and acceptor fluorescence,  $D/A$ . This method has been combined with 2PE microscopy (Fan et al., 1999; Okamoto et al., 2004) (Figure 4).

However, interpretation of intensity measurements is complicated by the fact that donor and acceptor absorption and emission spectra overlap (spectral bleed-through) and fluorescence intensities at any wavelength depend on relative concentrations (expression levels). Intensity measurements also fail to distinguish between a small fraction of interacting molecules with high  $Y_{\text{FRET}}$  and a large fraction of interacting molecules with low  $Y_{\text{FRET}}$ . Finally, in scattering tissues  $D/A$  can be further distorted by wavelength-dependent light scattering. Various methods have been devised to deal with spectral bleed-through and fluorophore concentrations (Wallrabe and Periasamy, 2005), but these methods are difficult to combine with 2PE microscopy in intact tissue (Yasuda et al., 2006).

FRET can also be quantified using measurements of the fluorescence lifetime, the average time elapsed between fluorophore excitation and photon emission (Lakowicz, 1999). Fluorescence lifetimes are on the order of nanoseconds. Fluorescence lifetime measurements can be easily combined with 2PE microscopy (2PEFLIM) to provide quantitative FRET imaging (see the legend of Figure 5 for details). These measurements typically involve donor fluorescence only. In the absence of FRET, the donor lifetime is relatively long. FRET cooperates with the usual decay processes to shorten the

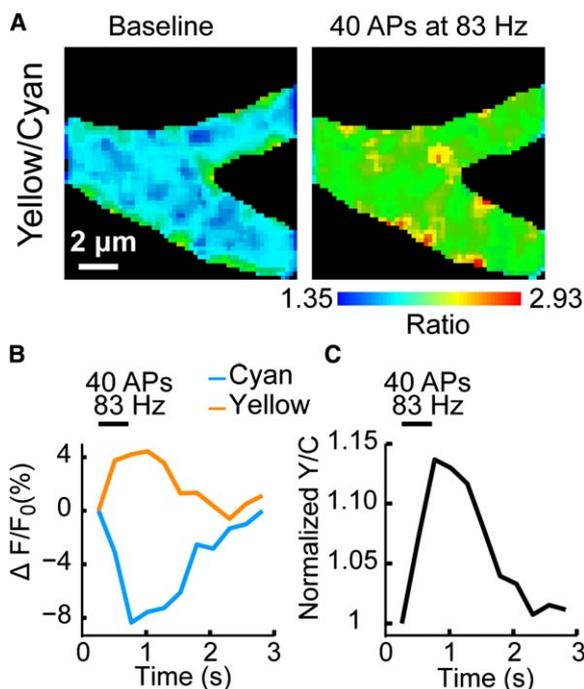


Figure 4. Two-Photon Excitation FRET Measurement

(A) FRET image of a CA1 dendrite expressing Yellow Cameleon 3.6 under baseline conditions and after a train of action potentials. Yellow Cameleon 3.6 (Nagai et al., 2004) was a gift from Atsushi Miyawaki. CFP was excited with a Ti:Sapphire laser running at 810 nm. The emission filters were CFP, 480/40; YFP, 535/50. (B) Time course of cyan and yellow fluorescence. (C) Time course of FRET ratio. (Tianyi Mao and Daniel O'Connor, unpublished data).

fluorescence lifetime. In most biologically relevant situations, where free donors and donors bound to acceptors coexist, the fluorescence decay curve will contain two exponentials. In each pixel, the fluorescence decay curves can be analyzed to derive the fraction of donor interacting with acceptor (binding fraction), perhaps the biologically most meaningful quantity (Figure 5 and legend).

With the help of suitable fluorescent probes, 2PEFLIM with time-correlated single-photon counting can quantify the binding fraction in single dendritic spines (Yasuda et al., 2006) (Figure 5). 2PEFLIM has been used to study the  $[Ca^{2+}]$ -dependent dynamics of Ras in small dendrites and spines (Yasuda et al., 2006). We expect 2PEFLIM to play a prominent role in dissecting neuronal signaling mechanisms in vivo.

