Photobleaching in Two-Photon Excitation Microscopy

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ABSTRACT The intensity-squared dependence of two-photon excitation in laser scanning microscopy restricts excitation to the focal plane and leads to decreased photobleaching in thick samples. However, the high photon flux used in these experiments can potentially lead to higher-order photon interactions within the focal volume. The excitation power dependence of the fluorescence intensity and the photobleaching rate of thin fluorescence samples ($\sim$1 μm) were examined under one- and two-photon excitation. As expected, log-log plots of excitation power versus the fluorescence intensity and photobleaching rate for one-photon excitation of fluorescein increased with a slope of $\sim$1. A similar plot of the fluorescence intensity versus two-photon excitation power increased with a slope of $\sim$2. However, the two-photon photobleaching rate increased with a slope $\geq$3, indicating the presence of higher-order photon interactions. Similar experiments on Indo-1, NADH, and aminocoumarin produced similar results and suggest that this higher-order photobleaching is common in two-photon excitation microscopy. As a consequence, the use of multi-photon excitation microscopy to study thin samples may be limited by increased photobleaching.

INTRODUCTION

Since its initial demonstration (Denk et al., 1990), two-photon excitation microscopy has proven to be an excellent technique for noninvasive imaging. The coupling of red photons to excite ultraviolet fluorophores takes advantage of the better penetration and relative harmlessness of the longer wavelengths. In addition, two-photon excitation microscopy reduces overall photobleaching by limiting it to the focal plane of the microscope (Denk et al., 1990, 1995). This reduction is especially important for imaging in thick samples, such as brain slices or pancreatic islets (Denk and Svoboda, 1997; Piston and Knobel, 1999). However, anecdotal and published evidence have indicated that photobleaching is more rapid under two-photon excitation than with conventional microscopy (Heikal and Webb, 1999; Sánchez et al., 1997).

For our quantitative NAD(P)H studies of pancreatic β-cells during glucose-stimulated insulin secretion, the photophysical properties of NADH were carefully measured under two-photon excitation. This study proceeded in the same manner as previous work on quantitation of GFP (Patterson et al., 1997), and included measurements of the photobleaching rate as a function of power. Because the number of fluorescent photons depends on the square of the excitation intensity and the photobleaching rate was expected to depend on the number of fluorophore excitations, we expected to find an intensity-squared relationship between excitation power and the photobleaching rate. However, these preliminary data on purified NADH suggested that the photobleaching rate depended not on the square of the intensity, but on a higher-order relationship.

To quantitate this phenomenon, the fluorescence intensities and photobleaching rates were measured as a function of the excitation power during one- and two-photon excitation of several fluorophores. These measurements were performed on aqueous microdroplets of fluorophore suspended in a hydrophobic medium as previously described (Patterson et al., 1997). To achieve relatively rapid photobleaching and minimize any effects due to excitation localization, most of these studies were performed in droplets that were compressed between a slide and coverslip to $\sim$1 μm thickness. Thicker droplets were also used to verify that the observed phenomena were not due to edge effects. Fluorescein was the fluorophore chosen for these initial experiments because the photobleaching properties of fluorescein have been previously studied (Song et al., 1995, 1996) and a comparison between one- and two-photon excited photobleaching could be made with the same dye.

Our results show that the one-photon excited photobleaching of fluorescein follows a near-linear relationship with the incident intensity, but that its two-photon photobleaching does not follow the expected intensity-squared dependence on power, but rather depends on $>$3 power of the excitation. Measurements of the two-photon excited photobleaching rates of several other fluorophores also revealed a dependence on higher orders of the excitation power. Preliminary experiments to determine the mechanism of this phenomenon were inconclusive, but the data from these experiments are presented in this short report. These mechanistic possibilities are discussed, as are the implications of this accelerated photobleaching for two-photon excitation microscopy of biological specimens.

MATERIALS AND METHODS

Two-photon excitation microscopy was performed using a previously described instrument (Piston et al., 1995) with a Zeiss 40$\times$ Plan Neofluar

Received for publication 16 August 1999 and in final form 6 December 1999.

