

# Ca<sup>2+</sup> Fluorescence Imaging with Pico- and Femtosecond Two-Photon Excitation: Signal and Photodamage

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**ABSTRACT** The signal and limitations of calcium fluorescence imaging using nonresonant multiphoton absorption of near-infrared femto- and picosecond laser pulses were examined. The fluorescence changes of various Ca<sup>2+</sup>-indicators induced by transient increases of the intradendritic calcium concentration were evaluated by evoking physiological activity in neocortical neurons in rat brain slices. Photodamage was noticeable as irreversible changes in the parameters describing the calcium fluorescence transients. At higher two-photon excitation rates, a great variety of irregular functional and structural alterations occurred. Thus, signal and observation time were limited by phototoxic effects. At lower excitation rates, photodamage accumulated linearly with exposure time. Femtosecond and picosecond laser pulses were directly compared with respect to this cumulative photodamage. The variation of the pulse length at a constant two-photon excitation rate indicated that a two-photon excitation mechanism is mainly responsible for the cumulative photodamage within the investigated window of 75 fs to 3.2 ps. As a direct consequence, at low excitation rates, the same image quality is achieved irrespective of whether two-photon Ca<sup>2+</sup>-imaging is carried out with femto- or picosecond laser pulses.

## INTRODUCTION

When imaging in scattering tissue, nonresonant two-photon excitation (TPE) fluorescence microscopy reportedly is superior to its single-photon excitation (SPE) counterpart (Denk et al., 1990, 1996). The quadratic dependence of the excitation on the illumination intensity confines the excitation to the focal region so that the mere generation of fluorescence in a raster fashion establishes a three-dimensional image. Importantly, ballistic and scattered emitted photons contribute equally well to the total signal. This allows the collection of a large fraction of fluorescence photons by external detectors resulting in a much higher collection efficiency. Moreover, TPE of visible dyes requires excitation wavelengths in the near-infrared where scattering coefficients are lower (Svaasand and Ellingsen, 1983) and, with rare exceptions, linear absorption is negligible. Weak endogenous absorption and spatial confinement of TPE inflicts considerably lower phototoxic stress than SPE, which is invaluable for live-cell imaging (Wokosin et al., 1996; Maiti et al., 1997). However, the nonlinear nature of TPE requires much higher intensities. The high intensities may well induce photodamage and irreversible sample modifications.

To provide an estimate for the intensities and power levels involved, let us first review briefly the power and

intensity levels in SPE and TPE microscopy, assuming typical wavelengths of  $\lambda_{\text{exc}} = 450$  nm and  $\lambda_{\text{exc}} = 870$  nm, respectively, and a numerical aperture  $\text{NA} = 1.0$ . The SPE rate is given by  $k_1 = \sigma_1 I / (hc/\lambda)$  whereby  $h$  is Planck's constant, and  $c$  the speed of light (Lakowicz, 1983). For a typical cross section of  $\sigma_1 = 10^{-16}$  cm<sup>2</sup>, an intensity  $I = 200$  kW/cm<sup>2</sup> is sufficient to reach  $k_1 = 5 \times 10^7$  s<sup>-1</sup>, which is close to saturation by ground state depletion. This intensity corresponds well to a continuous wave average power of  $\sim 140$   $\mu$ W at the focus. To obtain the same rate,  $k_2 = \sigma_2 [I / (hc/\lambda)]^2$ , with nonresonant TPE, for a typical TPE cross section of  $\sigma_2 = 10^{-49}$  cm<sup>4</sup>s (Xu and Webb, 1996), a much higher intensity of 5.4 GW/cm<sup>2</sup> is required. Given the same lenses and the wavelength needed for TPE is  $\lambda_{\text{exc}} = 870$  nm, this intensity corresponds to an average power of 12 W at the focus.

Although efficient continuous wave TPE microscopy is feasible for highly concentrated staining and large TPE cross sections (Booth and Hell, 1998; Hell et al., 1998), TPE microscopy usually requires pulsed lasers such as the mode-locked Ti:Sapphire, providing pulses of  $\tau \approx 100$  fs or  $\tau \approx 1$  ps pulse duration, at a pulse repetition rate  $f \approx 80$  MHz. For pulsed illumination, the  $n$ -photon absorption rate is given by

$$k_n = f \frac{\sigma_n}{(hc/\lambda)^n} \int_0^{T=1/f} I^n(t) dt, \quad (1)$$

where  $I(t)$  denotes the intensity. Because most power detectors are too slow to follow  $I(t)$ , the excitation rate is often expressed in terms of the time-averaged power value measured by the detector. For  $\tau \ll 1/f$ , the evaluation of the

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integral in the paraxial focusing approximation gives

$$\begin{aligned} k_n &= \frac{g^{(n)}}{(\tau f)^{n-1}} \left( \frac{\bar{I}}{hc/\lambda} \right)^n \sigma_n \\ &= \frac{g^{(n)}}{(hc/\lambda)^n} (I_{\text{peak}})^{n-1} \bar{I} \sigma_n \\ &= \frac{g^{(n)}}{(\tau f)^{n-1}} \left( \frac{\bar{P} \pi (NA)^2}{hc\lambda} \right)^n \sigma_n, \end{aligned} \quad (2)$$

where  $\bar{I}$  and  $\bar{P}$  denote the time-averaged intensity and time-averaged power, respectively. The constant  $g^{(n)}$  is the time-zero  $n$ -order temporal coherence. For hyperbolic-secant-squared shaped pulses,  $g^{(2)} = 0.59$ . When considering photodamage issues, it is important to bear in mind that the time-averaged values are not determining the absorption rate alone. They are normally used because they can be easily measured by a (slow) detector. The relationship between the peak intensity  $I_{\text{peak}}$  and the time-average intensity  $\bar{I}$  is  $I_{\text{peak}} = (\tau f)^{-1} \bar{I}$ . The proportionality of  $k_n$  to  $(I_{\text{peak}})^{n-1}$  reflects the fact that (only) nonlinear ( $n > 1$ ) processes benefit from high peak intensities.

Compared to the CW TPE case, pulsing reduces the required time-averaged power by a factor of  $(\tau f)^{-1/2}$  but, at the same time, increases the peak intensity by the same factor. By rearranging Eq. 2, one can calculate that, for the focusing conditions and assumed TPE rate  $k_2 = 5 \times 10^7 \text{ s}^{-1}$ , pulsed-mode TPE imaging with pulses of  $\tau = 1 \text{ ps}$ , and 100 fs would keep the time-averaged power  $\bar{P}$  down to 140 mW and 45 mW, respectively, at the expense of involving peak intensities of  $I_{\text{peak}} = 800 \text{ GW/cm}^2$  in the 1 ps, and even higher intensities of  $2.5 \text{ TW/cm}^2$ , in the 100 fs mode.

Standard Ti:Sapphire systems emit between 0.5 and 2 W of time-averaged power. Thus, the laser usually provides ample power to saturate nearly all dyes by TPE. In fact, the available power would allow an increase in the focal intensity by another order of magnitude. Evidently, dyes with 100 times lower cross sections could still be excited with saturating intensities. However, Eq. 2 indicates that the high peak intensities of  $>1 \text{ TW/cm}^2$  foster nonlinear ( $n > 1$ ) processes, some of which might be detrimental to the fluorophore or the sample. Such intensities can overwhelm the light specimen interaction before saturation by ground-state-depletion is reached.

Such evidence for irreversible fluorophore damage induced by TPE has indeed been found in single-molecule experiments (Eggeling et al., 1998). Photodamage in TPE microscopy has also been reported by several researchers in the field, often as a sudden localized onset of increased luminescence (König et al., 1999; Straub and Hell, 1998). It is reasonable to expect the limiting mechanisms to vary with the sample and fluorophore. In fact, such limits may well be a combination of linear and nonlinear processes, with each of them prevailing at a given power and intensity range. It will be important to establish the dependence of the damage on the excitation intensity as a function of the power order  $n$  of the (non-)linearity.

