Pulse-length dependence of cellular response to intense near-infrared laser pulses in multiphoton microscopes

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The influence of the pulse length, τ, of ultrashort laser pulses at 780 and 920 nm on cell vitality and cellular reproduction has been studied. A total of 2400 nonlabeled cells were exposed to a highly focused scanning beam from a mode-locked 80-MHz Ti:sapphire laser with 60-μs pixel dwell time. For the same pulse energy, destructive effects were more pronounced for shorter pulses. The damage behavior was found to follow approximately a P^2/τ dependence (P, mean power), indicating that cell destruction is likely based on a two-photon excitation process rather than a one- or a three-photon event. Therefore, femtosecond as well as picosecond pulses provide approximately the same relative optical window for safe two-photon fluorescence microscopy.

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Three-dimensional multiphoton laser microscopy, a novel, powerful optical section technique with submicrometer resolution, is based on nonresonant simultaneous absorption of two or three excitation photons in the subfemtoliter focal volume of a high-numerical-aperture (NA) objective.[1–4] Multiphoton excitation of intracellular fluorophores in living specimens utilizes excitation radiation in the near-infrared (NIR) spectral region of 700 to 1200 nm. With the radiation focused to diffraction-limited submicrometer spots, average milliwatt powers are sufficient to provide the required intensities of megawatts per square meter–gigawatts per square meter to excite approximately 300–600-nm electronic transitions (two-photon excitation) as well as approximately 200–400-nm transitions (three-photon excitation).[5] Owing to the lack of efficient one-photon absorbers in the 700–1200-nm spectral range in most of the cells, no photobleaching or photodamage in out-of-focus regions occurs, unlike in conventional fluorescence microscopy. The high penetration depth of the NIR excitation radiation provides the possibility of optical sectioning in deep tissue. Pinhole-free three-dimensional imaging with submicrometer lateral and axial resolution is possible owing to the tiny subfemtoliter excitation volume. Multiphoton excitation of intracellular fluorophores in living cells has been demonstrated by use of cw NIR microbeams[6–8] and more efficiently by use of femtosecond (fs) and picosecond (ps) laser pulses with kilowatt peak power but low-milliwatt average power.[1,9,10]

Nondestructive multiphoton imaging of living specimens is restricted to a certain optical window. The lower limit of this window is determined by the optical properties of the fluorophore (e.g., the molecular multiphoton cross section and the fluorescence quantum yield) and by the detector sensitivity, and the upper limit is determined by the onset of cell damage. Photothermal damage appears unlikely in most cells in which water is the major NIR absorber (e.g., in cells without the absorbers hemoglobin, melanin, or chlorophyll). That this is so was shown in cw 1064-nm microbeam studies in which Chinese hamster ovarian (CHO) cells labeled with a thermosensitive fluorophore were exposed to megawatt-per-square-centimeter intensities and gigajoule-per-square-centimeter fluence[11] as well as by temperature calculations for exposure to fs and ps pulses.[12]

However, destructive nonlinear photochemical effects as well as severe cell damage by optical breakdown effects can occur. With respect to the excitation wavelength, two-photon excitation of endogenous cellular absorbers, for example, by the coenzymes NAD(P)H and flavins as well as by porphyrins, can take place, which can result in the formation of destructive oxygen radicals and singlet oxygen (i.e., oxidative stress) and indirect DNA damage.[13] Three-photon absorption can result in DNA and protein excitation (a major absorption band of nucleic acids is at 260 nm; excitation of this band results in direct DNA damage). When light intensities in the range of some hundreds of gigawatts per square centimeter to some terawatts per square centimeter are used, intracellular optical breakdown and plasma formation can occur, resulting in intense white luminescence and severe morphological damage, such as cell fragmentation.[14] Irreversible cell damage, such as impaired cell division, caused by 150-fs scanning beams (730–800 nm, 80 MHz, 80-μs pixel dwell time) at average powers as low as 1–5 mW has been studied.[15]

In this Letter we report on the influence of pulse duration on CHO cells exposed to intense NIR excitation radiation in multiphoton microscopes. We provide evidence that cell damage depends strongly on pulse length.

We studied the influence of NIR excitation radiation at 780 and 920 nm provided by a tunable mode-locked Ti:sapphire laser (Coherent Mira; 80-MHz pulse repetition frequency, ≈1-W power, ≈120-fs pulse-length output). Radiation at 780 nm, which is also a typical NIR wavelength of compact solid-state laser systems (e.g., Coherent Vitesse and frequency-doubled fiber lasers), can excite a variety of endogenous chromophores, such