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0006-3495/00/04/2159/04 $2.00

1.3 NA objective and ~150 fs pulses of 700–780 nm laser light. One-photon excitation microscopy was performed with a 488-nm CW laser light on a Zeiss LSM 510. The laser power was adjusted with a variable neutral density filter (Newport Corp., Irvine, CA) for two-photon excitation or by changing the tube current in one-photon excitation. Scan times were chosen to eliminate effects of significant photobleaching during the pixel dwell time. In the case of fluorescein dextran, this meant scanning as rapidly as possible. Fluorescein dextran (10,000 MW), aminocoumarin dextran (10,000 MW), and Indo-1 pentopotassium salt were obtained from Molecular Probes, Inc. (Eugene, OR). NADH was obtained from Sigma (St. Louis, MO). 2-Aminoethanethiol hydrochloride (cysteamine or mercaptoethylamine) was obtained from Aldrich (Milwaukee, WI). Microdroplets for photobleaching were prepared in 1-octanol as previously published (Patterson et al., 1997). The fluorescein and aminocoumarin samples were attached to dextran to prohibit those dyes from slowly seeping into the octanol. To prepare the microdroplets, the fluorophores in phosphate-buffered saline, pH 7.3, were mixed 1:9 with octanol by pipetting for 5 s and then vortexing for 20 s. Larger droplets were allowed to sediment for 10–15 s and 3 μl of the solution were compressed to ~1 μm thickness between a slide and coverslip before being sealed with paraffin. These samples allowed us to photobleach in vitro in a volume small enough to alleviate the problems of diffusion in the bleached region. The mean pixel values from regions of interest encompassing the interior of the microdroplet were determined by quantitative image analysis using National Institutes of Health Image 1.62b7 (National Institutes of Health, Bethesda, MD) or with Zeiss LSM 510 analysis software. A mean pixel value of background outside the droplet was subtracted from each image. The decay in fluorescence intensity in the photobleaching experiments was fit to a single exponential equation ($A*e^{(-k*B)}$) for NADH and Indo-1 or to a double-exponential equation ($A*e^{(-k*B)} + C*e^{(-k*D)}$) for aminocoumarin dextran and fluorescein dextran using Kaleidigraph 3.0.4 (Abelbeck Software, Reading, PA).

RESULTS AND DISCUSSION

To ensure that fluorophore excitation and emission displayed the expected one- or two-photon dependence, fluorescence emission was measured as a function of excitation power. For photobleaching rate measurements, microdroplets were continuously irradiated with 488 nm (one-photon excitation) or 710 nm (two-photon excitation) laser light while collecting fluorescence images at 15-s or 1-min intervals, respectively. Time 0 fluorescence was normalized to 100% for each experiment and typical results from a two-photon excitation photobleaching experiment over excitation levels from 8.3 to 16.5 mW are shown in Fig. 1. One-photon photobleaching results are qualitatively similar (data not shown). In both cases, the intensity decreased exponentially under continuous irradiation, with the fluorescence half-life having an inverse relationship with excitation power. The rates presented in this paper were determined from exponential fits of the fluorescence decay, but regardless of how this decay was analyzed (i.e., by fitting multiple exponential decays, a single exponential decay, or simply reading the half-life from the plots shown in Fig. 1), the results of the excitation power dependence were the same.

A log-log plot of the initial fluorescence intensity versus power (*open squares* in Fig. 2 *A*) gave the expected slope for one-photon excitation (slope = 1.0 ± 0.1). Furthermore,

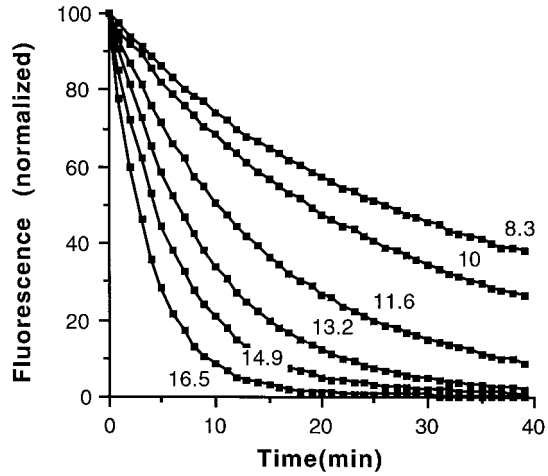

FIGURE 1 Fluorescein dextran (10 μM in pH 7.3) was suspended in ~1-μm-thick microdroplets and continuously irradiated with 710 nm mode-locked laser light at six different power levels (in mW units) while collecting images at 1-min time points.

the log-log plot of the photobleaching rate during one-photon excitation (488 nm) versus laser power yielded a line with a slope of 1.2 ± 0.1 (*filled squares* in Fig. 2 *A*), indicating that photobleaching closely followed the number of fluorescence photons. In this case, there does not appear to be a significant presence of higher-order photobleaching affects, which is in agreement with a previous one-photon excitation study that reported no change in the fluorescein dextran photobleaching yield over a range of excitation intensities (Widengren and Rigler, 1996).