According to Eq. 2, photodamage by linear absorption ( $n = 1$ ) depends solely on the average intensity  $\bar{I}$  and not on the peak intensity  $I_{\text{peak}}$ . If linear photodamage dominated, safe TPE would be possible only for high transient intensities such as can be obtained by minimizing the pulse width. If cubic or higher order ( $n \geq 3$ ) processes would dominate photodamage, increasing  $I_{\text{peak}}$  by reducing the pulse width would be disadvantageous. In the special case of a quadratic dependence, the photodamage and TPE rate will just be proportional to each other, and the attainable TPE rate would not depend on the pulse width.

As in the earlier stages of TPE microscopy, linear absorption was considered to be the limiting factor, so, keeping the pulses as short as possible was considered of particular importance (Denk et al., 1995). Prechirping units were applied to compensate for group velocity dispersion in the microscope (Soeller and Canell, 1996; Brakenhoff et al., 1995), so that the pulse could reach the specimen at its shortest possible duration ( $\sim 100 \text{ fs}$ ). However, recent two-photon imaging with increased  $\bar{P}$  through picosecond (7 ps) pulsed (Bewersdorf and Hell, 1998) and CW-excitation ( $\bar{P} = 200 \text{ mW}$ ) (Hell et al., 1998) revealed nonlinear optical effects as the major limitations and indicated that the reduction of the pulse duration is of secondary importance.

In a more recent study, a large population of live unstained Chinese hamster ovarian cells were exposed to Ti:Sapphire laser illumination and the impairment of cell replication quantified as a measure for photodamage (König et al., 1999). For pulses ranging from 2.2 ps down to 240 fs, the photodamage followed explicitly a quadratic power law; this rendered the pulse width irrelevant for optimizing for least photophysical damage. Whereas, for the 240-fs pulses, an average power of  $\bar{P} = 7.3 \text{ mW}$  was sufficient to impair cell viability, for the 2.2-ps pulses, an average power of  $\bar{P} = 20 \text{ mW}$  was required to produce the same effect. Taking into account the  $NA = 1.35$ , the corresponding focal intensities  $I_{\text{peak}}$  were  $0.35 \text{ TW/cm}^2$  for the femtosecond and  $0.1 \text{ TW/cm}^2$  for the picosecond pulses. Although these results are very valuable, the use of chirped laser pulses and the complexity of the damage mechanisms does not allow a general conclusion for other applications.

Due to its high penetration depth, TPE has been particularly successful in the imaging of  $\text{Ca}^{2+}$  dynamics in small neuronal compartments of live brain tissue (Svoboda et al., 1996; Yuste and Denk, 1995; Schiller et al., 1998; Koester and Sakmann, 1998; Denk et al., 1995; Yuste et al., 1999). Although the relevance of characterizing phototoxic effects has been recognized, information about the relationship between excitation power and intensity and photodamage in TPE microscopy is scarce. Finding out the optimal operational parameters for multiphoton imaging is of paramount importance to the proper use of this powerful imaging mode. Therefore, in our study, we investigated the limiting factors of TPE- $\text{Ca}^{2+}$  imaging in rat brain slices using various fluorescent  $\text{Ca}^{2+}$  indicators and pulse widths. In particular, we provide a quantitative description of the ob-

served photodamage and investigate the order of nonlinearity of the damage.

## MATERIAL AND METHODS

### Slice preparation

Acute neocortical slices were prepared from 13 to 14-day-old Wistar rats as described previously (Markram et al., 1997). Layer 5 pyramidal neurons in the somatosensory cortical area were identified using gradient-contrast (Dodt et al., 1998) infrared videomicroscopy. The bath solution contained 125 mM NaCl, 25 mM NaHCO<sub>3</sub>, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 25 mM Glucose, and 2 mM CaCl<sub>2</sub> (Biometra, Goettingen, Germany). The bath temperature was 32–34°C. All experiments were performed on basal dendrites 30–80  $\mu$ m away from the soma. To keep the attenuation of excitation laser power comparable, only structures 30–70  $\mu$ m below the slice surface were selected.

### Electrophysiology

Cells were filled with dyes by whole-cell recording. Patch pipettes were filled with 115  $\mu$ M K-gluconate, 20  $\mu$ M KCl, 10  $\mu$ M HEPES, 4  $\mu$ M ATP-Mg, 10  $\mu$ M phosphocreatine, 0.3  $\mu$ M GTP, and a dye, which was either Calcium Green-1 (CG-1, 100  $\mu$ M), Oregon Green 488 BAPTA-1 (OGB-1, 100, 200, or 500  $\mu$ M), Oregon Green 488 BAPTA-2 (OGB-2, 500  $\mu$ M), Magnesium Green (MG, 500  $\mu$ M), or Fluorescein (500  $\mu$ M). All dyes were purchased from Molecular Probes (Eugene, OR). Dyes were allowed to equilibrate for  $\geq 20$  min after establishing whole-cell recording before experiments started. Electrical recordings were made with a patch-clamp amplifier (EPC-7, List-Electronics, Darmstadt, Germany) operated in the current clamp mode. Initial access resistances were  $< 6$  M $\Omega$ . Action potentials (AP) were evoked by brief current injection (1 nA for 4–7 ms) into the soma.

### Two-photon laser scanning microscopy

A modified galvanometer scanning unit (TCS 4D, Leica Microsystems, Heidelberg, Germany) was adapted to an upright microscope (BX50WI, Olympus Optical Co., Tokyo, Japan) equipped with a 60 $\times$  objective (LUMPlanFL 60xW0.9IR, Olympus). For excitation femtosecond laser pulses and picosecond pulses that were nearly transform-limited in the focal plane were used. In a few cases, we also used femtosecond pulses chirped to picoseconds. The femtosecond laser pulses originated from a Ti:Sa-Laser (MIRA 900F, Coherent, Santa Clara, CA) pumped by a large-frame argon ion laser (Sabre ML14, Coherent). The nearly transform-limited picosecond pulses originated from a picosecond Ti:Sa-Laser (Tsunami, Spectra-Physics, Mountain View, CA). Both lasers were operated at  $870 \pm 5$  nm. Insertion of a mirror allowed a convenient change between the lasers during the experiment. When imaging in the line scan mode, signals were collected during both scan directions. External detectors were placed behind the objective and the condensor for signal collection. Transmission- and epifluorescence signals were recorded by photomultiplier tubes (PMT) (R6357, Hamamatsu Photonics, Herrsching, Germany) and digitized into 8-bit values. The signals were averaged off-line. In control experiments, a bandpass filter ( $525 \pm 25$  nm, Leica Microsystems) was inserted into the epifluorescence pathway.

### Analysis of calcium fluorescence transients

Fluorescence line scan images were analyzed using dedicated software. A line was scanned every 2.27 ms. Pixels of two lines between two positions enclosing the dendrite examined were averaged to obtain one time point. This resulted in a temporal resolution of 4.54 ms. Stimulation protocols began 150 ms after the start of the line scan (512 lines). Before stimulation, fluorescence was averaged for 100 ms to obtain the basal fluorescence,  $F_0$ .

A region distant from any indicator-containing structure was chosen for determination of the background fluorescence,  $F_B$ , which was subtracted. Fluorescence traces  $F(t)$  of a Ca<sup>2+</sup> transient evoked by an AP were fitted with a single exponential using a least-square fit routine (IGOR, WaveMetrics, Lake Oswego, OR). This fit rendered the amplitude of the fluorescence increase  $A_F$  and decay time constant  $\tau$ . Relative fluorescence changes were calculated as  $\Delta F(t)/F_0 = (F(t) - F_0)/(F_0 - F_B)$ .  $A_R$  was the amplitude of the relative fluorescence transient.