### Uncaging and Photochemistry

#### Glutamate and Calcium Uncaging

2PE photolysis of glutamate and  $Ca^{2+}$  can be used to perturb cells with exquisite spatial and temporal resolution (Denk, 1994; Denk et al., 1990). However, applying 2PE photolysis in biologically rich contexts has remained challenging, mainly because the cross-sections of most cages are small and need to be excited at relatively short wavelengths ( $\sim 700$  nm), at which multiphoton absorption by endogenous chromophores produces phototoxicity (Kiskin et al., 2002). Although suitable caged compounds are still rare, the last few years

have seen some notable developments (Table 1). In particular, MNI-caged glutamate provides a sufficiently large 2PE cross-section (0.06 GM) so that glutamate can be photoreleased in synaptic clefts to mimic unitary synaptic currents (Carter and Sabatini, 2004; Matsuzaki et al., 2001; Sobczyk et al., 2005). Stimuli can be repeated sufficiently often to allow fluctuation analysis of glutamate receptors at single synapses. MNI-caged glutamate has facilitated new types of experiments. The number and properties of glutamate receptors in single postsynaptic densities have been measured (Andrasfalvy et al., 2003; Carter and Sabatini, 2004; Matsuzaki et al., 2001; Smith et al., 2003; Sobczyk et al., 2005). Combining 2PE glutamate uncaging with 2PE calcium imaging has allowed the measurement of calcium influx through NMDA receptors and calcium-permeable AMPA receptors in single spines (Carter and Sabatini, 2004; Noguchi et al., 2005; Sobczyk et al., 2005) (Figure 6). 2PE glutamate uncaging has also been used to induce synaptic plasticity in individual spines (Matsuzaki et al., 2004). Stimulation of multiple spines can be used to study the mechanisms of synaptic integration in dendrites (Gasparini and Magee, 2006).

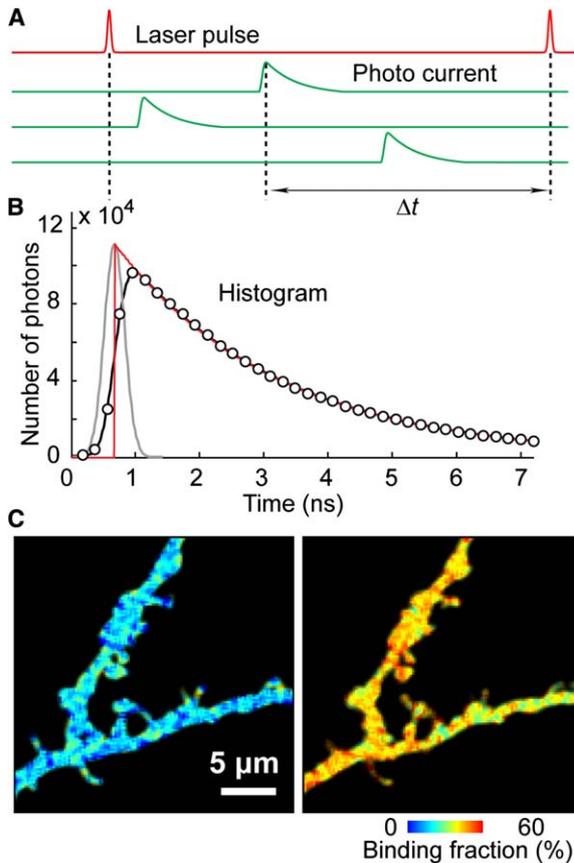
Photolysis of caged calcium can initiate calcium-sensitive signaling in presynaptic (Felmy et al., 2003a; Bollmann et al., 2000; Bollmann and Sakmann, 2005; Schneggenburger and Neher, 2000; Lou et al., 2005; Felmy et al., 2003b) and postsynaptic compartments (Yang et al., 1999) with exquisite spatial and temporal control. Although the development of caged calcium suitable for 2PE is still under development (Brown et al., 1999; Momotake et al., 2006), there have been some attempts to measure the effects of  $[Ca^{2+}]$  increases in astrocytes on cerebrovascular constrictions (Mulligan and MacVicar, 2004). Development of other compounds for 2PE uncaging will advance analyses of molecular processes in neurons at the single synapse level.

