Multiphoton Fluorescence Light Microscopy

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Advanced article

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Multiphoton fluorescence microscopy is a powerful imaging technique that depends on complex quantum mechanical interactions between photons and matter for fluorophore excitation. In conventional fluorescence microscopy, a fluorescent molecule is pumped to an excited state by absorbing a single photon. The molecule subsequently falls back to its ground state by emitting a less energetic photon. This is a linear process of absorbing and emitting energy in the form of single photons. By contrast, multiphoton microscopy is based on nonlinear interactions between light and matter, whereby multiple photons are absorbed to bring single fluorophore molecules to an excited state. Two-photon fluorescence microscopy is the most commonly used multiphoton imaging technique. In two-photon microscopy, the fluorescent molecule absorbs two photons simultaneously in a single event, and their combined energies provoke the electronic transition of the molecule to the excited state. Advantages of two-photon fluorescence, compared to typical single-photon epifluorescence microscopy, include reduced autofluorescence, deeper tissue penetration, inherent confocality and three-dimensional (3D) imaging as well as minimised photobleaching and photodamage. Thus, two-photon microscopy facilitates optical sectioning of thick biological specimens *in vivo*, which would not be possible with conventional imaging techniques. Recent advances in fluorescence microscopy have expanded the application spectrum and usability of multiphoton imaging, which has become an important and versatile tool in modern biomedical research.

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Introduction

The invention of two-photon fluorescence light microscopy (Denk *et al*., 1994) revolutionised three-dimensional (3D) *in vivo* imaging of cells and tissues. As shown in **Table 1**, progress in the development of two-photon microscopy, combined with other technological breakthroughs in past decades has rendered two-photon imaging methodologies accessible to the scientific community (Phan and Bullen, 2010). The theoretical basis of two-photon excitation was established by Goeppert-Mayer in 1931, and this photophysical effect was verified experimentally by Kaiser and Garret in 1963. Typical fluorescence requires a single photon of the appropriate energy to interact with a fluorophore (a molecule that fluoresces). The photon is absorbed and its energy causes the transition of the fluorophore to an excited electronic state. This higher electronic state is unstable and the molecule, within a short period of time (10^{-8}–10^{-9} s), returns to its ground state by emitting a new photon, which has less energy than the exciting photon. This excitation–emission process typically requires photons in the ultraviolet (UV) or blue/green spectral range. In two-photon excitation a fluorophore is excited by the simultaneous absorption of two photons (multiphoton absorption) (**Figure 1**). Each of these two photons has about half of the energy that is normally required to excite the molecule from its ground state. This process typically requires photons in the infrared spectral range. Since two-photon excitation depends on the simultaneous absorption of two infrared photons, the probability of two-photon absorption by a fluorescent molecule

Table 1 Milestones relevant to the development of two-photon microscopy

Year	Advance
1929	Maria Goppert predicts two-photon excitation
1962	Osamu Shimomura discovers aquoerin
1978	Thomas Cremer and Christoph Cremer develop the laser scanning confocal microscope
1983	Agard and Sedat develop deconvolution algorithms for image restoration and elimination of out-of-focus fluorescence
1990	Denk and co-workers develop the two-photon microscope
1991	Single-box Ti:Sapphire laser is introduced for two-photon excitation
1994	Martin Chalfie uses the Green Fluorescent Protein (GFP) as a genetic marker
1993	Bacskai and Tsien use FRET to resolve spatially dynamics of camp and protein kinase A subunits in *Aplysia* sensory neurons
1995	Tsien and colleagues engineer enhanced GFP (eGFP)
1996	Bio-Rad introduces the first commercial two-photon microscope
1996	Tsien and colleagues develop GFP variants: eCFP and eYFP
1999	Lukyanov and colleagues clone dsRed
2001	Denk and co-workers develop a miniature head-mounted two-photon microscope
2002	Miyawaki and colleagues clone Kaede, a green fluorescent protein that can be photoconverted to emit in the red upon exposure to UV light
2002	Tsien and colleagues generate the monomeric mRFP1 variant of dsRed
2004	Tsien and colleagues develop improved monomeric (mCherry) and tandem (tdTomato) variants of dsRed
2008	Gu and co-workers describe the first hand-held two-photon microendoscope
2009	Tsien and colleagues develop infrared fluorescent dyes derived from bacterial phytochromes

Adapted from (Agard and Sedat, 1983; Bacskai *et al*., 1993; Phan and Bullen, 2010).