## Pulse compression and diagnostics

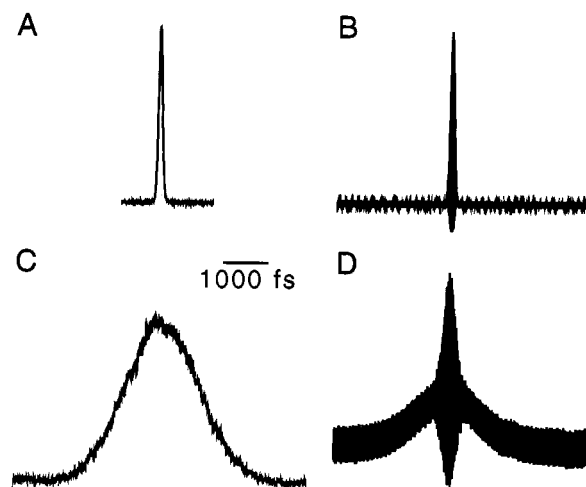
### Pulse width control

An external prism compressor of two SF10 glass prisms placed 82 cm apart was used for correcting first-order group velocity dispersion (GVD) of the femtosecond pulses (Kafka and Bear, 1987). This compensation allowed achievement of nearly transform-limited femtosecond pulses in the focal plane.

In a few experiments, the pulse length of the femtosecond pulsed mode Ti:Sapphire laser was prolonged by inserting 36 cm SF6-glass in the optical path. Depending on the spectral width of pulse, the induced chirp prolonged the pulses to between 1.4 and 2.8 ps (Fig. 1 B). The SF6-glass did not alter the spectrum.

### Interferometric autocorrelation

To determine the pulse width of the femtosecond pulses in the focal plane, the laser beam was split and directed into the two arms of a Michelson interferometer. The combined beam from the interferometer was fed into the multiphoton laser scanning microscope (Soeller and Canell, 1996) featuring a uniform solution of fluorescein as sample for TPE. Changing the path length allowed the determination of the second-order autocorrelation of the pulses (Fig. 1 A) (Diels et al., 1985). The pulse duration in the



**FIGURE 1** Determination of the pulse width by second-order autocorrelation. (A) Background free autocorrelation of pulses of a femtosecond mode Ti:Sapphire laser before entering the microscope; FWHM was  $112 \pm 6$  fs. Assuming Gaussian pulses, the pulse width amounts to 79 fs. (B) Interferometric autocorrelation in the objective focal plane showing an FWHM of  $114 \pm 5$  fs (pulse width 81 fs). (C) Background free autocorrelation of chirped pulses. To broaden femtosecond pulses to the picosecond regime, 36 cm of SF6 glass was inserted into the optical path. The background free autocorrelation signal had a width of 1.96 ps (pulse width 1.4 ps). (D) Autocorrelation of the pulses in the focal plane shows a strong chirp induced by the SF6-glass (FWHM  $\approx 2$  ps).

focal plane was defined as the full width at half maximum (FWHM). The data was fitted with a Gaussian to determine the FWHM.

### Background-free autocorrelation

Since the GVD induced by the microscope is not expected to change the pulse width of the chirped pulses or the picosecond pulses to a significant extent, we used an external, background-free autocorrelator (Model 409, Spectra-Physics) to obtain a better signal-to-noise ratio. Autocorrelation traces were monitored using a digital oscilloscope (DL 708, Yokogawa, Tokyo, Japan) that allowed convenient data storage. A Gaussian fit using least-square fit routines (IGOR, WaveMetrics) was performed and the FWHM was determined. We used the correction factor of 0.707 for Gaussian pulses for calculating the actual pulse widths from autocorrelation traces.

### Laser power determination

Average laser power was determined by a photodiode placed behind the objective. Diode measurements were calibrated with a power meter (Lasermate, Coherent) placed behind an aperture stop with the diameter and position of the pupil plane of the objective used. The transmission of the objective at 870 nm was taken into account.

## Determination of the photodamage rate $r$ at different pulse lengths

### Illumination protocol

A line 5  $\mu\text{m}$  in length was scanned repetitively every 2.23 ms for 400 s orthogonal to a basal dendrite. Taking the duty cycle of the scan (0.5) and the size of a basal dendrite into account (about 1  $\mu\text{m}$ ), cell structures were exposed for about 100  $\mu\text{s}$  every 1.115 ms. All data presented are given in terms of average laser power measured behind the objective. The incident intensities at the focus were attenuated by the scattering tissue. For estimating the TPE rates, the intensities have to be scaled by  $I(d) = I_0 e^{-\alpha d}$ , where  $d$  is the tissue depth. The parameter  $\alpha$  was, to our knowledge, not yet determined for brain slices.

### Determination of $r$

We monitored the basal fluorescence  $F_0$  from 20 line scans every 20 s during 400 s of constant light exposure. To correct for the differences in optical path length and excitation rates, we calculated the relative changes in basal fluorescence  $\Delta F_0(t)/F_0(t=0) = F_0(t) - F_0(t=0)/F_0(t=0)$  and fitted this with a line to obtain the rate of relative change in basal fluorescence,

$$r = \frac{\Delta F_0(t)}{F_0(t=0) \cdot \Delta t}.$$

This rate was taken as a quantitative measure for photodamage. Rates at different laser powers were fitted with  $r(\bar{P}) = A \cdot \bar{P}^x$ , where  $A$  and the exponent  $x$  were free parameters. When using a high-affinity dye (CG-1 or OGB-1), the changes in amplitude and decay time constant were also monitored.

### Comparing picosecond and femtosecond laser pulses in single cells

Experiments were performed on basal dendrites of labeled layer 5 cells. Dendrites were selected to be nearly at the same tissue depth ( $<10 \mu\text{m}$  variation). The rate of relative increase in basal fluorescence,  $r$ , was determined at different time-averaged laser powers,  $\bar{P}$ . At a given pulse length, normally 3–6 values of  $\bar{P}$  were used in random order. For each trial

of 400 s exposure, a location on the dendrite was chosen proximal to the trials executed before. This was done to avoid possible changes in AP-propagation and dye diffusion through damaged structures. The number of trials conducted in a single cell was limited by the lifetime of a dye-filled cell (60–90 min at the bath temperature of 35°C). Only two different pulse lengths were tested per cell, and the first pulse length was tested only at three different laser powers because of the limited lifetime. A cell was classified as stable when no significant changes in resting membrane potential, excitability, or calcium fluorescence dynamics at undamaged locations occurred. Trials were taken into account only as long as the cell was stable. Pulse length was varied across trials in a random order. We mainly used  $78 \pm 4$  fs pulses and 1.4–3.2 ps pulses, both nearly transform-limited in the focal plane. In some experiments, we also used chirped femtosecond pulses that had passed through 36-cm SF6, resulting in pulse lengths 1.4–2.8 ps. Pulse length of the picosecond laser systems was determined before and after each series of exposures (at 3–5 different laser powers) within 20 min. Femtosecond laser pulses were controlled only in the initial experiments before and after each cell because of the apparent stability in pulse length.

