# Two-Photon Fluorescence Microscopy: Basic Principles, Advantages and Risks

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## 1. Introduction

The application of two-photon excitation to fluorescence microscopy has become a powerful tool for studying biological function in live tissue and offers many advantages over conventional imaging techniques. Neuroscientists in particular have used this technology to image physiological functioning in microscopic and subcellular neural compartments. Neurons can be imaged deep within highly light scattering tissue with unparalleled spatial resolution and dramatically reduced photodynamic tissue damage and fluorophore photobleaching. In this chapter we describe the basics of two-photon excited fluorescence imaging. We review the biophysical principles of fluorophore excitation by the absorption of two-photons and the operation fundamentals of the mode-locked pulsed Ti:Sapphire laser that is essential for optimal two-photon excitation. The advantages of using focal volume excitation by two-photons are outlined and compared with conventional single-photon excitation for fluorescence imaging in thick, highly scattering brain tissue. Finally, a discussion of the potential caveats of using two-photon excitation to image physiological functioning is presented.

## 2. Biophysical Principles of Two-Photon Excited Fluorescence

Fluorescence is the process of photon emission by a molecule subsequent to the excitation of that molecule by absorption of a photon. It is the result of the molecule or fluorophore undergoing a three-stage process of 1; excitation, 2; internal conversion, and 3; emission(*1*) (figure 1A). Two-photon excited fluorescence differs from single photon excited fluorescence with regard to stage one of the three-stage fluorescence process. In two-photon excitation a fluorophore accomplishes the transition from its ground state to an excited state by the near-simultaneous ($\sim 10^{-16}$s) absorption of two photons. One photon excites the fluorophore to a 'virtual' intermediate state while the second photon further excites the fluorophore to the excited state(*2*). The two photons have approximately half the energy and double the wavelength of the photon required for a single photon excitation quantum event to occur. For example, two photons in the red region of the spectrum (~700nm) can combine their energies and excite a calcium ($Ca^{2+}$)-sensitive fluorophore such as Fura-2 that absorbs a single photon in the ultraviolet region (~350nm) (figure 1B).

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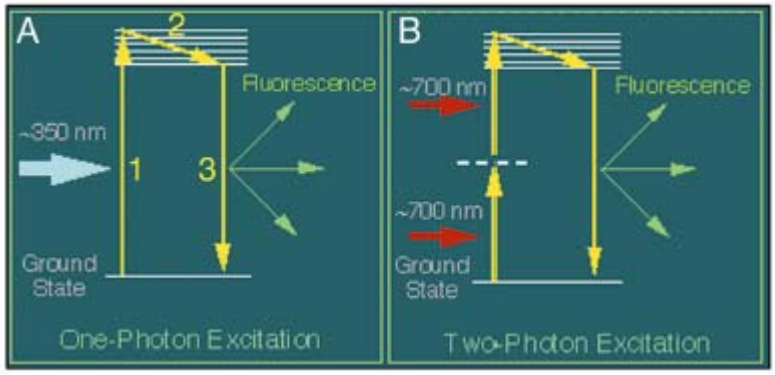


**Fig. 1** Jablonski Diagrams showing the three-stages of excitation, internal conversion, and emission (labeled 1-3 in A) involved in the process of fluorescence induced by the absorption of a single photon (A) or two photons (B). (Modified and reprinted http://www.bris.ac.uk/Depts/Anatomy/research/neuro/OneTwoPhoton/TwoPhoton.htm)