Similarly for the two-photon excitation case, the log-log plot of the initial fluorescence intensity versus power (*open squares* in Fig. 2 *B*) gave the expected slope for two-photon excitation (slope = 1.9 ± 0.2). However, the log-log plot of the fluorescein dextran photobleaching rate under two-photon excitation (710 nm) gave a slope of 3.1 ± 0.1 (*filled squares* in Fig. 2 *B*), indicating that photobleaching is not strictly coupled to the amount of fluorescence. While this phenomenon may indeed be fluorophore- and wavelength-dependent, similar photobleaching rate experiments on NADH (slope = 3.3 ± 0.1), Indo-1 (slope = 3.5 ± 0.1), and aminocoumarin dextran (slope = 5.1 ± 0.2) shown in Fig. 3 also found evidence of higher-order photon interactions. For each of these fluorophores the fluorescence intensity increased by the square of the excitation power as expected, so these higher-order photobleaching effects do not appear to arise from any direct ≥3-photon absorption. Since the fluorophores tested here show slopes >3, the higher-order photobleaching effects may be a common phenomenon with two-photon excitation.

Molecular diffusion also seems to have little effect on the higher-order dependence of two-photon photobleaching. First, the fluorescence samples were approximately the same thickness as the two-photon excitation volume (~1

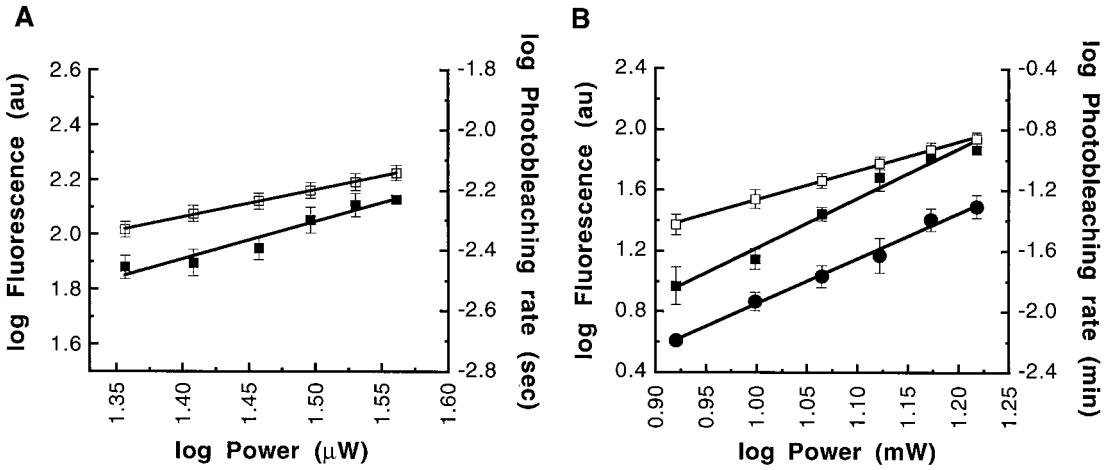

FIGURE 2 (A) The excitation dependence of fluorescein dextran fluorescence intensity (*open squares*) and the average photobleaching rate (*filled squares*) were determined for six levels of 488 nm laser light. (*B*) The excitation dependence of fluorescein dextran fluorescence intensity (*open squares*) and the average photobleaching rate (*filled squares*) were determined for six levels of 710 nm laser light. The excitation dependence of the average photobleaching rate was also determined for fluorescein dextran microdroplets of ~10 μm thickness (*filled circles*). Photobleaching experiments, as in Fig. 1, were fit to double-exponential equations to determine the average decay rate. Data are displayed as mean ± SE ($n \geq 5$).

μm). Therefore, all of the fluorescent molecules were continuously irradiated, negating diffusion into and out of the excitation plane. Second, fluorophores ranging in molecular weight from ~10,000 to ~700 exhibited the ≥3 dependence on photobleaching (Fig. 3). Last, photobleaching of thick (~10 μm) fluorescein dextran samples expectedly proved to be much slower than thin samples, but also displayed a rate dependence of 3.0 ± 0.1 on the excitation power (Fig. 2 *B*).