We assumed

$$\begin{aligned} r &= C \int_0^1 I^x(t) dt \\ &= A' \tau f (I_{\text{peak}})^x \\ &= A' \tau f \frac{\bar{P}^x}{(\tau f)^x} \\ &= A' \frac{\bar{P}^x}{(\tau f)^{x-1}}, \end{aligned} \quad (3)$$

with  $C$  and  $A'$  as appropriate constants. Given that the same power order  $x$  determines the photodamage within the investigated power and pulse length window, we can calculate  $x$  as

$$r_1 = r_2 \Rightarrow \frac{\bar{P}_1^x}{(\tau_1 f)^{x-1}} = \frac{\bar{P}_2^x}{(\tau_2 f)^{x-1}}.$$

Hence, we find

$$x = \frac{\log(\tau_1/\tau_2)}{(\log(\tau_1/\tau_2) - \log(\bar{P}_1/\bar{P}_2))}. \quad (4)$$

## Detection scheme

We used detectors placed at three different positions. One detector was the confocal, descanned detector with the pinhole removed. A second detector for capturing nondescanned epifluorescence was placed behind the water immersion objective (LumPlanFl 63W0.9IR, NA 0.9, Olympus). A third, external detector was placed behind the high NA = 1.4 oil condensor (see Fig. 2). We used the same type of PMT (R6357, Hamamatsu), supplied with the same voltage for all three detector positions. Because the sensitive area of the side-window PMTs is only  $8 \times 24$  mm, they required additional optics to achieve the scan-fields desired. For demagnification, we placed lenses with focal length of  $f = 12$  mm (for the transmission detector) and  $f = 20$  mm (epifluorescence external detector) in the corresponding planes. The virtual scanfield of the condensor PMT depends on the aperture (Fig. 2). In the system used, the virtual scanfield was about 1.2 mm. External detection through the NA = 1.4 oil condensor and the NA = 0.9 water immersion objective and descanned detection were compared using a fluorescence solution layer (Fluorescein). The number of photons and gain was calculated from the histograms to exclude variation of gain and dark current between the PMTs. The signal collected by the descanned detector



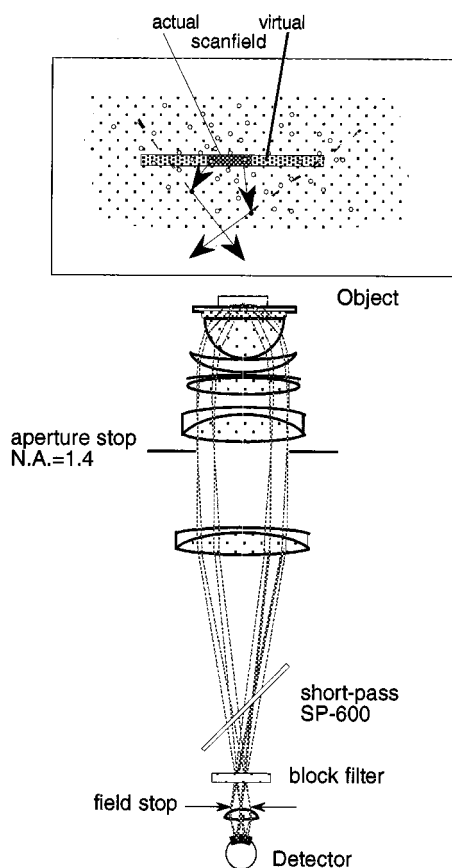


FIGURE 2 Nondescanned transmission collection. The optical elements including the condenser lenses are shown. The short-pass filter allowed IR-video imaging of the brain slices. The field stop used corresponded to a virtual scan-field of 1.2 mm.

was about 70% of the signal collected by the epifluorescence nondescanned detector. With the same fluorescence solution, the signal collected by the transmission detector was  $215 \pm 65\%$  (mean  $\pm$  SD,  $n = 18$ ) of the descanned epifluorescence signal. Because the situation might be different in highly scattering tissue, we also tested dye-filled (OGB-1, 200  $\mu$ M) neurons in brain slices. Here, the number of photons collected by the transmission external detection compared to the descanned epifluorescence detection ranged up to 450% ( $n = 2$ ).

## RESULTS

### Calcium dynamics of single back-propagating AP and cumulative photodamage

One major use of two-photon laser scanning microscopy is calcium imaging in small compartments of cells in highly scattering tissue. Therefore, we investigated the fluorescence dynamics in layer 5 neocortical neurons in rat brain slices filled with a fluorescent calcium indicator dye. Upon evoking an AP, the physiologically relevant transient increase of intradendritic free calcium was observed as a transient increase in fluorescence of the calcium indicator (Fig. 3 A). A single exponential function satisfactorily fitted the decay. The decay times varied between 50 and 400 ms, depending on the buffering by the dye (Helmchen et al.,

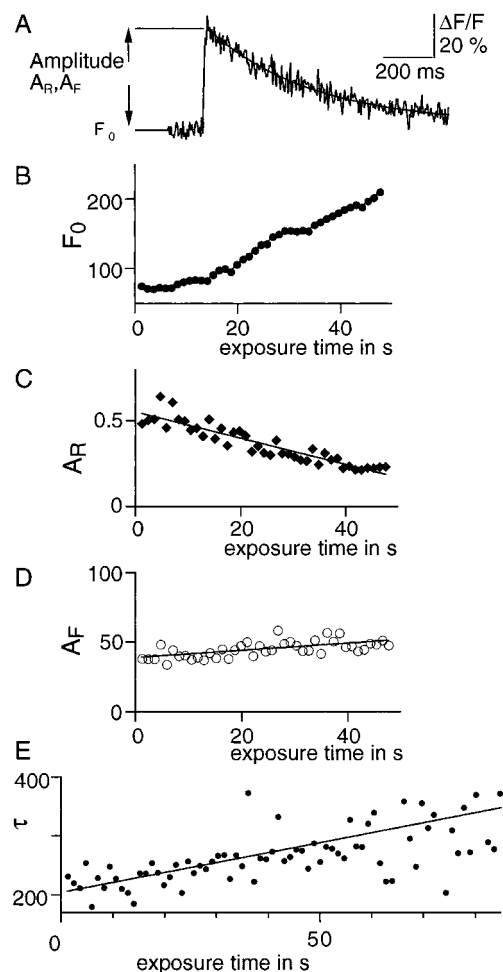


FIGURE 3 Changes in calcium dynamics at low excitation rates. (A) Somatic current injection (1 nA for 6 ms) evoked a back-propagating AP that elicited a calcium transient in the dendrites of a layer 5 pyramidal cell. The calcium transient was reported by a calcium indicator, OGB-1 (200  $\mu$ M). The trace presented is an average of five sweeps. The parameters describing this calcium fluorescence transient are basal fluorescence  $F_0$ , amplitudes  $A_R$  (and  $A_F$  for the relative fluorescence change), and decay time constant  $\tau$  (see Methods). (B–D) At an average laser power of 6.4 mW, changes in the parameters during 50 s of light exposure were observed. In the example shown, the basal fluorescence increased by nearly 300%, the relative amplitude  $A_R$  decreased. The absolute amplitude  $A_F$  changed only little. (E) The decay time constant increased from  $\sim$ 210 to  $\sim$ 320 ms. Different cell as in A–D, average laser power was 7.2 mW.

1996). The calcium fluorescence dynamics can be described by the following set of parameters: the basal fluorescence  $F_0$ , the amplitude of the fluorescence change  $A_F$ , and the decay time constant  $\tau$ . Changes in these parameters during prolonged laser light exposure were elected as measures of photodamage.

The relative fluorescence change  $\Delta F/F(t)$  was calculated and the amplitude  $A_R$  was monitored during up to 400 s of laser light exposure. Whereas the basal fluorescence  $F_0$  and decay time  $\tau$  increased with time, the amplitude of the relative fluorescence change  $A_R$  decreased (see Fig. 3, B–E). At low average laser powers, that is  $<3$ –7 mW (depending on the depth of the cell within the slice) for femtosecond

laser pulses and  $<8$ –24 mW for picosecond laser pulses, all changes were observed to be linear with exposure time. The changes were restricted to the illuminated area (see Fig. 4 *A*); they were cumulative and irreversible. This type of changes is further referred to as cumulative photodamage.

