Picosecond pulsed two-photon imaging with repetition rates of 200 and 400 MHz

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Summary
We show the viability of high-resolution two-photon fluorescence imaging of fixed and live cells by exciting the fluorophores with a train of near-infrared pulses with duration in the picosecond range. This is exemplified with a compact, diode-pumped Nd:YVO4 laser, emitting trains of 7-ps pulses at a wavelength of 1064 nm, with a repetition rate of 200 MHz at two separate outputs. Incoherent combination of the outputs enabled two-photon excitation with a repetition rate of 400 MHz. For a numerical aperture of 1·4 (oil), we used an average illumination power of up to 20–40 mW at the sample. The pulses were coupled into a beam scanning microscope, either directly or through a single mode glass fibre. Compared with standard femtosecond titanium–sapphire excitation conditions, our experiments were performed with a 2·5 or 5 times higher repetition rate, 30–70 times longer pulses and 10–35 times lower pulse peak intensity. The experiments indicate the possibility of significantly relaxing the temporal pulse width constraints for a series of applications.

Introduction
Two-photon excitation is evolving as an interesting alternative to single-photon excitation in biological fluorescence microscopy (Denk et al., 1995). Two-photon excitation is associated with a series of advantages comprising inherent three-dimensional imaging, reduced fluorophore bleaching in thick specimens and the possibility of collecting scattered fluorescent light from the focal region (Denk et al., 1990). The doubled excitation wavelength enables imaging of UV-excitable fluorophore distributions with standard visible optics and enables efficient filtering of the emitted light, so that a high signal-to-background ratio is achieved even for weak signals. The longer excitation wavelength penetrates deeper into scattering media and is generally more benign to live biological material (König et al., 1995b) which is also of great benefit to neurobiology (Denk & Svoboda, 1997). The use of two-photon excitation in 4 Pi-confocal microscopy provides a spatially well-defined point-spread-function ultimately leading to an unprecedented 3D resolution in the far-field (Hell & Stelzer, 1992; Hell et al., 1997).

The widespread use of two-photon microscopy has, however, been hampered by the expectation that it could be efficient only when used with lasers emitting ultrashort pulses with duration \( \tau \) in the hundreds or tens of femtosecond (subpicosecond) range, and at repetition rates \( f \) of 50–100 MHz (Denk et al., 1995). After the first demonstration with a colliding pulse mode-locked dye laser (Denk et al., 1990) featuring \( \tau \approx 100 \) fs and \( f \approx 100 \) MHz, the tunable titanium–sapphire laser \( \tau \approx 100 \) fs and \( f \approx 80 \) MHz became the light source of choice for routine two-photon biological microscopy (Curley et al., 1992; Stelzer et al., 1994; Denk, 1996). All-solid state approaches such as the Nd:YLF laser \( \tau \approx 350 \) fs and \( f \approx 100 \) MHz (Wokosin et al., 1996), the Cr:LiSAlF laser (Robertson et al., 1997) and the Cr:LiSrAlF laser (Svoboda et al., 1996) showed very good performance, but their operational parameters \( \tau \) and \( f \) did not significantly deviate from those of the standard Ti:sapphire laser.

In this paper we do not follow standard operational parameters. We deliberately use pulses of much longer duration. We also increase the repetition rate several fold. By doing this, we aim at extending the operational parameters of two-photon excitation microscopy to ranges which have hitherto been considered impractical. In addition, we aim at providing useful information about how strictly standard (subpicosecond) operational parameters have to be obeyed.