#### FRAP and Photoactivation of Fluorescence

To track molecular movements it is necessary to tag the molecules of interest in specific locations. A well-established method is to measure the fluorescence recovery after the photobleaching (FRAP). Using 2PE photobleaching, diffusion of biochemical substances through the spine neck has been measured (Majewska et al., 2000a; Pologruto et al., 2004; Sobczyk et al., 2005; Svoboda et al., 1996; Zito et al., 2004). Inversely, photoactivation of caged fluorophores (Mitchison et al., 1998; Svoboda et al., 1996) and photo-activatable XFPs (Lukyanov et al., 2005) can also be used to measure the diffusion and trafficking of tagged molecules (Bloodgood and Sabatini, 2005) (Figure 7). Photoactivation may offer advantages over FRAP. First, because most photo-activatable fluorescent proteins produce little fluorescence before photo-activation, the measurements have intrinsically higher signal-to-noise ratios: the number of photons required to detect a fluorescence change,  $\Delta F$ , from the baseline fluorescence  $F_0$ , is inversely proportional to  $(\Delta F/F_0)^2$  (Yasuda et al., 2004). Second, photoactivation may produce fewer free radicals and thus induce less phototoxicity.

#### Photoporation

The high peak intensities of light achievable with pulsed lasers have also been used to directly disrupt biological



**Figure 5.** Two-Photon Excitation Fluorescence Lifetime Imaging (A) Schematic illustrating the principle of time-correlated photon counting (Becker et al., 2001). The time between photons and the next laser pulse is measured. (B) Measured histogram of photon arrival times (open circles). The curve can be decomposed into the true fluorescence decay curve (red) and a pulse response function (gray) that reflects the finite response time of the detector (Yasuda et al., 2006). (C) 2PEFLIM images of dendrites expressing a FRET sensor for Ras activation before and after membrane depolarization (Yasuda et al., 2006). Each pixel can be expressed as the fraction of Ras bound to acceptor.

Fluorescence lifetime measurements typically involve donor fluorescence only. In the absence of FRET, the time course of the donor fluorescence,  $F$ , after a short pulse of excitation is

$$F = F_0 \exp(-t/\tau_D), \quad (1)$$

where  $\tau_D$  is the fluorescence lifetime of the donor in the absence of acceptor. When FRET occurs, the excited state lifetime of the donor is shortened.

Note that the fluorescence lifetime decreases when FRET occurs. The FRET efficiency, which is defined as the fraction of donor fluorescence quenched by acceptor, can be expressed in measurable quantities simply as

$$Y_{\text{FRET}} = 1 - \tau/\tau_D, \quad (2)$$

where  $\tau$  is the fluorescence lifetime of the donor bound to acceptor. Since only the donor fluorescence is involved, fluorescence lifetime measurements of FRET are independent of fluorophore concentrations and insensitive to wavelength-dependent light scattering. In biologically relevant situations at least two populations of donor co-exist: free donors and donors bound to acceptors. The fluorescence decay curve will contain two exponentials (Lakowicz, 1999):

$$F(t) = F_0 \cdot [P_{\text{AD}} \exp(t/\tau_{\text{AD}}) + P_{\text{D}} \exp(t/\tau_{\text{D}})], \quad (3)$$

where  $P_{\text{AD}}$  and  $P_{\text{D}}$  are the fraction of donor bound and unbound to acceptor, respectively ( $P_{\text{AD}} + P_{\text{D}} = 1$ ).  $P_{\text{AD}}$  is also called the binding

fraction. Illumination with a highly focused beam from a Ti:sapphire laser can cause small, reversible membrane perforations to cause DNA transfection (Tirlapur and König, 2002) or membrane depolarization (Hirase et al., 2002). Larger pulse energies, for example produced by regenerative amplifiers, can be used to perform microsurgery (Nishimura et al., 2006) and sectioning for anatomy (Tsai et al., 2003) down to the level of single axons (Yanik et al., 2004).