The two-photon cross section ($\sigma_{2p}$) is a quantitative measure of the probability of a two-photon absorption event occurring in a fluorophore(*3, 4*). It is a measure of fluorophore conversion efficiency from excitation photons to emitted fluorescence and defines the absorption wavelength and strength values. The unit of the two-photon cross section is the Goppert-Mayer or 'GM', where 1 GM=$10^{-50}$ $cm^4s$/photon. A related quantitative parameter commonly reported is the two-photon 'action' cross section ($\sigma_{2p}\varphi_F$). The action cross section is the product of the two-photon absorption cross section and the fluorescence quantum efficiency ($\varphi_F$) of the fluorophore and provides a direct measure of brightness(*3-5*). The quantum efficiency of the fluorophore is a measure of the emission efficiency defined as a ratio of the number of photons emitted to the number of photons absorbed. The two-photon action cross section spectra for some commonly used $Ca^{2+}$ indicators are shown in (figure 2A). Optimal two-photon excitation wavelengths cannot be determined simply by doubling the maxima of the single photon excitation wavelength. In fact, no quantitative predictions about two-photon absorption can be made from the onephoton excitation spectrum because the quantum-mechanical selection rules for two-photon absorption differ from those for single photon absorption(*6, 7*). However, cross sections have now been determined for a wide range of indicators and familiarity with indicator characteristics serves as an important guide in the selection of appropriate fluorophores for imaging experiments(*3, 4, 8*). Figure 2B shows a comparison of the two-photon cross sections of fluorescein, rhodamine B, and DiI with their corresponding one-photon absorption spectra.

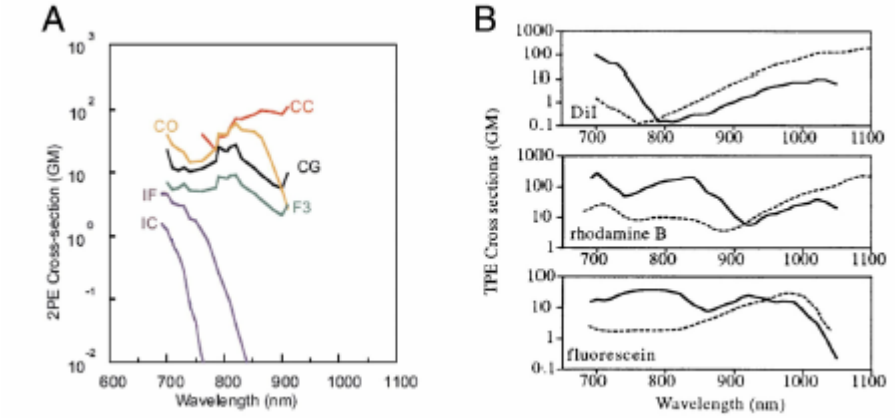


**Fig. 2** A) Two-photon action cross section spectra for some commonly used $Ca^{2+}$ indicators. Labeled $Ca^{2+}$ fluorophores are; IC=indo-1 with $Ca^{2+}$, IF=indo-1 without $Ca^{2+}$, F3=fluo-3 with $Ca^{2+}$, CG= calcium green-1 with $Ca^{2+}$, CO= calcium orange with $Ca^{2+}$, and CC=calcium crimson with $Ca^{2+}$. B) Comparison of two-photon cross

sections (solid lines) with their corresponding one-photon absorption spectra (broken lines) for DiI, rhodamine B, and fluorescein. Y-axis represents two-photon absorption cross sections for rhodamine B and fluorescein, and action cross section for DiI. Y-axis values are for one-photon results are in arbitrary units and the x-axis values represent twice the one-photon transition wavelengths. (Modified and reprinted from Xu 2002 and Xu *et al.* 1996).

## 3. The Two-Photon Laser

The probability of a two-photon absorption event occurring within a fluorophore is extremely low. An idea of the rarity of this process is illustrated in the following example. In bright sunlight the fluorescent molecule rhodamine B absorbs a photon through a 1-photon process about once a second and a photon pair by a 2-photon absorption every 10 million years(*9*). Therefore, although the two-photon phenomenon was predicted by Maria Goppert-Mayer in her doctoral dissertation in 1931(*10, 11*), the first investigations of this phenomenon only became possible with the advent of laser sources that provided the required high photon flux densities (*12*). Today pulsed lasers provide optimal two-photon excitation(*13*) improving the two-photon rate 100,000-fold, compared to continuous-wave laser operation at the same average power level(*9*). The Titanium:Sapphire crystal-based laser in particular has become the laser choice for biological laser scanning microscopy. This laser utilizes a titanium-doped sapphire crystal as a gain medium that allows wavelength tunability from ~700 - 1000nm providing two-photon excitation of fluorophores in the ultraviolet to green region of the light spectrum (~350 – 500nm) (figure 3).