The adaptation of standard parameters was based on the desire to have the highest possible rate of two-photon absorption at a given mean photon flux, i.e. time-averaged power, \( P_{ave} \) (Denk et al., 1995; Soeller & Canell, 1996), as the latter is considered to be the main limiting factor to...
two-photon imaging. Ultrashort pulsing can thus be motivated by the anticipated molecular absorption rate \( \phi_2(r,z) \), expected to increase dramatically with pulsed excitation:

\[
\phi_2(r,z) = 0.56 \frac{h^2(r,z)}{\sqrt{\langle h\omega \rangle^2}} \sigma_2 \approx 0.56 \frac{1}{\sqrt{\langle h\omega \rangle} F^2} \sigma_2 
\]

(1) \( h(r,z) \) is the intensity point-spread-function, \( h\omega \) is the photon energy with \( 2\pi \hbar \) being Planck’s constant of action, \( \omega = 2\pi c/\lambda \) the angular frequency, \( \sigma_2 \) the two-photon cross-section and \( \lambda \) the wavelength of light. \( F = (0.16/\text{NA})2\pi/2 \) approximates the focal area, and NA denotes the numerical aperture. The peak power of the pulse is given by \( P_{\text{peak}} = 0.56 P_{\text{ave}}(\tau f) \) and is thus increased over the average power by the factor \( \xi = 0.56/\tau f \) for temporally hyperbolic-secant shaped pulses.

For a given average power, mode-locking the Ti:sapphire laser boosts the signal by a factor \( \xi = 5 \times 10^4 \) over its continuous wave option (Hänninen et al., 1994). In practical systems \( \xi \) is slightly lower (\( \approx 3 \times 10^3 \)) because of dispersion broadening, which can, however, be compensated by appropriate prechirping of the pulses. A well compensated microscope can achieve pulses in the tens of femtosecond broadening, which can, however, be compensated by appropriate prechirping of the pulses. A well compensated microscope can achieve pulses in the tens of femtosecond regime at the sample (Müller et al., unpublished results), so that \( \xi = 10^5 \) is within practical reach. Equation (1) thus suggests that the shorter the pulse, the better the result, a recipe that appears to be bounded only by the concomitant increase in spectral width of the excitation light. The repetition rate \( f \) should not exceed the inverse lifetime of the dyes so as to allow the relaxation of the excited molecules between the pulses (Denk et al., 1995). With typical fluorescence lifetimes of \( 0.5 \text{–} 8 \text{ ns} \), repetition rates \( f \leq 125 \text{ MHz} \) should be appropriate. In any case, this suggests the use of pulse compression as a method to increase the total fluorescence signal.

The higher power required for two-photon excitation in conjunction with the belief that the average power would pose an upper limit has probably discouraged the investigation of longer pulses in two-photon excitation microscopy. Nevertheless, picosecond and CW two-photon excitation has been proposed and demonstrated by Hänninen et al. (1994, 1995). This proposal was based on the realization that Eq. (1) also implies that pulsing is helpful only in an operational range where the limits are set by effects that depend linearly on the excitation power. An example of limiting linear effects is the heating of water can be ruled out as a limiting factor for almost any two-photon microscopy application.

The recent acquisition of a beam scanning confocal microscope allowed us to resume initial investigations on super-picosecond pulsed two-photon imaging with the beam scanning conditions encountered in standard confocal microscopes. Our experiments thus differ from prior work (Hänninen et al., 1995) which involved a fundamentally longer pixel dwell time and stage scanning.

**Materials and methods**

For the experiments described in this paper, we used a pulsed diode-pumped Nd:vanadate (Nd:YVO4) laser with a repetition rate of 200 MHz and a fixed pulse width of 7·1 ps (GE 100, Time Bandwidth Products, Zurich, Switzerland). The laser was passively mode-locked by a semiconductor saturable absorber mirror (SESAM) (Keller, 1996). Lasing action occurs at 1064 nm which is a popular neodymium transition, also known from the Nd:YAG laser. The output coupler is arranged so that two independent, equally polarized output beams are formed. Each beam carries a maximum average power of 300 mW (Fig. 1). Output 2 can be used to prefocus the combined laser beam to compensate for the axial focal shift due to the high wavelength of 1064 nm.