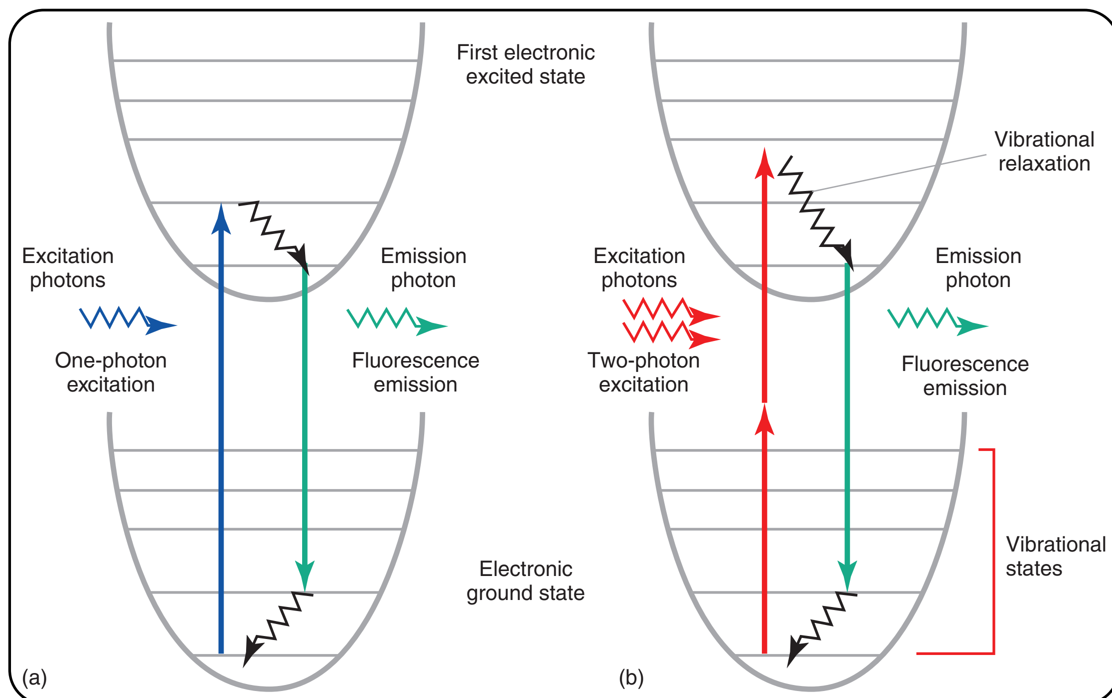

Figure 1 Jablonski diagram of one-photon (a) and two-photon (b) excitation, which occurs as fluorophores are excited from the ground state to the first electronic states. One-photon excitation occurs through the absorption of a single photon. Two-photon excitation occurs through the absorption of two lower-energy photons via short-lived intermediate states. After either excitation process, the fluorophore relaxes to the lowest energy level of the first excited electronic states via vibrational processes. The subsequent fluorescence emission process for both relaxation modes is the same.

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is a quadratic function of the excitation radiance. Under sufficiently intense excitation, three- and higher-photon excitation is also possible and deep UV microscopy based on these processes has been developed. **See also**: **The Development of Fluorescence Microscopy**

Design of a Multiphoton Microscope

Although the possibility of nonlinear excitation was first documented by Sheppard and Kompfer in 1978, two-photon microscopy was not practically demonstrated until 1990 (Denk *et al*., 1990). These early studies indicated that two-photon excitation processes could be exploited for 3D imaging. Indeed, inherent optical sectioning (confocality) is one of the most important properties of two-photon microscopes equipped with high numerical aperture objective lenses. In single-photon excitation of spatially uniform samples, fluorescence signals are generated equally from each z-section above and below the focal plane. By contrast, in two-photon excitation, over 80% of the total fluorescence signal is confined to the focal point region, approximately 1 μm thick. This sharp depth discrimination is the result of the inverse quadratic dependence of excitation probability on the spatial distribution of the excitation radiance. Appreciable two-photon fluorescence occurs only at the objective lens focal volume, where photon density is high; negligible fluorescence is excited outside of this volume (**Figure 2a**, **b**). The typical two-photon excitation point spread function when 960 nm excitation light is focused by a 1.25 NA objective has a full width at half-maximum of 0.3 μm in the radial direction and 0.9 μm in the axial direction (**Figure 2c**).