To rule out loading effects, the average laser power and pulse length were randomly varied. Also, the results did not correlate with loading time or distance to the soma. Most experiments were performed on dendrites close to the soma, where the dye concentration reached equilibrium within a few minutes (Helmchen et al., 1996). This is well below the time we waited after establishing the whole-cell recording before starting the experiments ( $>20$  min).

The increase of the basal fluorescence was also observable without back-propagating APs (OGB-2,  $n = 2$  and MG,  $n = 18$  with  $n$  representing the number of trials) and when a dye was used that was not sensitive to calcium (Fluorescein,  $n = 8$ ). The decrease of  $A_R$  could be explained by the increased basal fluorescence, because the amplitude of the absolute fluorescence change of the calcium fluorescence transient,  $A_F$ , remained constant (Fig. 3 *D*). The change of the basal fluorescence was observable with all dyes used. Qualitatively, these changes did also occur when intermittent laser light exposure instead of continuous exposure was used (e.g., 1 s exposure every 10–20 s). These characteristics allowed us to use the rates of change as quantitative measures of photodamage. The changes in basal fluorescence exhibited the highest S/N-ratio; therefore, the rate of relative change in basal fluorescence,  $r$  (see Methods), was taken as a measure for cumulative photodamage.

### Photodamage at high excitation rates

Aside from the cumulative photodamage described above, a broad spectrum of phototoxic effects was observed. When

calcium-sensitive dyes were used, sharp increases in  $F_0$  occasionally occurred in the absence of any electrophysiological signal. These sudden increases were irregular, depended nonlinearly on the applied exposure time, and were not localized to illuminated parts of the cell. In fact, they extended some micrometers along the dendrite. These effects were regularly observed at  $\bar{P} > 8$  and  $\bar{P} > 30$  mW for femtosecond and picosecond pulses, respectively. Increased photobleaching and other morphological alterations of spines and dendrites occurred at these laser powers, such as the formation of vesicular dye-filled structures (Fig. 4 *B*). For determination of  $r$ , we restricted the laser power intensity to lower values so that only the linear changes in basal fluorescence were observed. Experiments in which one of the other types of artifacts were observed were excluded from data. The damage rate  $r$  ranged up to 1.6%/s for  $\bar{P} < 8$  mW (femtosecond laser pulses) and  $\bar{P} < 30$  mW (picosecond laser pulses).

### Emission spectrum

A possible explanation for an increased level of fluorescence is the generation of a new chromophore from cellular compounds. Therefore, the emission spectrum was examined for possible changes. In some experiments, we compared the fluorescence from throughout the detectable spectrum ( $350 \text{ nm} < \lambda_{\text{em}} < 600 \text{ nm}$ ) with the fluorescence emitted at  $525 \pm 25 \text{ nm}$  and calculated the corresponding photodamage rates  $r$ . The dyes used have their emission maximum in the wavelength interval around 510–540 nm. The bandpass filter was used only for the epifluorescence signal. In this way, it was possible to compare fluorescence at 525 nm with the detectable fluorescence signal. The comparison was carried out for OGB-1 (100, 200, and 500  $\mu\text{M}$ ) and MG (500  $\mu\text{M}$ ). No significant difference between the two rates  $r$  could be observed. On average, the  $r$  at 525

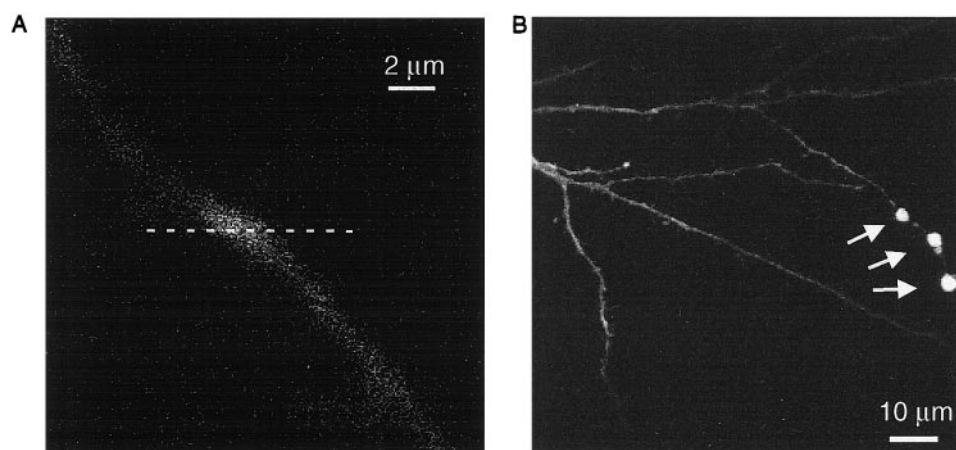


FIGURE 4 Photodamage at various excitation rates. (*A*) Image of a basal dendrite of a layer 5 pyramidal cell filled with OGB-1 (100  $\mu\text{M}$ ) showing a stationary and highly localized increase in basal fluorescence caused by repetitive line scans using femtosecond laser pulses ( $75 \pm 4 \text{ fs}$ ). The broken line indicates the position of the line scans performed before recording a stack of 10 images. Line scans were recorded with an average laser power of  $\bar{P} = 5.3 \text{ mW}$ , the xy-image with a lower power (about 3 mW). (*B*) Image of a basal dendrite showing vesicular, dye-filled structures (arrows) caused by repetitive line scan imaging with high average laser power ( $\bar{P} = 9.5$ –11.8 mW).

nm was  $95 \pm 6\%$  ( $n = 15$ ,  $\pm$  SE) of the  $r$  calculated from the total fluorescence. This indicates that the increase in basal fluorescence is not caused by the generation of a new chromophore. Furthermore, no changes could be detected in the background fluorescence  $F_B$  (all dyes and laser powers,  $n > 100$ ) supporting this point of view.

### Dependence of the photodamage rate $r$ on dye concentration

Cells at similar depth at close quarters were patched and filled by whole-cell recording with the same indicator at different concentrations (OGB-1, 200 and 500  $\mu$ M). In this way, it was possible to choose cell structures at very close quarters ( $<15 \mu$ m) and at equal depth containing different concentrations of dye. At a given average laser power, and thus, a constant TPE rate, the damage rate  $r$  was determined in both structures, and thus, at different dye concentrations. On average, we found  $r(500 \mu\text{M})/r(200 \mu\text{M}) = 104 \pm 15\%$  ( $n = 7$ ,  $\pm$  SE). Despite the 2.5-fold difference in concentration, no significant difference between the damage rates was found. This indicates that the measure of cumulative photodamage used did not depend on dye concentration; it suggests that  $r$  is a robust measure for photodamage.

### Photodamage and diffusion inhibition

The slower kinetics of the calcium fluorescence transients suggest changes in mobility in damaged areas. Therefore, we tested the diffusion properties of the dye upon evoking photodamage. For this purpose, cells ( $n = 4$ ) were filled with fluorescein (500  $\mu$ M) by whole-cell recording. At a location on a basal dendrite (e.g., the location marked by \* in Fig. 5), a significant change in basal fluorescence was induced by illumination. The patch pipette with fluorescein was withdrawn and the cell repatched, this time without any dye in the patch pipette. Washout of the dye molecules lead to a decrease of fluorescence in the cell. At a location nearby ( $<10 \mu$ m distance) and proximal to the damaged site, the fluorescence decreased exponentially with a time constant close to that anticipated for the dye to diffuse back through the dendrite into the pipette ( $\tau = 30$ – $70$  s, depending on the distance to the soma). However, in comparison to that, the fluorescence from within the damaged area decreased very slowly with a decay time  $>100$ -fold larger ( $\tau > 6000$  s, in all four cells examined). This drastic decrease in mobility indicates that a part of the dye molecules was bound and was no longer able to diffuse. Indeed, even the very slow decrease may have been caused mainly by photobleaching that is normally masked by diffusion of unbleached dye molecules into the observed area.

