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### Authors

König, K  
So, PT  
Mantulin, WW  
[et al.](#)

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# Cellular response to near-infrared femtosecond laser pulses in two-photon microscopes

K. König

*Institute of Anatomy II, Friedrich Schiller University Jena, 07743 Jena, Germany*

P. T. C. So, W. W. Mantulin, and E. Gratton

*Laboratory of Fluorescence Dynamics, University of Illinois, Urbana-Champaign, Illinois 61801*

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The influence of femtosecond near-infrared (NIR) microirradiation on cell vitality and cellular reproduction has been studied. Chinese hamster ovary cells exposed to a highly focused 150-fs scanning beam at 730, 760, and 800 nm (80 MHz, 80- $\mu$ s pixel dwell time) of  $\leq 1$  mW remained unaffected by the femtosecond microbeam. However, increased mean power led to impaired cell division. At  $\geq 6$ -mW mean power, cells were unable to form clones. They died or became giant cells. Complete cell destruction, including cell fragmentation, occurred at mean powers  $> 10$  mW. Cell death was accompanied by intense luminescence in the mitochondrial region. When we consider the diffraction-limited spot size in the submicrometer region, intensities and photon flux densities of 0.8-kW pulses (10-mW mean power) are of the order of terawatts per square centimeter ( $10^{12}$  W/cm<sup>2</sup>) and  $10^{32}$  photons cm<sup>-2</sup> s<sup>-1</sup>, respectively. Extremely high fields may induce destructive intracellular plasma formation. The power limitations should be considered during NIR femtosecond microscopy of vital cells and in the design of compact NIR femtosecond solid-state lasers for two-photon microscopes. © 1997 Optical Society of America

Two-photon microscopy, based on the application of near-infrared (NIR) ultrashort laser microbeams in laser scanning microscopes, has become a powerful tool in vital cell investigations and biotechnology.<sup>1,2</sup> Unlike in conventional microscopy, cellular chromophores are excited by simultaneous absorption of two photons with half the energy normally used for excitation. NIR photons can therefore effectively excite ultraviolet and visible transitions and can induce visible fluorescence. Two-photon excitation requires a high photon concentration in time and space that is given by diffraction-limited focusing of ultrashort laser pulses. In general, femtosecond laser pulses with watt and kilowatt peak power, low average power in the milliwatt range, and a megahertz repetition rate as well as high-numerical-aperture objectives are used in two-photon microscopy. Because of the square power dependence, two-photon excitation takes place in a subfemtoliter focal volume, which permits fluorescence scanning microscopy with inherent three-dimensional resolution. Because cellular absorbers have no efficient NIR transitions, no out of focus photobleaching or photodamage occurs. Two-photon microscopes are therefore considered as nondestructive tools in vital cell studies.<sup>1,2</sup> However, up to now we know of no systematic studies on cell vitality during the interaction of single vital cells with femtosecond NIR pulses.

Recently we reported on cell damage in optical traps by 70-mW cw NIR microbeams owing to two-photon excitation processes. Single human spermatozoa confined in 760-nm traps died within minutes. During this trapping period, the cells experienced NIR radiant exposures of gigajoules per square centimeter ( $10^9$  J/cm<sup>2</sup>). We found that the cell damage was more pronounced in 760-nm multimode cw traps than in

single-frequency traps because of transient power enhancement by longitudinal mode beating. In contrast, cells survived in traps with a trapping wavelength of  $\geq 800$  nm.<sup>3,4</sup> In this Letter we provide evidence that femtosecond microbeams at 730-, 760-, and 800-nm microbeams above certain power thresholds are able to influence the cellular metabolism, resulting in impaired cell division, uncontrolled cell growth, or cell fragmentation. Below these power thresholds, femtosecond NIR microbeams may act as nondestructive excitation beams in two-photon microscopy.

We studied the influence of femtosecond NIR excitation beams in two-photon scanning microscopes on cell vitality and cellular reproduction. Single Chinese hamster ovary cells were microirradiated with a NIR scanning beam of 150-fs pulse width, 80-MHz pulse-repetition frequency, and 80- $\mu$ s pixel dwell time per scan ( $512 \times 512$  pixels covering a  $35 \mu\text{m} \times 35 \mu\text{m}$  sample area) and a total of 10 scans per cell. The NIR beam was provided by a tunable Ti:sapphire laser (Mira 900, Coherent), coupled into a modified inverted Zeiss fluorescence microscope and focused to its diffraction-limited spot size by a  $63\times$  Zeiss Neofluar objective with a numerical aperture of 1.25.<sup>5</sup> Exposed cells ( $n = 300$ ) maintained in an incubator for 5–6 days were considered to be unaffected by NIR femtosecond microirradiation if clones consisting of  $\geq 50$  cells were produced. Cells irradiated with 730-, 760-, and 800-nm beams of a  $\geq 6$ -mW mean power at the sample were unable to form clones. These cells either were not able to exclude the deal-cell indicator Trypan Blue or became giant cells (uncontrolled growth). No cell was affected by  $\leq 1$ -mW microbeam irradiation. A 50% cloning efficiency was measured following exposure at 2–3 mW (Fig. 1).

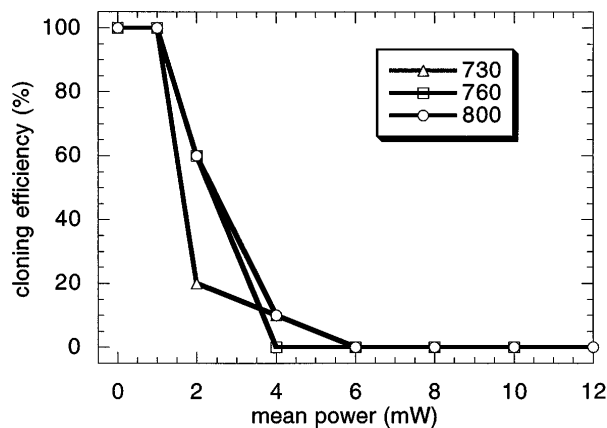


Fig. 1. Cloning efficiency versus mean laser power of 730-, 760-, and 800-nm femtosecond microbeams.

Luminescence imaging in the 400–700-nm spectral range during NIR microbeam exposure revealed autofluorescence in the mitochondria owing to the presence of fluorescent coenzymes. For mean excitation powers  $>2$  mW, the onset of an additional  $\approx 200$ -fold more-intense luminescence occurred in the mitochondrial region of the cell. At mean powers  $>10$  mW, the intense luminescence arose already during the first scan in all exposed cells. It was accompanied by complete cell destruction, including cell fragmentation. No cell exposed to NIR microbeams of a mean power  $\leq 1$  mW revealed such intense luminescence

even with over 100 scans, indicating a power dependence. Considering the diffraction-limited spot size in the submicrometer region, we note that intensities and photon flux densities of 0.8-kW pulses (10-mW mean power) are of the order of terawatts per square centimeter ( $10^{12}$  W/cm<sup>2</sup>) and  $10^{32}$  photons cm<sup>-2</sup> s<sup>-1</sup>, respectively. Therefore, extremely high fields may induce destructive intracellular plasma formation. Plasma induced by femtosecond laser pulses in other nonorganic materials at similar intensities has been reported.<sup>6</sup> The above power limitations should be considered during NIR femtosecond microscopy of vital cells and in the design of compact NIR femtosecond solid-state lasers (turnkey lasers) for two-photon microscopes.

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