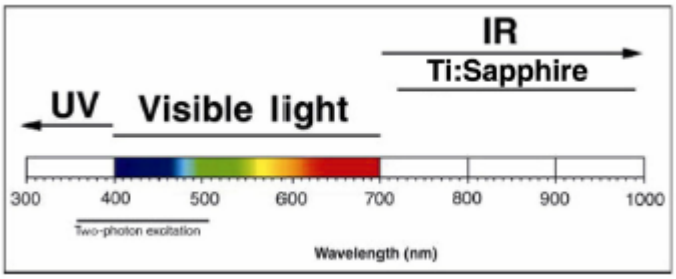


**Fig. 3** The Ti:Sapphire laser tunability range in the near-infrared (IR) wavelength region of the light spectrum allows for the excitation of fluorophores in the ultraviolet (UV) and visible regions (~350 - 500nm). (Modified and reprinted from Potter, SM1996).

It is the Ti:Sapphire crystal itself that produces the periodic sequence of high intensity pulses in this pulsed laser by a process known as Kerr lens mode-locking. The Kerr effect is an intensity-dependent change in the index of refraction of the crystal and only occurs when the intensity of the light is extremely high. As a laser beam is most intense at its center, the index of refraction of the crystal is changed such that high intensity light travels slower than low-intensity light at the edges of the beam and self-focusing of the beam results, much like light through a convex lens. Laser cavities can support numerous longitudinal modes of light, each with different frequencies operating with minor power fluctuations, none of which are sufficiently high enough to cause Kerr lens formation. A pulse evolves when a momentary large fluctuation of intensity above background noise is Kerr focused and highly amplified. The resulting pulse transiently depletes the population inversion set up in the gain medium and 'locks' the longitudinal modes in phase in the laser cavity so that there is destructive interference between propagating frequencies everywhere in the cavity except at one point where the waves add constructively(*14, 15*). The mode-locked pulse produced in a Ti:Sapphire laser is extremely brief (~$10^{-13}$s). The time between these femtosecond pulses is equal to one round trip in the optical cavity (~12ns), the inverse of which defines the high repetition rate (80-100MHz).

## 4. Focal Excitation

The Two-photon excitation process may be described as a chemical reaction where the fluorophore (F) is excited from it's ground state by the absorption of two photons (2)(P) to the excited state (F*).

$$F + 2P \rightarrow F^*$$

The rate of the reaction, here the excitation of the fluorophore, is proportional to $(P)^2$. Because two photons are required for each excitation event the probability of a fluorophore absorbing a photon pair is proportional to the square of the excitation intensity(*2, 16*). Consequently, unless the local photon flux is very high the probability of two-photon absorption is extremely low. Adequate photon flux for twophoton excitation is only possible by concentrating the photons both temporally using the pulses from the mode-locked laser and spatially by focusing through an objective lens of a microscope. At the focal point the photons are sufficiently 'crowded' enough to interact simultaneously with a fluorophore and generate an appreciable amount of two-photon excitation. Outside the focal point, the photon density is not high enough to allow for two of them to be within the absorption cross section of a single fluorophore at the same time and excitation does not occur (figure 4).