An important detail was revealed by another observation. In two experiments (of four cells), the decay of fluorescence distal to the damaged site was significantly slower than its proximal counterpart: 288 versus 65 and 150 versus 72 ms, in spite of close ( $10 \mu$ m) proximity between the locations.

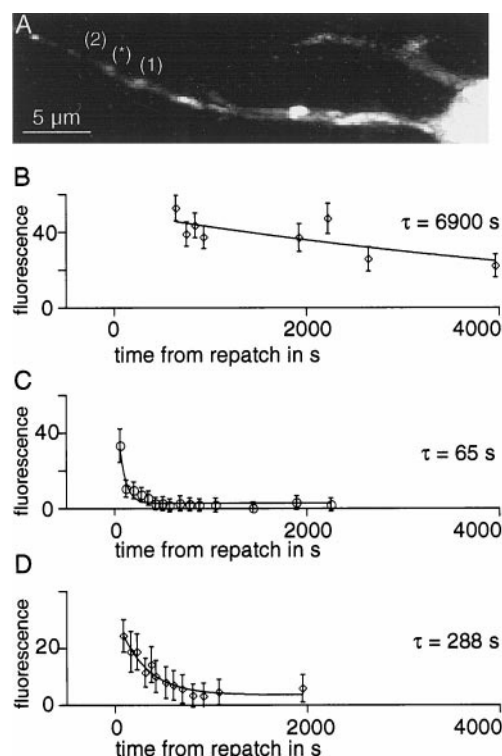


FIGURE 5 Changes in diffusion after eliciting photodamage. (A) Image of a layer 5 pyramidal cell filled with fluorescein (500  $\mu$ M) by whole-cell recording. At a certain place on a basal dendrite (indicated by \*) a line was scanned for 65 s at an average laser power (10 mW, 80-fs pulses) that induced an increase in basal fluorescence (image recorded before this procedure). (B) Decrease in fluorescence by washout after repatching. The cell was repatched with a pipette containing no dye. The decrease in fluorescence due to the washout of the dye was monitored. In the damaged area (\*) the decay of fluorescence was very slow, indicating that diffusion was inhibited. The decay time  $\tau = 6900$  s was more than 100-fold larger than in the unaffected area (I). (C) Proximal (I) to the damaged area, the washout of the dye occurred with a regular diffusion constant ( $\tau = 65$ ). (D) The decay of fluorescence was monitored also at a place (2) distal to the damaged area, where the dye had to diffuse through the damaged area (\*) for washout. The decay time constant ( $\tau = 288$  ms) was 4 times higher than in (I).

That means that unaffected dye diffused more slowly through damaged areas. This difference is consistent with the hypothesis that damaged areas were changed structurally in correspondence with the observed alterations in calcium fluorescence kinetics.

### Dependence of the photodamage rate $r$ on pulse length

Changing the pulse length is well suited for estimating the predominant order of the damage process at a given TPE rate. By adjusting the pulse length and peak intensity so that  $\tau \cdot I_{\text{peak}}^2$  is kept constant, the TPE rate can be kept constant while higher- and lower-order processes are being changed. Conversely, if the damage rate does not depend on  $\tau \cdot I_{\text{peak}}^2$ , then the damaging process must be a two-photon mechanism. The influence of the pulse duration on the damage rate

$r$  was tested (see Methods) by altering the pulse width from 75 fs to 0.8–3.2 ps. The excitation rates used were kept low to avoid the additional, irregular photodamaging mechanisms occurring at higher excitation rates. At these excitation rates,  $r$  ranged up to 0.25%/s. For 22 cells,  $r$  was measured as a function of the average laser power for femtosecond and picosecond laser pulses within a small power interval. For equal  $r$ , the exponent  $x$  could be derived by means of Eq. 4. No difference was found between chirped ( $n = 5$ ) and nearly transform-limited laser pulses ( $n = 17$ ) regarding the calculated exponents; therefore all data were pooled. We found an average exponent of  $x = 1.997 \pm 0.38$  ( $n = 22$ ,  $\pm$  SD, Fig. 6), indicating a two-photon-induced photodamaging mechanism at low excitation rates.

### Dependence of the photodamage rate $r$ on excitation rate

It has been speculated that higher-order processes like three-photon excitation contribute to photodamage at the peak intensity and power levels used for imaging. One might ask if, at the low excitation rates used, potential third-order components were masked by noise. Therefore, we tested the dependence of the photodamage on the excitation rate for femto- and picosecond laser pulses, separately. For a given pulse length,  $r$  was measured at different average laser powers for femtosecond (Fig. 7A) or picosecond laser pulses (Fig. 7B). The dependence on  $\bar{P}$  was fitted to determine the exponent  $x$  (see Methods) using Eq. 3. We tested 25 cells. Since no differences between the calculated exponents using different calcium dyes (MG:  $n = 10$ ; OGB-1: 100  $\mu$ M,  $n = 2$ ; 200  $\mu$ M,  $n = 3$ ; 500  $\mu$ M,  $n = 2$ ; OGB-2: 500  $\mu$ M,  $n = 3$ ) and chirped ( $n = 11$ ) and nearly transform-limited ( $n = 13$ ) picosecond laser pulses were observed, all data were pooled. For femtosecond laser pulses, the average exponent was  $x = 2.5 \pm 1.0$  (mean  $\pm$  SD,  $n = 16$ ). For picosecond laser pulses, the average exponent was  $x = 2.2 \pm 1.0$  (mean  $\pm$  SD,  $n = 12$ ). Since calcium indicators were used in these experiments, it was possible that the changes in basal fluorescence resulted from changes in

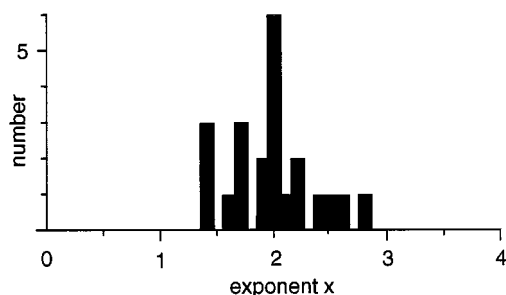


FIGURE 6 Dependence of cumulative photodamage on pulse length. Using Eq. 4, the order of excitation for the damage process was calculated by comparing similar damage rates  $r$  in a pulse length interval from 75 fs to 3.2 ps at low TPE rates. The histogram shows the results of 22 measurements with a mean of  $1.997 \pm 0.38$  (mean  $\pm$  SD).