Fig. 1. Experimental set-up (A and B). In set-up A the picosecond-laser is coupled into the beam scanning confocal microscope by a single mode fibre (1a) with a collimator lens (2a) at the end. The parallel laser beam is then coupled into the microscope by a dichroic mirror (4). The numbers denote the scan mirror (5), scan lens (6), tube lens (7), objective lens (8) and the sample (9). While the scattered laser light is blocked by a colour glass (10) (KG3), the fluorescent light is detected by the regular photomultiplier tubes (11). In set-up B the two laser outputs are combined with a λ/2 plate (1b) and a polarization beam splitter cube (2b). In this way a pulsed laser source with a repetition rate of 400 MHz is obtained. A telescope consisting of two lenses (3b) is used to prefocus the combined laser beam to compensate for the axial focal shift due to the high wavelength of 1064 nm.
be coupled into a single mode fibre through a gradient index lens. The beam divergence was specified to < 1.6 mrad. The laser does not require cooling of any kind, and can be operated from the main power supply.

Imaging was performed using an inverted confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany). The microscope scans the beam in the focal plane by a fast \( x \) and slow \( y \) tiltable mirror. The specimen stage is movable along the optical axis \( z \) in 40-nm steps. The detection unit featured three photomultipliers that were placed behind a single detection pinhole, which, however, was removed to allow nonconfocal detection. The photomultipliers were operated in an analog (current) mode. The infrared laser beam was coupled into the microscope using a

Fig. 2. XY-images of a pollen grain at different axial positions. The stack was taken with the fibre-coupled picosecond-laser emitting at \( \lambda = 1064 \text{ nm} \) (set-up A) at a total pixel dwell time of 8 \( \mu \text{s} \) (eight medium scans). The average power of the laser in the focus was about 20 mW.
dichroic mirror with high transmissivity in the visible, and the reflection centred at 1064 nm.

We had the option of either feeding the beam into the microscope through a glass fibre or coupling it directly into the microscope with a mirror. In the initial experiment (set-up A), we used a 2-m-long single mode fibre (Fig. 1). The pulse length of the laser is not significantly elongated by group velocity dispersion in the fibre because of the small spectral bandwidth of the picosecond pulses (<0.3 nm). Fibre-coupling leads to stable and reliable imaging conditions. Alternatively, both laser outputs were combined by polarization combination and coupled directly into the microscope (set-up B). For this purpose, we tilted the polarization of 'output 2' with a λ/2-plate. A polarization beam splitter cube was then applied to superimpose the tilted beam onto that of 'output 1'. The beams were carefully co-aligned. Owing to the cavity design of the laser one of the beams is delayed by about half a repetition period (i.e. 2.5 ns) to the other, so that we obtained a single train of pulses having a repetition rate of 400 MHz.

We experimented with three standard objective lenses supplied by the microscope manufacturer: a water-immersion lens 63× 1.2NA W PL APO and two oil-immersion lenses 100× 1.4NA oil PL APO 1.4–0.7, and 40× 1.2NA oil PL APO 1.25–0.75. Being standard objective lenses, they were not originally designed for 1064 nm. We observed that the focus was axially offset with respect to the focus of the visible light, e.g. by about 1 μm for the 40× lens. In view of the square-law dependence of axial imaging, the high internal magnification of the intermediate optics of the microscope can lead to a slight confocalization through internal vignetting. We counteracted the shift by prefocusing the beam before it entered the microscope. The entrance aperture of the lenses were very slightly overilluminated, thus making a reasonable compromise between the economic use of the laser light and amplitude flatness at the entrance pupil. Adequacy of overexpansion was derived from axial resolution measurements and the image brightness. The body of the microscope was of the standard type: being optimized for the visible range it was only partially transparent for the exceptionally long wavelength of 1064 nm: the transmissivity of the 100× oil-immersion lens was determined to 43% at 1064 nm. An absorbing

Fig. 3. Power dependence of the fluorescence of (a) a solution of rhodamine 6G in immersion oil and (b) pollen fluorescence when excited at 1064 nm. The measurements were performed with set-up B. The data show the power dependence for both outputs separately, their sum as well as their 400-MHz counterpart. The dashed line indicates a quadratic power dependence.