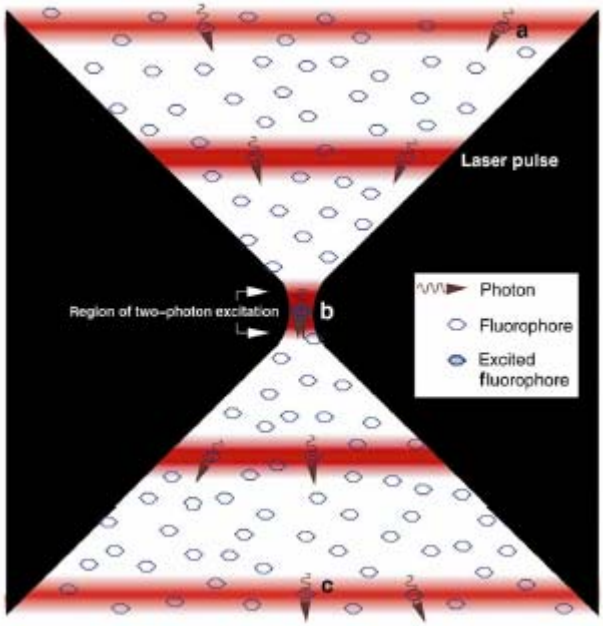


**Fig. 4** Schematic representation of two-photon fluorophore excitation that occurs at the focus of a microscope with mode-locked laser pulses (red). Photons (red symbols) located outside the focal region (a and c) have no chance of simultaneously interacting within the cross section of a single fluorophore (blue hexagons) because the photon flux density is not high enough. Sufficient photon 'crowding' only occurs at the focus and this high photon density allows for two photons to be within the cross section of a single fluorophore and interact simultaneously causing fluorophore excitation (stippled blue hexagon (b)). (Reprinted from Piston DW 1999).

In a thick sample such as a brain slice, with a spatially homogeneous distribution of fluorophores, twophoton absorption is limited to an ellipsoid (0.3μm in diameter and 1μm long for a 700nm wavelength) volume of approximately 0.1femtoliters for an objective lens with a numerical aperture = 1.4(*7*). With conventional confocal laser scanning microscopy the absorption of a single-photon is used to excite a fluorophore to a higher energy state making the fluorescence yield linearly dependent on the excitation intensity. The consequence of this is that fluorescence is not restricted to the focal plane but is generated throughout the hourglass-shaped excitation beam path resulting in photodestruction of the fluorophore and photodynamic damage to the specimen (figure 5) (*7, 17*). Imaging structures deep within

the tissue also become impossible because the excitation beam is attenuated by fluorophore absorption above the focal plane (in addition, depth penetration is severely compromised because the shorter wavelength excitation photons are scattered much more than the excitation wavelengths used for two-photon excitation)(*18*).

A powerful application of localized excitation is the use of two-photon excitation for the photolysis of caged molecules(*19*). A caged molecule is an inactive derivative that can be converted into a biologically active form when certain chemical bonds are broken or photolyzed (*20*). With brief flashes of light photolabile $Ca^{2+}$ chelators for example can be used to rapidly increase intracellular free $Ca^{2+}$ concentrations(*21-24*) ($Ca^{2+}$ acts as a unique signaling molecule for a large number of important cellular

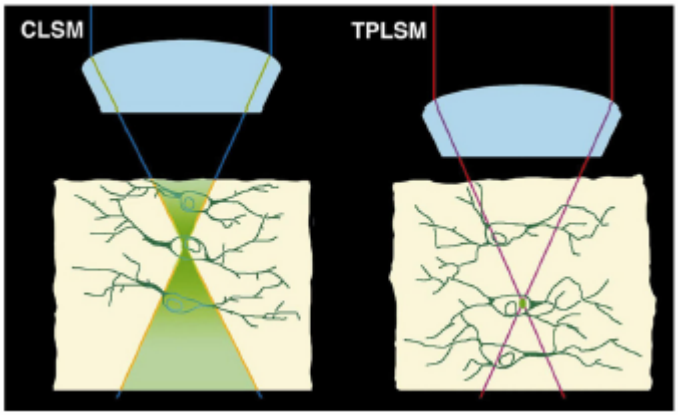


**Fig. 5** Comparison between the fluorescence obtained a neural tissue preparation using conventional single-photon confocal laser scanning microscopy (CLSM) and two-photon laser scanning microscopy (TPLSM). Fluorescence is generated throughout the hourglass-shaped excitation beam path with (CLSM) whereas with (TPLSM) fluorescence is limited to the focal region. As a result, photodynamic tissue damage and fluorophore photobleaching only occurs within this region of the neuron illustrated. The longer wavelengths used in TPLSM also penetrate deeper within the tissue. (Reprinted from Potter, SM 1996).

processes(*25*)). Photolysis of caged $Ca^{2+}$ using two-photon excitation can selectively release $Ca^{2+}$ within femtoliter volumes(*26-30*). This provides a means of controlling intracellular events with unmatched spatial resolution. With single–photon excitation the same amount of illumination occurs in all planes above and below the focal volume. As a result the same total amount of uncaging and therefore $Ca^{2+}$ release occurs in all planes. In our lab we use two-photon photolysis to selectively uncage $Ca^{2+}$ within localized regions of single astrocytes at depths greater than 100µm in the brain slice preparation (figure 6).