Two-photon excitation efficiency is maximised when laser light is focused to a diffraction-limited volume. **Figure 3** shows a typical two-photon microscope design. A critical component in a two-photon microscope is its light source; a high-radiance light source on the order of 10^{10}–10^{12} W cm^{-2} is required for efficient excitation. Two-photon microscopy utilising continuous-wave lasers has been demonstrated. Adequate radiance level can in principle be achieved by focusing light from a 1 W continuous-wave laser to a 10^{-9} cm^2 diffraction-limited focal volume. However, the high average laser power is a concern for pigmented biological samples with appreciable one-photon absorption. To offset considerable specimen photodamage, high repetition rate (100 MHz), ultrafast (femtosecond or picosecond pulse widths) lasers, such as titanium-sapphire and Nd:YLF lasers, are the most widely used light sources in modern implementations of multiphoton microscopy. The higher peak power

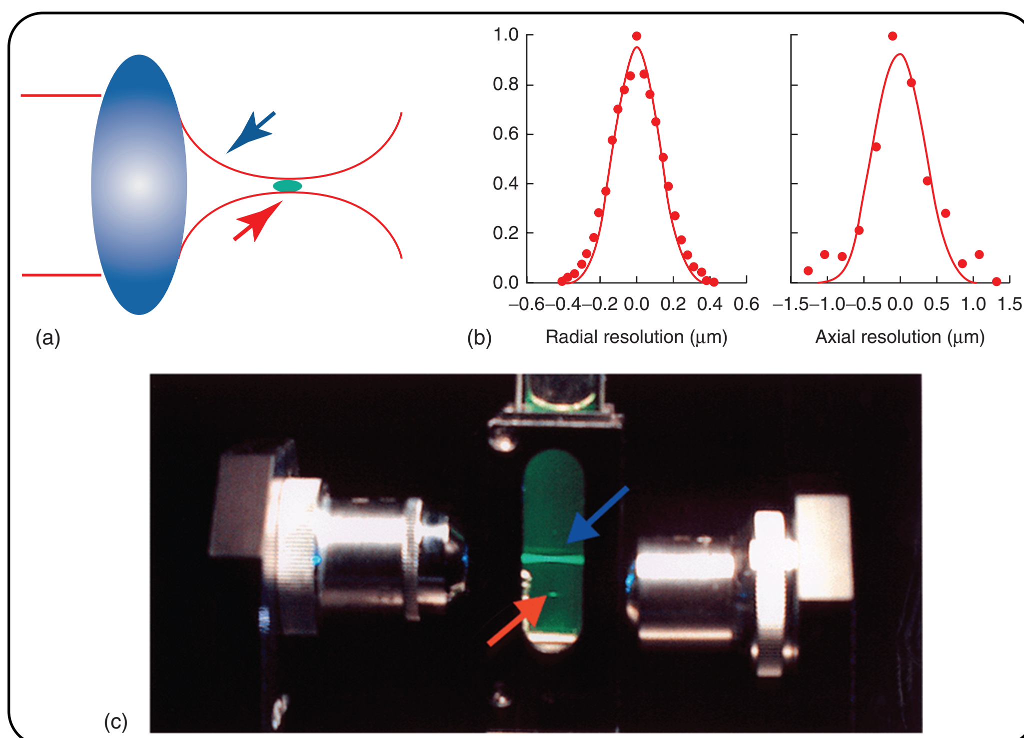

Figure 2 A schematic representation of the localisation of two-photon excitation. (a) Infrared light (blue arrow) is focused by an objective lens and fluorescence (red arrow) occurs only at the focal volume. (b) A detailed excitation profile of the two-photon excitation volume. The full width at half-maximum of the excitation profile is 0.3 μm along the radial direction (left) and is 0.9 μm along the longitudinal direction (right) at a laser wavelength of 960 nm. (c) A demonstration of the localisation of two-photon excitation volume. Fluorescein solution is excited by one-photon excitation (blue arrow) via a 0.1 numerical aperture objective; fluorescence excitation is observed throughout the path of the laser beam. For two-photon excitation using a second objective with the same numerical aperture (red arrow), fluorescence excitation occurs only from a 3D localised spot.