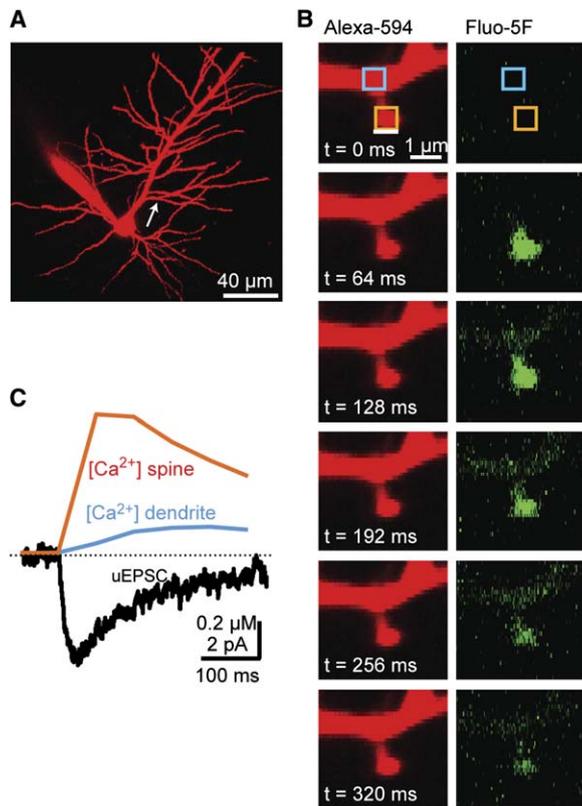
### Imaging Neuronal Populations

Single-unit studies employing tungsten microelectrodes have revolutionized our understanding of the functional organization of the brain. However, extracellular recordings have major drawbacks. Only one (or a few) neuron is interrogated at a time, and the cell type and location are not well defined. In addition, only neurons that respond vigorously to the stimulus of interest are usually studied, biasing the sample. 2PE microscopy, in combination with fluorescent probes of neuronal function, has the potential to overcome these problems.

Direct measurement of membrane depolarization can be achieved with voltage-sensitive dyes (Grinvald and Hildesheim, 2004). However, imaging with single-cell resolution has remained challenging except in the most favorable circumstances (Baker et al., 2005). Alternatively, action potentials open voltage-gated calcium channels (VGCCs) and cause  $\text{Ca}^{2+}$  influx into the cytoplasm, which can be readily detected using  $[\text{Ca}^{2+}]$  imaging (Helmchen et al., 1996; Svoboda et al., 1997). Populations of neurons can be loaded with membrane-permeable  $\text{Ca}^{2+}$  indicators in vitro (Yuste et al., 1992) and in vivo (Stosiek et al., 2003). Using these techniques it has been possible to track the dynamics of populations of individual

fraction. Thus, one can derive the fraction of the donor population bound to acceptor from the fluorescence decay curve. It should be noted that sensors for fluorescence lifetime measurements have different design criteria than those for intensity-based methods (Yasuda et al., 2006).