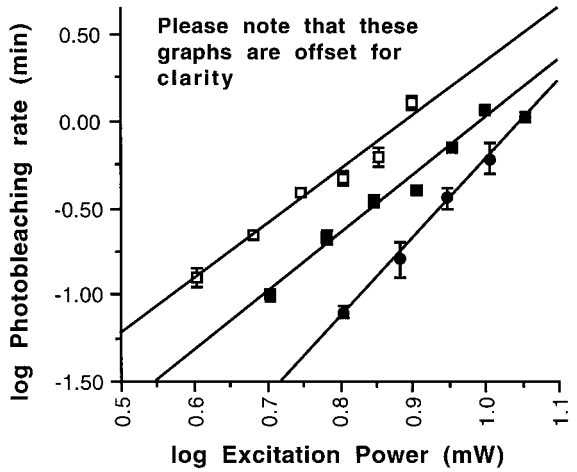

FIGURE 3 Two-photon excitation photobleaching experiments as in Fig. 2 *B* were performed on aminocoumarin dextran (*filled circles*; slope = 5.1 ± 0.2), NADH (*open squares*; slope = 3.3 ± 0.1), and Indo-1 (*filled squares*; slope = 3.5 ± 0.1). Data for NADH and aminocoumarin dextran have been offset from Indo-1 for display and are plotted as mean ± SE ($n = 5$). The actual excitation levels in these experiments range from 4.2 to 8.3 mW.

The photobleaching data are not due to artifacts of fluorophore saturation or pulsewidth because the two-photon excited fluorescence intensity measurements show a strict quadratic dependence on the excitation power. Based on typical two-photon excited saturation values, our experiments have been carried out with intensities about an order of magnitude below the saturation level. Furthermore, fluorophore saturation would cause a deviation from the intensity-squared dependence, and no such deviation was observed. Likewise, any significant variation of the ~150-fs pulsewidths as a function of power would also affect the fluorescence signal. Again, since no deviation was observed in the intensity-squared dependence of the fluorescence, we can conclude that pulsewidth variations did not influence the bleaching results.

We have investigated whether photon interactions with the excited triplet state are involved in the mechanism for the accelerated photobleaching. Indeed, the fluorescein triplet state has been shown to be involved in photobleaching (Song et al., 1996). However, log-log plots of fluorescein dextran photobleaching versus power in the presence of mercaptoethylamine (MEA), a triplet state quencher (Song et al., 1996), still resulted in a slope of 3.2 ± 0.1 (data not shown). The MEA did not affect the fluorescence of the fluorescein dextran (data not shown), so these data argue that although the triplet state may be involved in fluorescein photobleaching, it is not associated with the higher-order effects observed with two-photon excitation.

These data led us to conclude that the fluorophore is initially excited by simultaneous two-photon absorption, and then one or more photons interact with the excited molecule to increase the probability of photobleaching.

Such a sequential mechanism was proposed for the intensities used in one-photon laser scanning microscopy (Tsien and Waggoner, 1995), although no strong evidence of increased bleaching has been observed in conventional confocal microscope experiments. This sequential absorption model has previously been suggested in a photobleaching study of rhodamine 6G (Widengren and Rigler, 1996) and rhodamine B (Sánchez et al., 1997). To address this issue, we varied the wavelength of the two-photon excitation. Photobleaching experiments on fluorescein dextran and NADH indicate that excitation wavelength does not seem to affect the power dependence of the two-photon photobleaching. Log-log plots (data not shown) of the fluorescence intensities displayed the expected quadratic power dependence, and the log-log plots of photobleaching versus power both showed a $\geq$3-photon dependence (fluorescein dextran = 3.1 $\pm$ 0.1; NADH = 3.0 $\pm$ 0.2). However, if the dye is excited away from its excitation maximum, then there will be more incident photons per excitation. In the case where a single extra photon interacts with the two-photon excited state to accelerate bleaching, we would expect even faster photobleaching per fluorescent photon at the less efficient excitation wavelength. For NADH, the two-photon cross-section at 760 nm is about one order of magnitude less than at 710 nm, and the overall photobleaching rate was decreased as expected from their two-photon cross-sections (Xu et al., 1996). However, for matched fluorescence intensities the photobleaching at 760 was not faster, but rather three times slower than it was at 710 nm. This indicates that a simple extra photon model is not sufficient to describe the phenomenon, but that possibly a higher-order resonance absorption is involved.

