

Cell damage in near-infrared multimode optical traps as a result of multiphoton absorption

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We report on cell damage of single cells confined in continuous-wave (cw), near-infrared (NIR) multimode optical traps as a result of multiphoton absorption phenomena. Trapping beams at NIR wavelengths less than 800 nm are capable of damaging cells through a two-photon absorption process. Cell damage is more pronounced in multimode cw traps compared with single-frequency true cw NIR traps because of transient power enhancement by longitudinal mode beating. Partial mode locking in tunable cw Ti:sapphire lasers used as trapping beam sources can produce unstable subnanosecond pulses at certain wavelengths that amplify multiphoton absorption effects significantly. We recommend the use of single-frequency long-wavelength NIR trapping beams for optical micromanipulation of vital cells. © 1996 Optical Society of America

A novel micromanipulation tool in vital cell biology is optical traps. Single-beam gradient force traps are created when cw laser beams are focused with an objective of high numerical aperture (NA). They are based on the generation of pico-Newton forces that are due to the change in momentum that occurs during laser beam–cell interaction.^{1,2} To avoid cellular heating and competitive thermal forces, one should use a trapping wavelength in the near-infrared (NIR) spectral region. As a result, a large number of trapping studies have been conducted with cw Nd:YAG lasers operating at 1.06 μm . With the increased availability of tunable Ti:sapphire and compact diode laser sources, however, there has been substantial interest in developing optical traps at shorter NIR wavelengths, at which water absorption can drop by as much as a factor of 10.³ This can be particularly beneficial in the case of motile cell studies in which it is essential to produce trapping forces that are higher than the adenosine triphosphate–driven intrinsic motility forces. Under these conditions, laser powers of 50–150 mW are typically required for confinement of highly motile spermatozoa.⁴ Spermatozoa trapping has been proclaimed to be a useful, novel method in laser-assisted *in vitro* fertilization.^{5–7} When high-NA objectives (NA \sim 1.3) are used to focus 50–150-mW cw radiation to diffraction-limited spots ($d \sim \lambda/\text{NA}$), trapping intensities of 15–40 MW/cm² (750–1064 nm) and photon flux densities of $\sim 10^{26}$ cm⁻² s⁻¹ are generated. With typical 10^{-50} cm⁴/s molecular two-photon absorption coefficients,⁸ these photon flux densities are sufficient for inducing two-photon excitation processes.

Recently we reported what we believe to be the first observation of multiphoton excitation in optical traps. We found that NIR trapping beams induce two-photon excited fluorescence of endogenous and exogenous fluorophores in single motile sperm cells. In addition, we detected cell damage by 70-mW, 760-nm trapping beams from a cw Ti:sapphire laser.⁹ In this

Letter we provide evidence that cell damage occurs in single-frequency traps at NIR wavelengths of <800 nm and that trap-induced cell damage is more pronounced in multimode cw traps. Trapping NIR beams of <800 nm are capable of two-photon excitation of endogenous cellular absorbers,⁹ such as reduced pyridine coenzymes, with electronic transitions in the UV. It is well known that reduced coenzymes and other endogenous chromophores in excited electronic states can produce cytotoxic oxygen radicals and singlet oxygen through charge transfer and energy transfer (type-I and type-II photo-oxidation), resulting in cell damage by oxidative stress.^{10–12}

Our cw NIR beam system consisted of a multimode, Ar⁺-laser-pumped Ti:sapphire ring laser (Coherent Innova-100/899-01) operating in spatial filtered TEM₀₀ mode. The Ti:sapphire laser contained a three-plate birefringent filter with an average linewidth of ~ 12 –20 GHz, which permitted tuning from ~ 700 to 1000 nm.¹³ The NIR beam was coupled to an inverted fluorescence microscope (Zeiss Axiovert 135) equipped with thermoelectrically cooled color and slow-scan CCD cameras (Zeiss ZVS-47DEC; Princeton Instruments ST135/TE576). For vitality tests, motile spermatozoa were stained with a live–dead fluorescence assay kit¹⁴ and trapped with a 70-mW trapping beam (power measured after the objective) at different wavelengths. The trapping beam was used as a fluorescence excitation source. Fluorescence (green from live cells; red from dead cells) was detected in the range of 400–700 nm with appropriate combinations of dichroic, bandpass, and source-blocking short-pass filters. At the beginning all trapped sperm cells exhibited a two-photon excited green fluorescence spot (<0.5 μm in diameter) in the cell head, indicating the trapping beam position and that the cell was alive.

Green cellular fluorescence remained in optical traps at wavelengths of 800 nm or higher for up to 10 min. In contrast, fluorescence color transition from green to red occurred at NIR trapping wavelengths of <800 nm,

indicating lethal damage. Interestingly, 760-nm traps induced the most efficient cell killing (within 1 min of trapping); see Fig. 1.