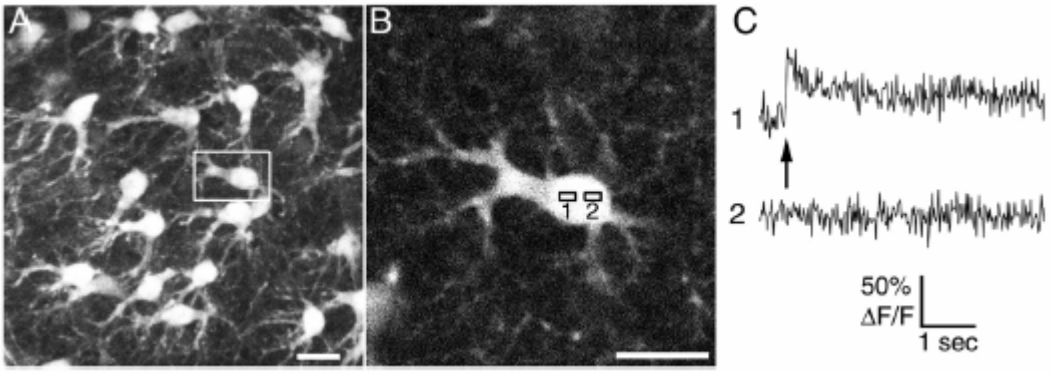


**Fig. 6** Two-photon photolysis of caged $Ca^{2+}$ A) Two-dimensional projection of a 71µm image stack (26 images separated by a 2.84 Z-plane step) showing the fluorescence from astrocytes loaded with the $Ca^{2+}$ indicator rhod-2. (The most superficial slice was >50µm below the surface of the slice.) B) Zoomed up single plane image from the

stack showing the single astrocyte from the rectangle box in (A). Rectangle 1μmX2μm boxes marked 1 and 2 within the astrocyte show the regions of interest from which the transients in (C) were derived during high-speed image acquisition. The astrocyte was co-loaded with the $Ca^{2+}$ cage DMNP-EDTA(*31*). C) Transients were acquired simultaneously from the boxed regions in (B). The photolyzed region (1) produces a increase in $Ca^{2+}$ fluorescence after the flash (arrow) that is limited to this area. No increase is seen in region (2) separated by just 1μm. Scale bars = 10μm.

## 5. Fluorescence Detection

The quadratic dependence of the excitation probability on excitation light intensity is what makes two-photon excitation so useful for fluorescence microscopy because fluorescence is generated *only* within the tiny focal volume and thus all fluorescence constitutes useful signal (*32*). This is in sharp contrast to single-photon excited confocal microscopy where only a tiny fraction of the total fluorescence comes from the focal volume and thus can be utilized for image generation. Here the fluorescence emanating from the focal plane must be selected by blocking the out-of-focus fluorescence with a confocal pinhole placed before the light detector. In highly light scattering tissues such as a brain slice the use of confocal apertures are particularly wasteful because emitted photons scattered on their path out of the tissue become indistinguishable from out-of-focus fluorescence. As a result, only emitted photons that have ballistic trajectories may contribute to the signal, while scattered photons, often the majority, are rejected by the detector pinhole(*9*). Compounding the problem, in highly scattering tissue a proportion of the photons emitted out of the plane of focus end up finding their way though the confocal pinhole anyway which adds to the background of the image and decreases image contrast(*18*). Two-photon excited fluorescence makes much more efficient use of the photons generated. Because fluorescence is limited to the focal plane with two-photon excitation all fluorescence photons whether leaving the preparation on scattered or ballistic trajectories, are 'in focus' and may be collected without the use of a confocal pinhole at the detector (figure 7). This dramatically improves the signal-to-background ratio compared with standard confocal microscopy.