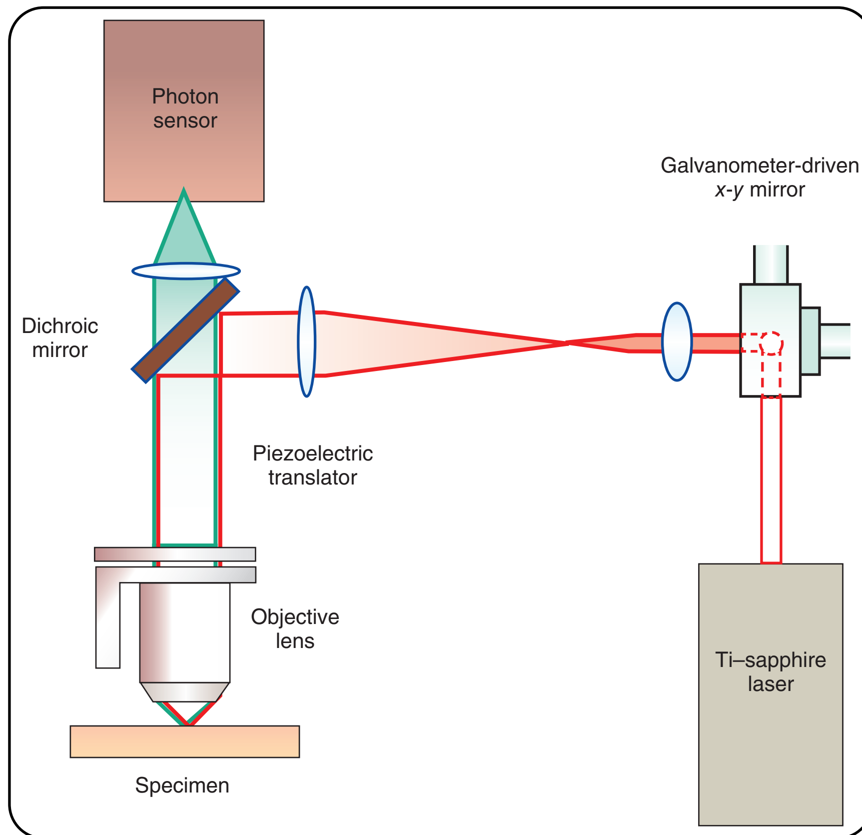

Figure 3 A schematic drawing of typical components in a two-photon microscope. This system typically consists of a high-peak-power pulsed laser, a high-throughput scanning microscope and high-sensitivity detection circuitry.

and the lower duty cycle of these lasers minimise average power deposition in biological samples, while maximising two-photon excitation efficiency.

Figure 3 shows a laser excitation beam directed into the microscope via an epiluminescence light path. The excitation light is reflected by a dichroic mirror to the microscope objective and is focused in the specimen. Two-photon induced fluorescence is generated at the diffraction-limited volume. Images are constructed by raster scanning the fluorescent volume in three dimensions using a galvanometer-driven x–y scanner and a piezo-objective z-driver. Emission signals are collected by the same objective and transmitted through a dichroic mirror along the emission path (epifluorescence). An additional barrier filter is also required to further attenuate scattered excitation light. High-sensitivity detection electronics, such as single-photon counting circuitry, are used to ensure maximal detection efficiency and signal dynamic range.

Fluorophores for Multiphoton Microscopy

One-photon and two-photon excitation are fundamentally different quantum-mechanical processes and obey very different selection rules. Thus two-photon absorption spectra are not direct derivatives of the respective one-photon spectra. As a result, the two-photon excitation spectrum of a fluorophore, scaled to half the wavelength is typically not equivalent to its one-photon excitation spectrum. Ideally, wavelength scans are performed to derive two-photon absorption spectra for each fluorescent dye or protein and determine the optimal excitation wavelength for maximum brightness (Dickinson *et al*., 2003). A wide range of fluorescent proteins have been systemically characterised for their two-photon absorption properties (Drobizhev *et al*., 2011; Tillo *et al*., 2010). The emission spectrum of a fluorophore, in the absence of ground-state heterogeneity, is independent of the excitation mechanism, since the molecule relaxes to the same excited state through vibrational mechanisms before emission. Therefore, fluorophore emission spectra remain essentially unchanged between one- and two-photon excitation modalities.