Fig. 4. Edge response of a rhodamine 6G solution in immersion oil taken with a 100× 1.4NA objective and the 400-MHz-set-up (set-up B). The intensity plot was created by averaging an x–z image in the x-direction. The derivative corresponds to the z-response to an infinite plane. By independently measuring three z-responses, the axial resolution was determined to 940 nm ± 30 nm (FWHM).
filter (KG3, 2 mm thickness, Schott, Mainz, Germany) with an optical density $> 3$ at 1064 nm was placed in the detection path to suppress scattered laser light. The detected light passed the dichroic mirror as well as the KG3 filter which leaves visible light largely unattenuated.

Experiments

We started off by imaging test objects, namely a thick layer of a highly concentrated solution of rhodamine 6G dissolved in oil, as well as a sample of mixed pollen grains (Carolina Biological Supply Company, B690) (Fig. 2). Both samples displayed a pronounced signal which remained largely unaffected by the occasional insert of an additional KG3 filter. This indicated that laser light was suppressed whereas light from the sample could pass through without major losses. To further investigate the nature of excitation, we measured the power dependence of the fluorescence signal both for the rhodamine solution as well as the fluorescence of pollen grains. The measurements were
performed by using direct coupling (set-up B) offering both repetition rates. The fluorescence signal was measured for each version of illumination: output 1, output 2 (both at 200 MHz) and the 400-MHz version. We denoted the corresponding fluorescence yield by $W_{1/200}$, $W_{2/200}$ and $W_{400}$, respectively. Irrespective of the pump power, SESAM modelocking ensured a constant pulse duration.

Figure 3 shows the fluorescence signals $W_{1/200}$, $W_{2/200}$ and $W_{400}$ vs. the excitation power for a solution of rhodamine in oil in (a), and the fluorescence of pollen grains in (b) on a double-logarithmic scale. The data points for the rhodamine layer were obtained by recording $xz$-images and subsequent averaging. The beams of outputs 1 and 2 produced nonequal power values at the focus of the objective lens. Hence, the power range covered by each of the three measurements is different; the light from output 1 is weaker than its counterpart. For example, the power dependence of $W_{400}$ follows a slope of $2.07 \pm 0.1$ for the rhodamine sample. For the pollen grains we obtained $2.35 \pm 0.15$. The high repetition rates of 200 and 400 MHz pose the question of saturation by depletion of the ground state of the fluorophores. Therefore, we calculated the sum of the fluorescence generated by both outputs, $W_{1/200} + W_{2/200}$, and displayed it in Fig. 3 (crosses) along
with the measured 400-MHz fluorescence signal, $\Psi_{400}$ (circles).

In a second experiment the axial resolution of the system was measured for the 100× 1·4NA oil objective and the 400-MHz version (set-up B) by using the rhodamine 6G solution. An edge response was recorded by taking an $xz$-image with the microscope (Fig. 4). The image was averaged in the lateral $x$-direction and differentiated to obtain the axial response to a plane. The FWHM of the differentiated signal was measured as $940 \pm 30 \text{ nm}$.

The 3D-stack of $xy$-images of the pollen grain (Fig. 2) nicely demonstrates the sectioning capability of the multiphoton microscope; in the case of the fibre-coupled version (set-up A) using the 100× objective lens. The pixel dwell time was $8 \mu s$ (i.e. eight ‘medium scan’ frames as specified by the microscope manufacturer) and the average power at the focus, $P_{\text{ave}} \approx 20 \text{ mW}$. The dwell time was achieved by accumulating a number of images taken at the same location. It can also be seen that the intensity decreases with increasing depth in the sample. The deepest nominal depth was $32 \mu m$.