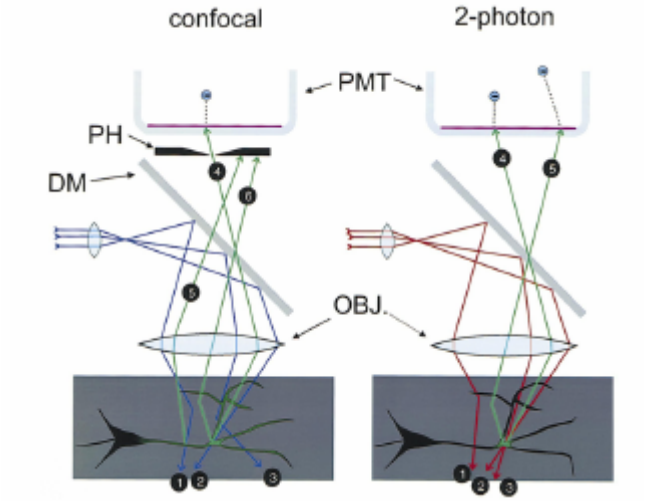


**Fig. 7** Fluorescence detection comparison between confocal and two-photon imaging modes in a highly scattering brain tissue preparation. Numbers (1-6) indicate the path of photons during excitation (blue and red lines) and fluorescence (green lines) for confocal and two-photon scenarios. The shorter wavelength photons used with confocal imaging have a greater chance of being scattered as they enter the slice (3) and fluorescence (and photodamage and photobleaching) occurs throughout the slice (green region). Because excitation is restricted to the focal point with two-photon excitation all photons ballistic (4) and scattered (5) can be collected at the photomultiplier (PMT). Fluorescence generated from single-photon excitation must maintain ballistic trajectories (4), scattered photons (6) although originating at the focus are excluded by the pinhole. (Reprinted from Denk and Svoboda 1997).

## 6. Caveats

To fully exploit the benefits of using two-photon excitation for fluorescence imaging in living preparations an understanding of the potential risks are necessary. Because of the nonlinear relationship between excitation probability and excitation light intensity with two-photon excitation photodynamic tissue damage and fluorophore photobleaching are restricted to the focal volume. This is one of the great advantages over conventional single-photon excited confocal microscopy where the linear dependence of photon absorption on excitation intensity results in photodamage occurring throughout the excitation beam path. Nevertheless, photodamage within the focal volume may be just as significant for two-photon as it is for single-photon excitation and compromise biological viability of the specimen(*7*). Fluorophore photobleaching may in fact be more rapid with two-photon excitation (*33*). The rate of fluorophore photobleaching follows a near-linear relationship with the incident intensity for single-photon excitation. However, the rate of two-photon photobleaching does not follow the expected intensity squared dependence, but rather depends on >3 power of the excitation indicating the presence of higher order photon interactions and accelerated photobleaching(*34*). Fluorophores may not only undergo complete destruction and become nonfluorescent from photobleaching(*35*), but may remain fluorescent and unable to accurately report molecular changes. For example, in the dendrites of neocortical neurons basal $Ca^{2+}$ fluorescence has been shown to increase linearly with cumulative two-photon exposure at low laser powers (<10mW) (*36*)(figure 8A). The increased basal fluorescence is thought to result from irreversible photo-induced changes in the fluorophores that bind them to the cellular matrix or membranes. The bound fluorophore is molecularly altered and no longer able to report changes but remains fluorescent, which raises the absolute concentration of the fluorophore as the mobile fraction equilibrates (*36*). The rise in basal fluorescence severely compromises true reporting of $Ca^{2+}$ dynamics, as the amplitude of the relative fluorescence changes will be greatly underestimated even if the amplitude of the absolute fluorescence changes remain unaltered (*36*) (figure 8C+D). In addition, cumulative two-photon exposure at low laser powers may induce photodamage independent of the fluorophore. Intracellular environments may become viscous from photo-induced protein-protein and proteincytoskeleton binding severely compromising physiological functioning. This results in a slower decay time constant of the $Ca^{2+}$ fluorescence transient as fluorophore molecules have reduced mobility and hampered diffusion through damaged areas (*36*) (figure 8E). At higher excitation intensities (>10mW) severe damage occurs. $Ca^{2+}$ fluorescence signals may abruptly rise indicating a sudden increase in $Ca^{2+}$ concentrations (*37, 38*) and morphological alterations such as the formation of vesicular fluorophore-filled structures(*36*) or 'blebbing' occur (Figure 8F). A final point to consider is the excitation wavelength dependence of photodamage. Two-photon excitation of endogenous fluorophores such as reduced nicotinamide adenine dinucleotide (NADH) (*39*) and the flavins (*40, 41*) can result in the formation of reactive oxygen species that cause oxidative damage and reduced viability (*6, 42, 43*). A general feature of the two-photon action cross sections of the endogenous fluorophores is that >50% of their peak values occur at wavelengths less than 800nm (*44*). Indeed, cellular damage has been shown to occur to a much greater extent through a two-photon absorption process at wavelengths < 800nm (*45*) while, long-term imaging (images every 2.5mins for over 24hours) has been performed at a wavelength of 1,047nm without compromised viability(*46*).