The difference between one- and two-photon photobleaching will, of course, depend on the excitation levels used. At the lowest excitation intensities, photobleaching rates between one- and two-photon excitation will be comparable, but at the power levels that are typically used in biological imaging (3–5 mW at the sample for $\sim$150 fs laser pulses) this difference can easily be a factor of 10. Such increased photobleaching would be substantially detrimental in only a subset of experiments, particularly for thin-sample (<5 μm) and single-molecule studies. However, since the lowest possible light exposures are used in live cell fluorescence microscopy experiments, photobleaching is generally minimized. In fact, for the duration and excitation level that we normally use for NAD(P)H imaging, $<$5% of the fluorophore would be photobleached in the absence of diffusion. While elimination of this small amount of photobleaching would be desirable, it would not significantly improve the experimental results. Of course, there are still other tremendous advantages of two-photon excitation for imaging of thick samples, such as better sample penetration, decreased overall fluorophore photobleaching, and red illumination of ultraviolet dyes, that make it a very desirable imaging technique (Centonze and White, 1998; Denk and Svoboda, 1997).

This work was supported by National Institutes of Health Grant DK53434 and National Science Foundation Grant BRI 98-71063 (to D.W.P.). During part of this work, G.H.P. was a National Institutes of Health trainee (GM08320). This work was performed using resources of the Cell Imaging Shared Resource supported by the Vanderbilt Cancer Center (CA68485) and Diabetes Research and Training Center (DK20593).

REFERENCES

Centonze, V. E., and J. G. White. 1998. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. *Biophys. J.* 75:2015–2024.

Denk, W., D. W. Piston, and W. W. Webb. 1995. Two-photon molecular excitation in laser-scanning microscopy. *In* Handbook of Biological Confocal Microscopy. J. B. Pawley, editor. Plenum Press, New York. 445–458.

Denk, W., J. H. Strickler, and W. W. Webb. 1990. Two-photon laser scanning fluorescence microscopy. *Science.* 248:73–76.

Denk, W., and K. Svoboda. 1997. Photon upmanship: why multiphoton imaging is more than a gimmick. *Neuron.* 18:351–357.

Heikal, A. A., and W. W. Webb. 1999. One-and two-photon time-resolved fluorescence spectroscopy of selected fluorescent markers: photobleaching, triplet-, and singlet-state dynamics. *Biophys. J.* 76:260*a*. (Abstr.).

Patterson, G. H., S. M. Knobel, W. D. Sharif, S. R. Kain, and D. W. Piston. 1997. Use of the green-fluorescent protein (GFP) and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73:2782–2790.

Piston, D. W., B. D. Bennett, and G. Ying. 1995. Imaging of cellular dynamics by two-photon excitation microscopy. *Microsc. Microanal.* 1:25–34.

Piston, D. W., and S. M. Knobel. 1999. Quantitative imaging of metabolism by two-photon excitation microscopy. *Methods Enzymol.* 307: 351–368.

Sánchez, E. J., L. Novotny, G. R. Holtom, and X. S. Xie. 1997. Room-temperature fluorescence imaging and spectroscopy of single molecules by two-photon excitation. *J. Phys. Chem. A.* 101:7019–7023.

Song, L., E. J. Hennik, I. T. Young, and H. J. Tanke. 1995. Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy. *Biophys. J.* 68:2588–2600.

Song, L., C. A. G. O. Varma, J. W. Verhoeven, and H. J. Tanke. 1996. Influence of the triplet excited state on the photobleaching kinetics of fluoroscein in microscopy. *Biophys. J.* 70:2959–2968.

Tsien, R. Y., and A. Waggoner. 1995. Fluorophores for confocal microscopy: photophysics and photochemistry. *In* Handbook of Biological Confocal Microscopy. J. B. Pawley, editor. Plenum Press, New York. 267–279.

Widengren, J., and R. Rigler. 1996. Mechanisms of photobleaching investigated by fluorescence correlation spectroscopy. *Bioimaging.* 4:149–157.

Xu, C., R. M. Williams, W. Zipfel, and W. W. Webb. 1996. Multiphoton excitation cross-sections of molecular fluorophores. *Bioimaging.* 4:198–207.