Figure 5 shows a pollen grain imaged with the 400-MHz version (set-up B) of our microscope. The data were visualized by means of a 3D-volume rendering software (T3D, Fortner). The image is based on 32 $xy$-images of $512 \times 512$ pixels. The pixel dwell time was $1 \mu s$ (i.e. one ‘medium scan’) and was recorded with an average power of $83 \text{ mW}$ at the entrance pupil, i.e. $P_{\text{ave}} \approx 35 \text{ mW}$ at the sample.

Figure 6 shows a series of laterally scanned images showing the autofluorescence signal of the cyanelles of a living glaucocystis alga at different axial positions. The stack was taken with the 400-MHz set-up of the picosecond-laser (set-up B). The average power of the laser in the focus was about $30 \text{ mW}$. The sample was flowing in water and thus a relatively short pixel dwell time of $1 \mu s$ was necessary. The recording of one image took about $1·7 \text{ s}$.

In Fig. 7, two images out of a time-series of 32 are displayed, showing the cytoplasmic streaming in acetabularia recorded with set-up A. The recorded signal is autofluorescence of the chloroplasts. The total recording time of one image was $1·7 \text{ s}$, the average power $P_{\text{ave}}$ at the sample was $\approx 20 \text{ mW}$ and the repetition rate $200 \text{ MHz}$. The images were taken with a time difference of $15 \text{ s}$. The pixel dwell time was $2 \mu s$ (four ‘medium 2’ scan). A formation of floating chloroplasts is marked by a circle. To provide a feel for the data, the inset shows an unaveraged intensity line profile along a single line in the image.

Figure 8 shows cat kidney cells imaged with the fibre-coupled 200-MHz version. The average power at the sample was about $20 \text{ mW}$ and the pixel dwell time $16 \mu s$ (16 averaged frames at ‘medium scan’).
Fig. 9. Three axially offset $xy$ images of a neuron in a rat brain slice.

Fig. 10. An overlay of two images of *P. ciliatum* taken simultaneously with two parallel detectors taken with the fibre-coupled 200-MHz set-up.
Discussion and conclusion

Our experiments allow two important conclusions. The first is the viability of two-photon imaging with pulses of several picosecond duration: that is 30–70 times longer than in standard two-photon microscopy. The second is that two-photon imaging at 1064 nm excitation can be carried out without significant compromise in spatial resolution. This is remarkable since standard objective lenses and a microscope body optimized for the visible range were used. The wavelength of 1064 nm further extends the range of useful wavelengths to well above 1 μm (Wokosin et al., 1996) and is, to our knowledge, the longest wavelength that has been used in multiphoton excitation microscopy so far.

The axial resolution of the 100× (NA = 1.4) oil-immersion lens of 940 ± 30 nm (FWHM of 2-response) compares well with the theoretical prediction of 930 nm (Hell et al., 1995). This indicates good aberration correction of the intermediate optics and the objective lens used in the near infrared, even at 1064 nm. The sketch in Fig. 1 shows that our set-up used a descanned detection, i.e. the fluorescence light passed through the intermediate optics of the system so that the scanning spot rested in the plane of the (here absent) confocal pinhole. Descanned detection allows the use of all the filter combinations supplied by the manufacturer and is insensitive to ambient light. However, in spite of the removal of the pinhole, the high magnification of the system still leaves open the possibility of a slight confocalization of the system, caused for instance by the combination of the large magnification and potential axial chromatic aberrations. Detailed comparisons using non-descanned detection of the microscope used are underway in our laboratory.

The wavelength of 1064 nm is well known from the Nd:YVO₄ laser and allows the use of a whole range of available optimized optical components such as lenses, prisms, retarders and filters. A further technical advantage is the fact that photomultipliers are almost insensitive at this wavelength and are therefore insensitive to scattered laser light. The large difference between excitation and detection supports efficient filtering. Further advantages of the 1064-nm wavelength include the anticipated reduced scattering and the reduced susceptibility to object-induced aberrations. For instance, the effect of spherical aberrations due to refractive index mismatch scales with the wavelength, so that a 33% greater penetration depth is expected for mismatched samples (Hell et al., 1993). It has been shown that large pollen grains are highly scattering media whose two-photon excitation images are superior to their single-photon confocal counterparts (Potter, 1996). The slight reduction in brightness of the xy-images in the stack of the pollen grain (Fig. 2) could be due to residual scattering but partially also due to an intensity drop caused by refractive index mismatch.