Technological developments in multiphoton microscopy have generated considerable interest in the development and characterisation of new fluorophores with properties that would allow harnessing the powers of new imaging modalities. Recently, infrared fluorescent proteins derived from bacterial phytochromes were described (Shu *et al*., 2009). The use of such fluorescent proteins increases the capacity for deeper tissue penetration because these proteins both absorb and emit wavelengths that are less affected

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by light scattering and tissue opacity. Moreover, photoactivatable fluorescent proteins markers such as Kaede (Ando *et al*., 2002), eosFP (Wiedenmann *et al*., 2004), Dendra (Gurskaya *et al*., 2006) and kikume Green–Red (kikGR; Habuchi *et al*., 2008) have been developed that shift from green to red fluorescence upon exposure to UV light. This type of optical marker can be used for single-cell marking and tracking for long periods of time (Hatta *et al*., 2006; Kohli *et al*., 2011). **See also**: **Green Fluorescent Protein (GFP)**

In addition to fluorescent proteins, the use of small organic fluorescent molecules, coupled with multiphoton imaging, is becoming indispensable in modern biomedical research. Such fluorescent probes can provide information about the localisation and quantity of biomolecules of interest. The advantage of these probes over the fluorescent proteins is that they can be used directly with biological specimens without the need of prior genetic engineering. New fluorescent probes with improved properties are becoming available, designed for a variety of target biomolecules. For example, there is a variety of fluorescent probes that can be used for the detection of lipid rafts (Kim *et al*., 2007a), specific organelles (Kim and Cho, 2013), various metal ions such as Ca^{2+}(Kim *et al*., 2007c), Mg^{2+} (Kim *et al*., 2007b), Zn^{2+} (Singh *et al*., 2015) and other analytes including reactive oxygen species (ROS; Soh, 2006), reactive nitrogen species (RNS; Gomes *et al*., 2006), anions (O'Neil and Smith, 2006) and saccharides (Cao and Heagy, 2004). Despite the fact that numerous probes have been developed so far, none of them is ideal for all applications. The next generation fluorescent probes are anticipated to exhibit increased photostability, brightness and a wide range of defined intracellular localisation patterns. **See also**: **Fluorescent Analogues in Biological Research**; **Fluorescent Probes Used for Measuring Intracellular Calcium**

Comparison of Conventional, Confocal and Multiphoton Microscopy

Conventional light microscopy is an important tool in the field of biology. Brightfield and darkfield microscopy are some of the simplest microscopy techniques. In brightfield microscopy the sample is illuminated by white light and contrast is obtained by the differential absorbance of the transmitted light. The simplicity of brightfield microscopy makes it a very popular imaging method. In darkfield microscopy, while the whole sample is illuminated by light, transmitted light is rejected and only scattered light is collected by the objective lenses. This microscopy technique can be used to enhance the contrast in unstained and live biological samples. Conventional microscopy approaches are limited by the scattering and shallow penetration of light, allowing observation of only relatively thin samples. Thus, ability to resolve microscopic structures in optically thick samples is limited because the image at the focal plane is blurred by out-of-focus noise. The invention of confocal microscopy in the 1960s and two-photon microscopy in the 1990s has started to address 3D imaging needs. Confocal microscopy solves the problem of blurring by rejecting signals that come from above and below the focal plane. This is achieved optically, by using a focused scanning laser beam to illuminate the sample and by placing a pinhole aperture in front of the electronic photon detector. **See also**: **Confocal Microscopy**; **Fluorescence Microscopy**; **Light Microscopy – Brightfield and Darkfield Illumination**; **Single-Molecule Light Microscopy**

Two-photon microscopy has a number of additional advantages. Firstly, in two-photon microscopy light of longer wavelength is used. Near infrared wavelengths are poorly absorbed and less scattered in biological samples. This allows penetration of the excitation light deeper into the specimen. Therefore, two-photon microscopy permits examination of thick biological samples. Also, the use of infrared wavelengths reduces photodamage and photobleaching, since photons carry less energy and fluorophores are excited only at the focal plane. This is particularly important when a specific region needs to be imaged repeatedly over a long period of time. Furthermore, because in two-photon microscopy excitation only occurs at the focal plane, confocality is inherent, alleviating the requirement for pinholes and the consequent reduction of sensitivity due to photon loss. Finally, two-photon microscopy can be used to trigger controlled and localised photochemical reactions in a specimen without unwanted, spurious effects outside of the focal plane (Denk *et al*., 1994). **See also**: **Cell Staining: Fluorescent Labelling of the Golgi Apparatus**; **Fluorescence Resonance Energy Transfer**