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as amino acids, by a three-photon process and can excite coenzymes, porphyrins, and cytochromes by a two-photon event. Pulses at 920 nm can excite intracellular absorbers mainly by a three-photon process and flavins and porphyrins by two-photon absorption. The absorption coefficient of water is ≈7 times greater at 920 nm than at 780 nm. The NIR beam used here was coupled to an inverted confocal-laser scanning fluorescence microscope (Zeiss Model LSM 410). The parallel beam was expanded to fill the back aperture of a 40× Zeiss Neofluar bright-field objective (NA, 1.3). To vary the pulse length in the range 120–1000 fs we stretched the pulses with a folded SF14 prism pair (Fig. 1). Blazed gratings (750 nm; 651 mm^{-1}) were used to obtain ps pulses (1–4 ps). The pulse length and the bandwidth of the chirped laser pulses were measured with an autocorrelator (APE Berlin) before the beam entered the microscope, which was characterized by a typical dispersion value of ≈5000 fs^2 (NIR).[16] Transmission of, e.g., 120-fs pulses through the microscope resulted in an ≈170-fs pulse length at the sample. Cw radiation was provided by interruption of mode locking (no additional etalon was used). The mean laser power was measured in air after transmission through the objective (a Coherent Fieldmaster LM2 powermeter was used). Assuming a FWHM beam size at the focal spot of $\lambda/(2NA)$, 10-mW mean power of 150-fs pulses at 780 nm corresponds to ~0.8-kW peak power and 1.2-TW/cm^2 peak intensity.

Nonlabeled CHO cell pairs (which underwent one division) in sterile 2-ml cell chambers (silicon frame between coverslips) were microirradiated with the scanning beam ten times at a pixel dwell time of ≈60 μs per scan (16 s/frame; 512 × 512 pixels, zoom 2, covering a 160 μm × 160 μm sample area). With a typical cellular area of 700 μm^2, the cell dwell time was ≈400 ms per scan. With a cell concentration of ~20,000 ml^{-1}, the typical distance of the cell pairs was ≈150 μm. Exposed cells, marked with a diamond on the outside of the coverslip window of the chamber and maintained in an incubator for 48 h, were considered to be unaffected by the NIR scanning beam if clones consisting of >8 cells (more than two cell divisions) were produced. Cell vitality was determined by postexposure incubation of a live–dead viability kit (Molecular Probes) consisting of the live-cell fluorophor calcein (2 μM; fluorescence maximum, 517 nm) and the red fluorescent dead-cell indicator ethidium homodimer-1 (4 μM; fluorescence maximum, 617 nm). A total of 2400 exposed cells were studied.

A strong dependence of cell damage on pulse length was found, as can be seen in Fig. 2(a). Cell damage was less pronounced with increasing pulse length. For example, loss of cell viability (in all exposed cells) occurred at mean powers of 7.3 ± 1.0 and 20 ± 2 mW for 240-fs and 2.2-ps pulses, respectively. The loss of viability, as indicated by the onset of propidium iodide, was accompanied with highly localized intense white luminescence, followed by carbonization (black areas). Irreversible cell damage, such as failed cellular reproduction, occurred at lower power levels, as determined by a sensitive cell-cloning assay. A 50% cloning efficiency (50% of cells failed normal cell division) was found at mean powers P_{50} of 4.5 ± 0.5 (240 fs) and 7.3 ± 1.0 mW (2.2 ps). Therefore a ninefold increase of pulse length requires an approximately

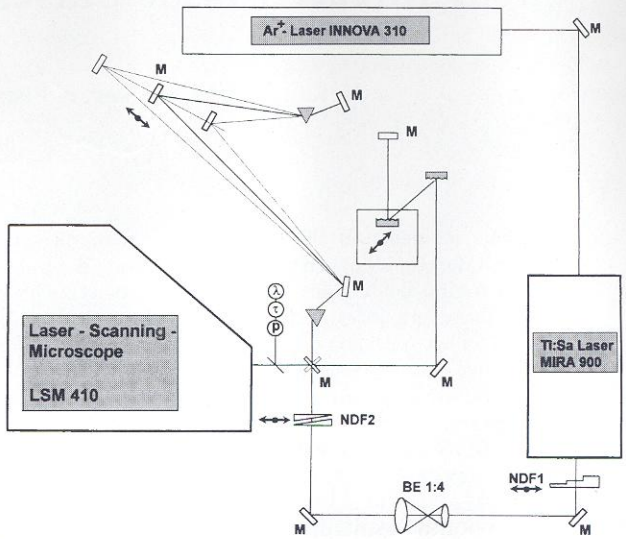

Fig. 1. Scheme of the multiphoton laser scanning microscope, with an out-of-cavity pulse-stretching unit consisting of two SF14 prisms and two blazed gratings: NDF1, NDF2, neutral-density filters; BE, beam expander; M's, mirrors; P, powermeter.