The power dependence curves of Fig. 3(a,b) carry important information about the performance of the 7-ps, high-repetition-rate two-photon microscope at 1064 nm. The curves do not show a significant decline of the slopes, thus indicating that saturation played only a minor role in these experiments. This conclusion is further substantiated by the comparison of $\Psi_{1/200}$ + $\Psi_{2/200}$ (crosses) with $\Psi_{400}$ (circles). Whereas $\Psi_{1/200}$ + $\Psi_{2/200}$ imply temporal gaps of 5 ns between the pulses, $\Psi_{400}$ involves gaps of 2·5 ns. Given a typical fluorescence lifetime of a few nanoseconds ($\approx$ 3·5 ns for rhodamine 6G), one would expect $\Psi_{400}$ to be up to 50% lower than $\Psi_{1/200}$ + $\Psi_{2/200}$, in the worst case. However, both values coincided within a maximum deviation of 7%, thus supporting the view that saturation did not play a significant role. These findings agree with our qualitative observation that most of our imaging applications could have been done at a higher average power at the sample if this had been available. If it was possible to reach saturation by ground state depletion, the lifetime of dyes could be inferred from the ratio ($\Psi_{1/200}$ + $\Psi_{2/200}$)/ $\Psi_{400}$ in a similar manner to the double-pulse method proposed by Brakenhoff and co-workers (Müller et al., 1995; Buist et al., 1997).

The all-solid state Nd:YVO₄ laser was attractive to us since, besides allowing us to demonstrate first picosecond operating conditions and fibre-delivery, it operated reliably and did not require any cooling. The narrow spectrum of the pulse (0·3 nm as opposed to 8–10 nm in a femtosecond system) facilitates fibre-coupling of the laser into the microscope because the lower peak-power involves less self-modulation of the pulses in the fibre. While reliability and all-solid state features are also offered by other lasers such as the Nd:YLF the Nd:YVO₄ is available (in Germany) at one-third of the cost of the former.

A disadvantage of the 1064-nm wavelength, and in general of wavelengths above 1 μm, is the fact that the coatings of microscope components are not optimized for this infrared wavelength range. Although our microscope was surprisingly well corrected for aberrations, it
transmitted only about 15–18% of the total laser light onto the back aperture of the objective lens. For our experiments this drawback ultimately set limits to the available two-photon excited signal. Therefore, although the images produced feature a reasonable signal-to-noise ratio, many of them could probably have been recorded somewhat faster by a 100-fs titanium–sapphire laser operating at around 800 nm. The reasons for the lower signal in the experiments reported herein are due to the particular constellation of limited available power, 7 ps pulse width, and unusually long excitation wavelength. Owing to a blue-shift in the two-photon excitation spectrum, at the wavelengths of around 800 nm, two-photon excitation cross-sections of many popular dyes (Xu et al., 1996) are usually higher at 700–850 nm than what can be expected at 1064 nm. Therefore, the lower signal observed in the images cannot be attributed to the longer pulses per se but is catalysed by the poorer cross-sections and limited power available at the focus.

An increase in power could be achieved by adapting the coatings of the microscope or by increasing the laser output power. As we were unable to reach saturation or other limitations in the fluorescence photon flux we cannot emphasize the application of this particular laser. Nevertheless, the Nd:vanadate might well turn out to be a good compromise in performance and cost for a series of applications at present.