Limitations of Multiphoton Microscopy

Despite the potential advantages of two-photon microscopy, some limitations and drawbacks remain to be solved. Compared with confocal microscopy operating at UV or blue–green excitation wavelengths, two-photon microscopy minimises photobleaching and photodamage. Unlike in confocal microscopy, where the sample is illuminated in its entire volume, photobleaching and photodamage are limited to a sub-femtolitre volume at the focal point. This reduction in photodamage volume results in a dramatic increase in the viability of the biological sample. However, two-photon excitation causes considerable photobleaching at the focal plane. Although, in single-photon microscopy photobleaching depends mainly on the excitation wavelength, in two-photon microscopy photobleaching depends on the photon density at the focal point (Kalies *et al*., 2011; Patterson and Piston, 2000). Also, in pigmented samples thermal damage is observed at the focal plane because of local heating from absorption of infrared light at high laser power. In addition, photodamage can be caused by two-photon or higher-photon excitation of endogenous and exogenous fluorophores similar to that of ultraviolet irradiation. Fluorophores may also act as photosensitisers in photooxidative processes. Photoactivation of these fluorophores results in the formation of reactive oxygen species that trigger successive biochemical damage cascades in cells (Konig *et al*., 1996). Furthermore, in specific settings, it is possible that near infrared excitation light may interact linearly with chromophores in the sample. For example, this could happen in plant tissues, where the photosynthetic complex absorbs near infrared radiation (Ustione and Piston, 2011). Finally, specific two-photon applications suffer

from important drawbacks, including poor temporal resolution and low signal-to-noise- ratio. For example, it is difficult to evaluate and correlate fluorescent changes of calcium indicators to neuronal spikes, both in terms of monitoring the exact numbers of action potentials and timing between them (Gobel and Helmchen, 2007; Grewe and Helmchen, 2009). However, improvements and new developments in speed acquisition photodetector technology, redesigning and generation of new synthetic or genetically encoded molecular indicators are steadily overcoming these limitations (Cheng *et al*., 2011; Duemani Reddy *et al*., 2008; Grewe *et al*., 2010; Looger and Griesbeck, 2012).

Applications and Possibilities for Two-photon Microscopy

Two-photon microscopy is expected to have an impact in areas such as physiology, neurobiology, embryology and tissue engineering, for which imaging of highly scattering tissue is required. Two-photon microscopy has been used successfully to study the development of model organisms such as *Danio renio* (Carvalho and Heisenberg, 2009; Yaniv *et al*., 2006), *Caenorhabditis elegans* (Filippidis *et al*., 2009; Ji *et al*., 2008) and *Drosophila melanogaster* (Rebollo and Gonzalez, 2010). These are areas where traditional confocal microscopy fails because photodamage substantially reduces the viability of the specimens. The main advantage of two-photon microscopy is its ability to maintain the resolution and contrast deep within scattering tissues. This allows direct visualisation of cells and their network dynamics, *in situ*, while they are maintained embedded in their natural environment. In addition, monitoring of cellular function, responses and organelles after systemic manipulations is greatly facilitated (Anzalone *et al*., 2014; Chen *et al*., 2011; Kawakami *et al*., 2013; Niell and Smith, 2005; Ohki *et al*., 2005; Sheffield and Dombeck, 2015). Two-photon microscopy can also be coupled with optogenetics and photorelease of caged molecules, including neurotransmitters among others, for precise spatiotemporal manipulations, simultaneous regulation and monitoring of cellular activity within intact neuronal circuits (Bernstein *et al*., 2012; Deisseroth, 2011; Ellis-Davies, 2009; Fenno *et al*., 2011; Peron and Svoboda, 2011). Moreover, *in vivo* two-photon imaging allows studies of structural and functional changes in an organism over long period of time. The development of a miniaturised fibre-optic two-photon microscope has opened new possibilities for *in vivo* imaging of freely moving and behaving animals (Engelbrecht *et al*., 2008; Flusberg *et al*., 2005; Helmchen *et al*., 2001). Clinically, two-photon microscopy may find an application in noninvasive optical biopsy, for which high-speed imaging deep into tissues is required. This prerequisite has already been addressed by video rate two-photon microscopy (Lee *et al*., 2011). **See also**: ***Caenorhabditis elegans* as an Experimental Organism**; **Zebrafish as an Experimental Organism**

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