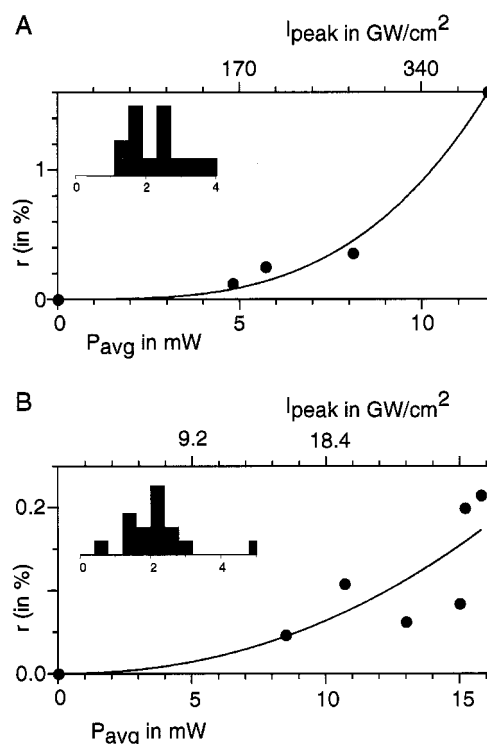


FIGURE 7 Dependence of cumulative photodamage on average laser power. (A) The graph shows four measurements of the rate of relative change  $r$  (in %) of basal fluorescence at different time-averaged powers  $\bar{P} = 4.8$ –11.8 mW (dots). Measurements were carried out in the same cell and at different locations in similar depths on a basal dendrite of a layer 5 pyramidal cell filled with MG (500  $\mu$ M). Resting membrane potential was  $-67$  mV and pulse length was  $78 \pm 5$  fs. The continuous line shows a fit using Eq. 3, yielding the function  $r = 0.0004\%/s \cdot (\bar{P}/\text{mW})^{3.35}$ . The upper left insert shows a histogram of evaluated exponents for femtosecond laser pulses. (B) The graph shows measurements of  $r$  (in %) at different time-averaged powers  $\bar{P} = 8.5$ –15.8 mW (dots) from a different cell. The dye used was MG (500  $\mu$ M), resting membrane potential was  $-64$  mV and pulse length was  $\sim 1.4$  ps. The continuous line shows a fit using Eq. 3, yielding the function  $r = 0.00042\%/s \cdot (\bar{P}/\text{mW})^{2.18}$ . The upper left insert shows the histogram of exponents for picosecond laser pulses.

resting calcium levels. Therefore, control experiments with fluorescein (500  $\mu$ M) were performed. The average exponent was  $2.5 \pm 0.5$  (mean  $\pm$  SD,  $n = 5$ ) for femtosecond laser pulses. This indicates that the results did not depend on whether a calcium indicator or an unspecific dye was used.

### DISCUSSION

To fully exploit the benefits of two-photon laser scanning microscopy, detailed knowledge is required about power limitations and potential photodamage. Various criteria have been proposed to quantify photodamage, strongly depending on the particular biological application of multiphoton imaging. In multiphoton imaging of cultured cells, photodamage has been related to the decrease in cell viability and quantified through the observation of the metabolic state (Sako et al., 1997; König et al., 1999). In the case of calcium imaging of neuronal cells, photodamage can be



observed as nonphysiological functional and morphological changes. At low excitation rates, changes in AP-evoked calcium fluorescence transients occurred. The observed changes are independent of the occurrence of APs, clearly localized to areas exposed to the laser, proportional to the time elapsed, and irreversible. The fact that the basal fluorescence increased linearly with time allowed us to define the rate of basal fluorescence increase as a dependable measure of photodamage. The damage rates are presented in terms of average laser power. The quantification in terms of TPE rates would be useful but compromised by several assumptions, such as scattering in brain slices, refractive mismatch-induced aberrations, etc. Nevertheless, we want to give an estimate of the orders of magnitudes involved; this is done in the Appendix.

A possible explanation of the increase in the basal level of fluorescence is that some dye molecules undergo changes binding them to the cellular matrix or membranes. Hence the basal fluorescence increased, because the mobile fraction of dye equilibrated again, raising the absolute concentration of dye. In contrast, the absolute fluorescence increase  $A_F$  evoked by an AP remained constant, indicating that the bound dye molecularly altered and no longer reported to calcium changes. This is supported by a previous report for another dye, fura-2, where a similar intermediate photoproduct with altered characteristics was found (Becker and Fay, 1987). Based on these assumptions, one can calculate that the maximal number of photons a dye molecule emits before it binds is  $<10^6$  (see Appendix). In our case, the generation of a new chromophore is unlikely because we did not observe a significant spectral difference between the fluorescence from the photodamaged and undamaged areas. We also did not observe an unexpected onset of fluorescence in cell structures that did not contain dye molecules. The steady increase in basal fluorescence used for quantification of cumulative photodamage should not be confused with the abrupt increases of basal fluorescence observed at higher intensities when a calcium-sensitive dye was used. The latter might be caused by calcium entering through a leaking external or internal membrane.

In addition, we found a hampered diffusion of dye molecules through damaged areas and a slower decay of the calcium fluorescence transients. This might be explained by a reduced mobility of the dye molecules. If this interpretation is right, it indicates much more severe changes than only the binding of dye molecules to intracellular structures. One possible interpretation is that the intracellular environment was heavily changed and became viscous, presumably by the binding of many proteins to each other and the cytoskeleton, comparable to what happens in fixation procedures. Such a severe change would impair the physiology of the affected cell structure because proteins cannot fulfil their functions anymore.

Several observations indicate the physiological relevance of the measure used for the cumulative photodamage. The increase in the decay time of the calcium fluorescence transients and the reduced diffusion through and out of

damaged cell parts suggest that the photodamage affects physiological parameters that are independent of the fluorophore. Furthermore, the laser intensities of excitation correlate to those reported where photodamage in unstained cells has been examined (König et al., 1999).

The investigation of the role of the pulse length is of particular interest because it reveals the dependence of the photodamage mechanism on the excitation intensity. If we just consider the TPE rate (Eq. 2), we find that, to achieve the same two-photon fluorescence as the femtosecond ( $\tau_1 = 100$  fs) illumination, the picosecond ( $\tau_2 = 2$  ps) pulse train requires a time-averaged power  $\bar{P}$ , or time-averaged intensity  $\bar{I}$  that is higher by a factor of  $\sqrt{\tau_2/\tau_1} \approx 4.5$  but a peak power, or peak intensity, that is lower by the same factor. (Note that, in Eq. 2, the TPE rate is proportional to the product of average and peak power). Our experiments show that this variation in pulse length, average, and peak power can really be carried out and that calcium signals can be equally well observed with picosecond laser pulses (Fig. 8). This is in accordance with previous findings (Hänninen et al., 1994; Hell et al., 1998; Bewersdorf and Hell, 1998; Schönle and Hell, 1998; Jenei et al., 1999; König et al., 1999) and sufficient to prove that the mechanism responsible for the cumulative photodamage is not caused by a linear absorption mechanism such as heating through single-photon absorption. In fact, at low excitation rates, the measurements revealed a quadratic exponent,  $x = 2.0 \pm 0.4$ , indicating that cumulative photodamage is dominated largely by two-photon absorption. Hence, for a given photodamage rate  $r$ , the femtosecond pulses involved a higher peak power  $I_{\text{peak}}$  in connection with a lower average power, whereas the picosecond pulses used a higher average power in conjunction with a lower peak power  $I_{\text{peak}}$ , just as suggested by Eq. 2. As a good rule of thumb, the rate of photodamage induced is determined by the product of the squared intensities times the pulse length:  $\tau \cdot I_{\text{peak}}^2 \propto (P \cdot \text{NA}^2)^2/\tau$ . This relationship indicates that higher time-averaged power can be afforded if the aperture is lower or the pulse length is longer. In other words, for low excitation rates, the photodamage is just proportional to the two-photon fluorescence rate.

Although, at low excitation rates, we found a quadratic nature for the damaging mechanism, at higher rates, we measured exponents  $>2.0$ . This might indicate that higher-order effects that are too weak to be observed at low intensities may become more important at higher excitation rates. The data presented are indeed consistent with this assumption, but this interpretation is difficult to test due to the onset of additional mechanisms of damage. These irregular artifacts observed at higher excitation rates could not be quantified and might have affected the estimation of the exponents when evaluating the dependence of the photodamage rate  $r$  on the average laser power.