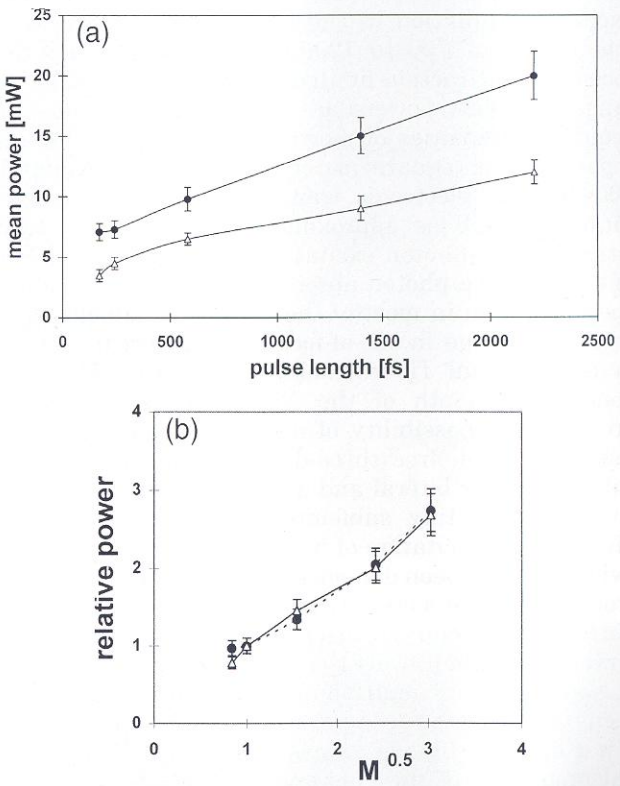

Fig. 2. (a) Mean power of the 780-nm pulses used to induce cell damage versus pulse length. ●, mean power at which the onset of visible morphological damage occurs (the bars indicate standard deviation); △, mean power at which cloning efficiency is reduced to 50%. (b) Relative power versus the square root of pulse broadening M. Curve fitting reveals a nearly linear relation ($y = 0.82x + 0.13$, $R = 0.99$). The symbols are defined in (a).

threefold-greater mean power for the same damage effect to be induced. This power value corresponds to the square root of pulse broadening and reflects a damage process based on two-photon excitation [Fig. 2(b)], in which the excitation efficiency is indirectly proportional to the pulse length and proportional to power squared. Because two-photon fluorescence excitation follows the same P^2/τ relation, the shift from 240 fs to 2.2 ps reduces the fluorescence signal by a factor of 9 and requires that a threefold-greater mean power be used to maintain the image quality in two-photon fluorescence microscopy. We were able to image the fluorescence of a variety of fluorophores, e.g., fluorescent microbeads (Fluoresbrite carboxy YG 6 μm; Polysciences), using a two-photon excitation process with minimum mean powers $P_{\min}$ in the ranges 350–450 μW (240 fs) and 1.2–1.5 mW (2.2 ps) in the descanned mode. Therefore, two-photon fluorescence imaging appears to be a safe, novel optical section technique within a certain window of mean power. In the case of CHO cells and our descanned detection system, we found a relatively narrow window covering ~1 order of power magnitude ($P_{50}/P_{\min} \approx 10$). The ratio was approximately the same for both fs and ps pulses. Within this window, we were able to scan CHO cells at a mean power of 2 mW and a 170-fs pulse length for more than 1 h without phototoxic effects.

Interestingly, cell damage, such as impaired cell division, also occurred during scanning of cellular regions that do not include nuclear DNA. Therefore, photoinduced failure in cellular reproduction does not require direct nuclear DNA damage, e.g., by three-photon excitation. Using the Ti:sapphire laser in cw operation at 780 nm, we were not able to harm the cells, even with as much as 25 mW of power.

Changing the wavelength to 920 nm resulted in fewer destructive effects, indicating that linear photothermal effects owing to water heating are not responsible for cell damage. 200-fs pulses induced cell death and 50% cloning efficiency at 9 and 6 mW, respectively. We were able to image three-photon excited fluorescence of 3-μM Hoechst 33342-labeled living CHO cells with as little as 3 mW of power (200 fs). The cells exposed to 920-nm light remained alive. Although a three-photon event requires higher photon flux densities, three-photon imaging within the safe window is possible. The fluorescence excitation efficiency will follow the relation $I \sim P^3/\tau^2$. We were not able to reduce the cloning efficiency to 50% with 1-ps pulses at 920 nm and 11-mW mean power.

In conclusion, ultrashort pulses in the fs and ps regions as well as cw microbeams can be used within certain optical windows for nondestructive nonlinear imaging of living specimens. For two-photon fluorescence imaging, fs and ps pulses seem to cover the same relative window determined by the optical properties of the fluorophores and the detector sensitivity and by the upper limit of two-photon-induced cell damage. For three-photon imaging, which requires higher photon flux densities, the relative window is narrower and will decrease with increasing pulse length. Therefore, for efficient nondestructive three-photon imaging fs pulses are recommended.

The presence of the narrow relative optical window for safe cell and tissue monitoring should be considered in the performance of multiphoton experiments on living specimens, in the development of compact solid-state ultrashort laser sources and multiphoton microscopes, and in the design of multiphoton fluorophores.

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