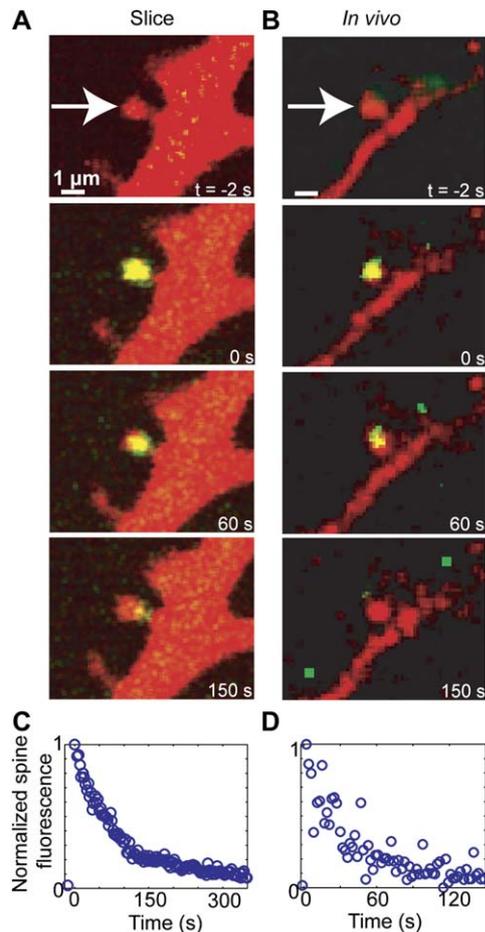
Methods for fluorescence lifetime measurements fall broadly into two groups: time-domain and frequency-domain (Gratton et al., 2003; Lakowicz, 1999). Time-domain methods directly measure the fluorescence decay after a brief excitation pulse. They are typically implemented using time-correlated photon counting (TCSPC) (A), where the timing between laser and photon pulses is measured. This method has high sensitivity, since each photon contributes to the signal. However, because photon counting rates saturate at fairly low levels ( $\sim 10^6$  photons per second) (Becker et al., 2001; Lakowicz, 1999) TCSPC-based imaging may be too slow for some applications (e.g., 1000 photons per histogram in a  $128 \times 128$  pixels image will require  $\sim 15$  s of acquisition time). TCSPC is naturally and easily combined with 2PE since the laser is already pulsed (Becker et al., 2001; Jakobs et al., 2000; Peter et al., 2005; Straub and Hell, 1998). Therefore TCSPC lends itself to high-sensitivity imaging in scattering tissues (Yasuda et al., 2006). Using frequency-domain methods, the fluorescence lifetime is obtained by modulating both excitation intensity and detector gain typically with a sinusoidal function (Philip and Carlsson, 2003; Piston et al., 1992; So et al., 1995; Squire et al., 2000). In contrast to TCSPC, this methods allows unlimited counting rates and thus potentially better temporal resolutions. However, frequency-domain methods are wasteful since only a fraction of detected photons contribute to the signal (Philip and Carlsson, 2003). When measuring fluorescence lifetimes in small subcellular compartments such as dendrites, dendritic spines or axonal boutons (C), the number of photons available is limited and TCSPC methods are preferable (Gratton et al., 2003).



**Figure 6. Simultaneous 2PE Glutamate Uncaging and 2PE Microscopy  $[Ca^{2+}]$  Imaging at Single Spines**  
(A) A neuron loaded with a  $[Ca^{2+}]$  indicator (Fluo-5F; green) and a  $Ca^{2+}$ -insensitive dye (Alexa 594, orange).  
(B) High-magnification time-lapse image of a region of interest (white arrow in [A]). 2PE photolysis of glutamate is indicated by the white bar and occurred immediately after the first image.  
(C) NMDA-receptor-mediated current measured at the soma (black) and changes in calcium concentration (regions of measurement indicated by boxes in [B]).  $\Delta G/R = 1$  corresponds to  $[Ca^{2+}] = 220$  nM. Adapted from Sobczyk et al., 2005.

neurons in vitro (Cossart et al., 2003, 2005) and in vivo (Kerr et al., 2005; Stosiek et al., 2003) (Figure 8). For example,  $[Ca^{2+}]$  imaging can reveal the fine-scale organization of the developing zebrafish tectum (Niell and Smith, 2005), cortical maps in the cat visual cortex (Ohki et al., 2005), and olfactory responses in the zebrafish olfactory bulb (Yaksi and Friedrich, 2006).

A drawback of  $[Ca^{2+}]$  indicators is that it is not straightforward to relate fluorescence changes to number of spikes or spike timing. First, the coupling between activity and  $Ca^{2+}$  accumulations depends on a number of factors that are difficult to control:  $Ca^{2+}$  influx per action potential varies from cell to cell; the  $[Ca^{2+}]$  change depends on the amount of  $[Ca^{2+}]$  indicator trapped in the cell and the size of the compartment imaged;  $Ca^{2+}$  can enter the cell not only through VGCCs but also through synaptic receptors and through calcium-induced calcium release. Second, the relationship between fractional fluorescence changes and  $Ca^{2+}$  accumulations by itself is complex: resting  $Ca^{2+}$  levels vary from cell to cell and compartment to compartment; membrane-permeable forms of  $[Ca^{2+}]$  indicators are often trapped in intracellular compartments, producing