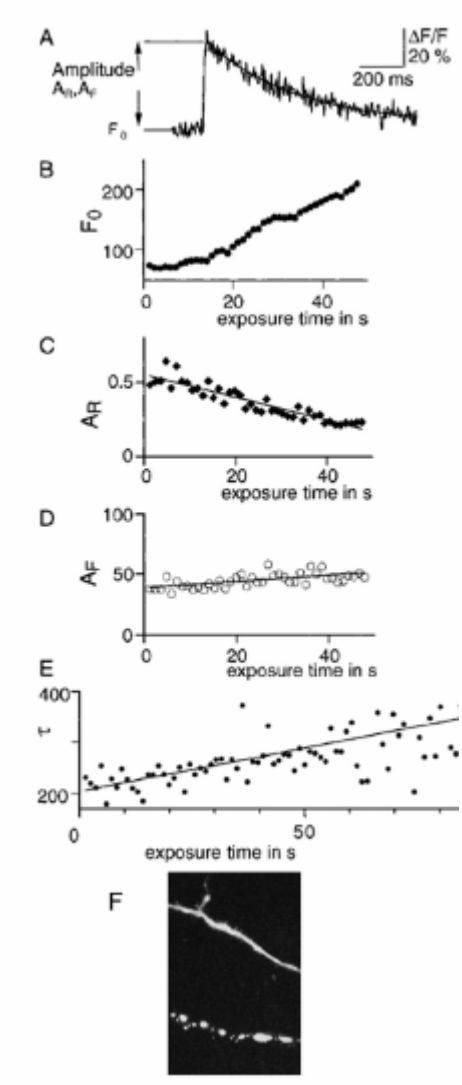


**Fig. 8** Cumulative two-photon exposure at low intensity alters $Ca^{2+}$ fluorescence dynamics. A) Average of 5 $Ca^{2+}$ fluorescence transients in the dendrite of a cortical pyramidal neuron evoked by back-propagating action potentials. Increases in the basal fluorescence $F_0$ with increasing exposure time (B) decreases the relative amplitude ($A_R$) of the fluorescence change (C), while the absolute amplitude ($A_F$) of the fluorescence transient remains unchanged (D). E) The decay time constant (τ) increases linearly with two-photon exposure time from ~210ms to ~320ms. B-D the average laser power was 6.4mW and 7.2mW for E. (A-E Reprinted from Koester *et al*. 1999). F) Two-photon image of two dendrites of a CA1 pyramidal neuron in the hippocampus. The bottom dendrite was scanned 5 times at ~25mW, the upper dendrite was not scanned prior to the image taken.

## 7. Conclusion

Laser scanning microscopy employing two-photon excitation has become the imaging choice for studying physiological functioning in highly light scattering brain tissue. The focal excitation resulting from the absorption of two-photons provides inherent three-dimensional resolution without the need for a confocal pinhole and the nonlinear dependence of photon absorption on the excitation light intensity eliminates background fluorescence and allows for efficient collection of both scattered and ballistic emitted photons. The longer excitation wavelengths used with two-photon imaging dramatically increase tissue penetration depth and allow for long-term imaging without compromised tissue viability. Optimal operational parameters such as the selection of fluorophores with large cross sections, matching laser wavelength for fluorophore absorption, maintaining excitation light intensity to the lowest possible level, and efficient fluorescence detection will minimize tissue photodamage and improve viability.

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