In summary, the aim of this work was to clarify to what extent possible light-specimen interactions influence physiologically significant parameters in multiphoton calcium imaging. Furthermore, we investigated the role of peak

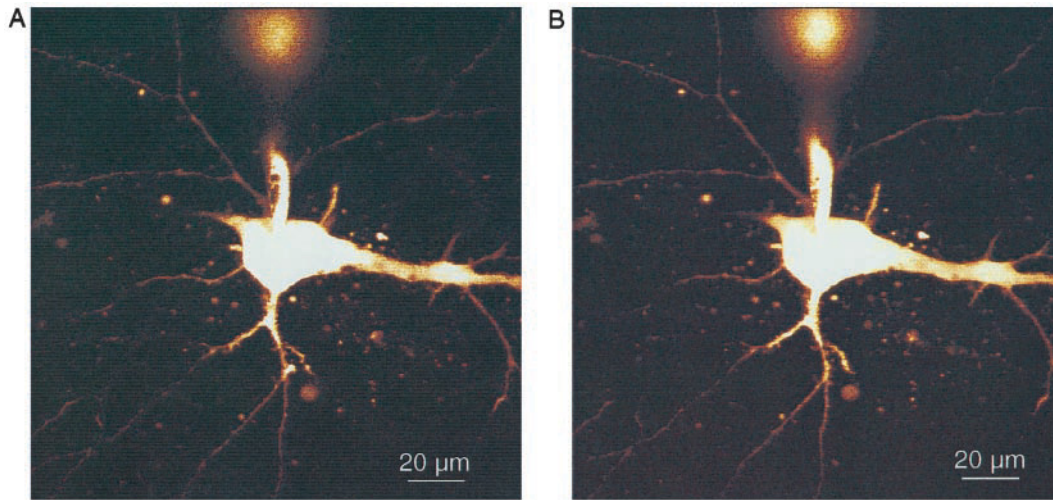


FIGURE 8 Comparison of femto- and picosecond mode imaging. Projections of stacks of 25 images of a layer 5 pyramidal neuron filled with OGB-2 (500  $\mu$ M) by somatic whole-cell recording; excitation wavelength was  $\lambda = 870$  nm. (A) Pulse length used was  $\tau = 1.4$  ps and average laser power was  $\bar{P} \approx 30$  mW. (B) Pulse length used was  $\tau = 74 + 4$  fs (nearly transform-limited) and average laser power was  $\bar{P} \approx 7$  mW. Similar two-photon laser scanning rates were achieved in both cases.

intensity, excitation rate, and pulse length in photodamage. For low excitation rates, we established the rate of increase of basal fluorescence as an index of photodamage. This parameter enabled us to examine the order of the damaging process by changing the pulse length. The observed exponent of  $2.0 \pm 0.4$  indicates two-photon absorption as the predominant damaging mechanism. This means that photodamage in TPE imaging is proportional to the signal and, thus, that signal can only be obtained at the expense of photodamage. When imaging over a longer time, low excitation rates must be used to avoid too heavy changes. This results unfortunately in a lower S/N-ratio. One way to overcome this is to optimize the collection efficiency (see Methods: Detection scheme). One should keep this in mind when applying multiphoton imaging to live cells. For low excitation rates, the results indicate that, within a window of 75 fs to 3.2 ps, the pulse width is irrelevant for two-photon calcium imaging.

Nevertheless, the use of multiphoton imaging provides several advantages over confocal microscopy in highly scattering tissue. The possibility of using ballistic photons for signal generation allows the capture of a much larger fraction of the fluorescence photons than in confocal microscopy, and the spatial confinement of excitation inflicts considerably less phototoxic stress to the cell. By carefully adjusting safe intensity levels for imaging, these benefits can be fully exploited.

## APPENDIX: AN ESTIMATE OF THE PHOTOSTABILITY

This model is based on the assumption that the increase in basal fluorescence is caused by binding of dye molecules to intracellular structures or membranes. Then, two pools of dye molecules exist, one of free, mobile dye molecules repetitive excitation will shift a dye molecule to the pool of

bound, immobile dye molecules. The pool of the mobile molecules is assumed to equilibrate by diffusion within a time (about ms) much shorter than the observation time (seconds), and its concentration is, therefore, approximated by a constant value. It would be useful to have an estimate of the average number of excitations before a dye molecule is shifted from the mobile pool to the immobile pool. Cross sections are difficult to measure for TPE, thus, in most cases, the product of cross section and quantum efficiency  $\sigma_2 q_e$  is given (Xu and Webb, 1996). For this reason, we can also calculate only the number of TPE fluorescence photons that will be emitted before a dye molecule gets bound. The estimate is based on several assumptions, like the attenuation of excitation light by tissue and collection efficiency, but it will give a feel for the orders of magnitude involved.

The model assumes that a cylindrical dendritic element of 1  $\mu$ m diameter and 0.5  $\mu$ m length is illuminated. The volume  $V = \pi(0.5 \mu\text{m})^3 = 4 \cdot 10^{-19} \text{ m}^3$  contains  $B = c \cdot V = 5 \cdot 10^4$  dye molecules at a dye concentration of  $c = 200 \mu\text{M}$ . We assume typical values encountered in the experiments: a gray value of  $G = 90$  per pixel, a pixel dwell time of  $t_p = 4 \mu\text{s}$ , the time the structure is illuminated during one cycle:  $t_i = 100 \mu\text{s}$  (see Methods), and a line repetition rate of  $f = 440$  Hz. At the voltage used for the PMTs,  $HV = 920$  V, the gain was about  $w = 4.0$ . Based on this, the number of photons collected in the epifluorescence detection channel is  $n_c = (t_i/t_p) \cdot (G/w) \cdot f = 2.5 \cdot 10^5 \text{ s}^{-1}$ . Given the detection efficiency of  $\epsilon = \Phi \cdot Q_e = 0.1 \cdot 25\% = 0.025$ , where  $\Phi$  is the collection efficiency of the system and  $Q_e$  the quantum efficiency of the PMTs, the number of photons emitted per molecule per second is  $n_{em} = (n_c/\epsilon) \cdot (1/B) = 2 \cdot 10^2 \text{ s}^{-1}$ . Another way to get an estimate for  $n_{em}$  is to use Eq. 2. A typical value of  $\bar{P} = 3$  mW that is attenuated to 50%,  $\sigma_2 q_e = 10^{-50} \text{ cm}^4 \text{ s}$  is assumed. Using Eq. 2, the number of photons emitted per molecule per second is  $n_{em} = 4 \cdot 10^3 \text{ s}^{-1}$ . This number deviates from the first number by one order of magnitude. Nevertheless, both numbers are used as upper and lower limits.

If dye molecules in both pools exhibit the same quantum efficiency and cross section, the number of photons emitted before a dye molecule changes pool is given by  $n_{em}/r$ . Given a typical value for  $r = 0.4\% \text{ s}^{-1}$  found for  $\bar{P} = 3$  mW, the average number of photons that a dye molecule emits before it binds is  $0.5 \cdot 10^5$  to  $1 \cdot 10^6$ . This number of  $n_{em}/r$  is independent of  $\bar{P}$  because  $n_{em}$  and  $r$  scale both with  $\bar{P}^2$ .

This simple model overestimates the number of emitted photons because photobleaching is not considered. Photobleaching will reduce the number of the dye molecules in the bound, immobile pool. The rate  $r$  is then decreased by the rate by which the pool of immobile, but still

fluorescent, dye molecules is depleted by photobleaching. Consequently, the real  $r$  is higher than the observed one, and thus,  $n_{em}/r$  is lower. In fact, the number of useful photons might be very low. Under certain conditions photobleaching was observed in our experiments and it will therefore play a role. Fluorescein is known to have a high bleaching rate (about  $3 \cdot 10^{-5}$ , (Hirschfeld, 1976)). This might explain the observation that the damage rates  $r$  observed with fluorescein were lower than the damage rates observed with other dyes.

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