It is interesting to calculate the equivalent of average power that would have been required with a femtosecond laser with the working parameters of $f' = 100$ MHz, focal $\tau = 300$ fs (Nd:YLF by Microlase, Strathclyde, U.K.). Evaluating Eq. (1), we find that

$$P_{ave}^{100\text{fs}} = \frac{P_{ave}^{7\text{ps}}}{(0.3 \times 10^{-12} 1 \times 10^{8})/(7 \times 10^{-12} 2 \times 10^{8})}$$

For example, the 20 mW (at 200 MHz) of focal average power used would correspond in efficiency to 2.9 mW of the 300-fs system (at 100 MHz). The comparison also supports our previous findings that our experiments were mainly limited by the power available. It is also interesting to calculate the equivalent average power for a pulse width $\tau'' = 2$ ps and $f'' = 100$ MHz (also Nd:YLF). In this case one would have $P_{ave}^{100\text{fs}} = 0.39 P_{ave}^{7\text{ps}}$. Hence a lesser increase in power, by a factor of 2.58, is expected to compensate for the relative pulse width broadening to 2 ps. Therefore images that are taken with an average power of about 10 mW with 300-fs pulses can most likely be taken at 25–8 mW with 2-ps pulses of the Nd:YLF. However, picosecond two-photon imaging is probably difficult to pursue in applications in which up to 300 mW of average power of 100–300 fs are required, such as very deep imaging of brain slices with long-working-distance low-aperture lenses (Denk & Sweboda, 1997).

 Naturally, the acceptable power levels depend on the application, excitation wavelength and aperture. For high-aperture lenses, two-photon imaging with pulses in the 100–300 fs range has been reported up to 20–40 mW. The use of 3–6 mW was reported as being sufficient for wavelengths around 700–800 nm. For higher power, i.e. higher focal intensities, damaging mechanisms such as sudden onsets of strong (auto-) luminescence, i.e. bright spots, were observed occasionally for some samples (König et al., 1996). Nonlinear optical effects have been suggested to be responsible for this phenomenon (König et al., 1995a).

We made similar observations in the cat kidney sample and in muscle samples in which we also saw sudden onsets of strong luminescence, albeit occasionally. Future developments will therefore include the increase of the average power and pulse width variations in order to find out the limitations of operation. While we used a higher average power, our experiments were carried out at a significantly lower peak intensity. This increases the potential risk of optical trapping. In the present experiments no trapping effects were observed.

Equation (1) indicates that a higher repetition rate leads to a reduction of the total fluorescence yield. While this is certainly true for a given average power and for excitation rates below the saturation level, this will hold only if the maximum obtainable fluorescence flux is limited by ground state depletion rather than nonlinear optical effects, such as multiphoton photolysis. The latter scenario, however, is supported by recent experiments with Coumarine 120 in water at the single-molecule level (Eggeling et al., 1997). It is still a general rule, the fundamental increase in repetition rate pursued here would probably be the method of choice for increasing the image brightness in multiphoton microscopy. Shortening the pulse in two-photon microscopy would increase the risk of detrimental effects, especially of third order and higher.

To date, the operational limitations of two-photon microscopy are still under investigation. The measurements and imaging evidence in this paper provide useful information for determining the functional parameters of multiphoton excitation. Our work differs from most prior investigations by purposely using longer pulse widths rather than making the pulse short with pulse compressors. The success in imaging with 7 ps is encouraging as it indicates the possibility of increasing the average power and reducing the peak intensity in two-photon microscopy. Without generalization for all applications, we think that the usefulness of external pulse width compression becomes questionable and should be considered carefully before application. Moreover, our images provide initial evidence that the pulse duration can be prolonged into the 1–3 ps regime in high-aperture two-photon microscopy. Longer
pulses will be beneficial if the two-photon microscopy is limited by three and higher order induced limiting effects. This is more likely to take place at shorter wavelengths. Our results suggest that, if the power is available, at shorter two-photon excitation wavelengths (750–900 nm), pulses of a few picoseconds should yield bright images.

In conclusion, we have extended the operational range of two-photon microscopy to the use of 20–30 times longer pulses, a longer wavelength (1064 nm) and a hitherto unachieved 400-MHz pulse repetition rate. The present results should stimulate further research on two-photon excitation microscopy with longer (picosecond) pulse duration.

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References