**Figure 7. 2PE Activation of Photoactivatable GFP**  
(A and B) Images of actin tagged with pa-GFP (Patterson and Lippincott-Schwartz, 2002) in a cultured brain slice (A) and in the barrel cortex in vivo (B). Cells were also expressing mCherry (Shaner et al., 2004) as a volume marker. Spines indicated with the arrows were activated using 780 nm light. Imaging was with an Ytterbium-doped laser running at 1030 nm (R. Weimer and N. Gray, unpublished data). (C and D) The time course of green fluorescence in the stimulated spines.

variable static baseline fluorescence;  $Ca^{2+}$  indicators respond sublinearly to  $[Ca^{2+}]$  increases. It is sometimes possible to detect saw-tooth-shaped fluorescence transients corresponding to single spikes (Cossart et al., 2005; Helmchen et al., 1996; Maravall et al., 2000; Svoboda et al., 1997), but the properties of the noise sources in the intact brain may not allow single spike detection reliably in vivo (Kerr et al., 2005; T. Sato and K.S., unpublished observations).

Genetically encoded  $[Ca^{2+}]$  indicators (GECI) may offer an alternative to synthetic indicators. GECIs have been used in combination with 2PE in *Drosophila* (Wang et al., 2003, 2004b) and mice (Hasan et al., 2004). With current GECIs, their slow response kinetics, small dynamic range, and nonlinear behavior only add to the problems discussed for synthetic indicators above (Pologruto et al., 2004). However, progress in indicator development (Diez-Garcia et al., 2005; Guerrero et al., 2005; Mank et al., 2006; Reiff et al., 2005; Palmer et al., 2006; Nagai et al., 2004) and data analysis tools (Yaksi

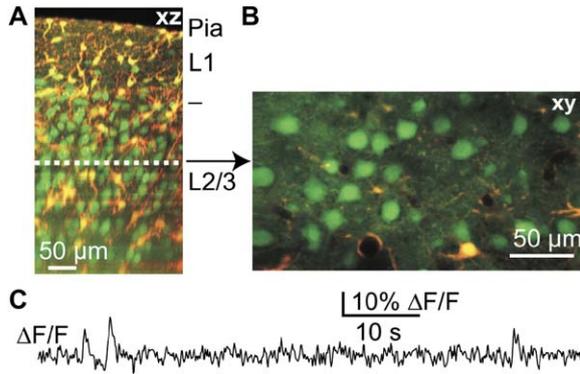


Figure 8. Imaging Neuronal Populations

(A and B) In vivo images of neurons (green, bulk-labeled with Oregon Green Bapta-1 AM) and astrocytes (red-yellow, bulk-labeled with sulforhodamine) (Kerr et al., 2005).

(C) Time course of fluorescence fluctuations in a single neuron.  $[Ca^{2+}]$  transients in response to individual spikes can be detected (Kerr et al., 2005). (Reprinted and modified from Kerr et al., 2005, copyright 2005, National Academy of Sciences, USA).

and Friedrich, 2006) are rapid, and substantial advances can be expected over the next few years.

### Conclusions and Outlook

2PE microscopy is a maturing technology that is contributing to discoveries in neurobiology on many spatio-temporal scales. The limits of 2PE microscopy and the underlying mechanisms are becoming understood. This knowledge is driving technical advances to overcome these limits. 2PE microscopy will continue to benefit from developments in optical physics and optoelectronics. Careful manipulation of the spectral components of the excitation light will allow 2PE microscopy to penetrate deeper in tissue with reduced photodamage (Oron and Silberberg, 2005; Zhu et al., 2005). Adaptive optics may help to compensate for scattering to further improve imaging depth (Feierabend et al., 2004; Neil et al., 2000). A marriage between micromechanical approaches, new fiber optics (Gobel et al., 2004; Ouzounov et al., 2002), and laser scanning microscopy (Wang et al., 2004a) will allow the development of tiny head-mounted 2PE microscopes for imaging in behaving animals, including mice (Flusberg et al., 2005; Helmchen et al., 2001). The development of biocompatible synthetic fluorophores with ultra-large cross-sections would have a profound impact on neuroscience research. It may be possible to mutate or evolve fluorescent proteins for brightness under 2PE (Zacharias and Tsien, 2006). These and other advances will continue to enhance the impact of 2PE microscopy on neuroscience research.

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