To prove whether the pronounced cell damage effect at 760 nm has its origin in biological or in laser output characteristics, we varied the source power and measured the fluorescence intensity of a Rhodamine 123 solution placed in a sealed chamber on the microscope stage. We found a quadratic dependence of fluorescence intensity on laser power for 800-nm excitation (exponent 2.03); however, absolute fluorescence intensities were 3–10-fold lower than those for 760-nm excitation at the same power. Using 760-nm beams, we were able to employ as little as 5 mW of power (measured after the objective) to excite fluorescence. This efficiency difference did not appear to be a simple consequence of wavelength-dependent variations in absorption cross section or mean photon flux density. As a result, we examined the temporal characteristics of our cw Ti:sapphire laser beam. Measurements of the laser output with a 1-GHz avalanche photodiode (Model 1651, New Focus) and a 1-GHz digital oscilloscope (DSA 601, Tektronix) clearly showed the presence of ultrashort pulses (Fig. 2). Pulse widths were in the subnanosecond region; however, the actual pulse width and height were difficult to determine because of detector bandwidth limitations. Pulse-repetition frequencies f were found to be variable and appeared at only integral multiples of 180 MHz. Although Fig. 2 shows $f = 180$ and $f = 360$ MHz for 800 and 760 nm, respectively, modulation stability was not predictable, and we have observed frequency oscillations at every wavelength. The 180-MHz base frequency corresponds to c/p , where c is the velocity of light and p is the cavity length of this ring laser (~ 1.7 m). Variations in 760-versus-800-nm pulse amplitude are also illustrated in Fig. 2. In contrast to the random pulse-frequency behavior, pulse amplitude was clearly dependent on wavelength, and the highest peak powers were always obtained when the birefringent filter position was optimized for the 760-nm output.

The shortest possible pulse width can be determined in the extreme case of mode locking from the number of allowed modes N and the round-trip cavity time T . For a 180-MHz modulated output and linewidths of up to 20 GHz, 111 longitudinal cavity modes can contribute to pulse generation. Thus, under optimal conditions, longitudinal mode beating could produce pulses as short as $T/N = 50$ ps. Although our laser was not optimized for mode locking, wavelength-dependent pulse variations suggest that partial mode locking occurred at 760 nm. Because two-photon absorption is proportional to the square of the instantaneous peak intensity, two-photon effects are most efficient at 760 nm.

To confirm that the pronounced cell damage at this wavelength is based on laser output characteristics, we introduced an étalon into the Ti:sapphire laser cavity. The étalon-modified laser output was single frequency (20-MHz linewidth), and there was no longitudinal mode beating visible in the photodiode output. Under these conditions, power-dependence curves were obtained for Rhodamine 123 that were lower in intensity

but virtually identical in function form, as in the case of the multimode cw laser. Two-photon excited fluorescence was clearly visible in optically trapped sperm cells when both 760- and 800-nm single-frequency beams were used. We found that the introduction of the étalon suppressed the biological effect but that cell death could not be avoided at short-wavelength NIR traps. When we used 760-nm true cw traps, cell damage occurred 6–7 times later (406 ± 160 s) than when multimode beams (65 ± 20 s) with identical power densities were used.

Phototoxic effects produced by two-photon excitation of the extracellular fluorophores of the live–dead assay kit, e.g., through photodynamic action, can be excluded. The two-photon excitation volume of $<1 \mu\text{m}^3$ (Refs. 15 and 16) is within the sperm head of typical dimensions $5 \mu\text{m} \times 3 \mu\text{m} \times 2 \mu\text{m}$. Intracellular accumulation of dead-cell fluorophore propidium iodide occurs only after cell death. To exclude any cell damage effects as

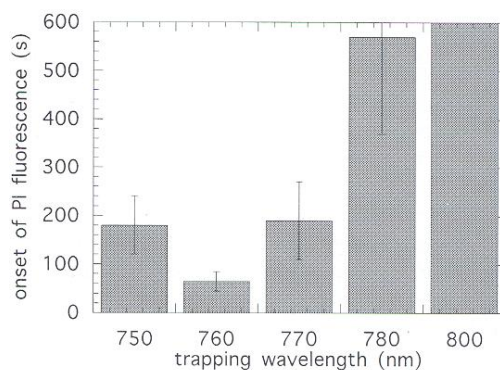


Fig. 1. Onset of intracellular propidium iodide (PI) fluorescence (cell death) in optically trapped spermatozoa ($n = 20$, 70 mW) in the case of multimode cw traps. The maximum trapping period was chosen to be 600 s. No cell survived in the 750-, 760-, and 770-nm traps. A time of 600 s for the onset of propidium iodide fluorescence was used for cells unaffected during trapping (all cells in 800-nm traps and some cells in 780-nm traps).

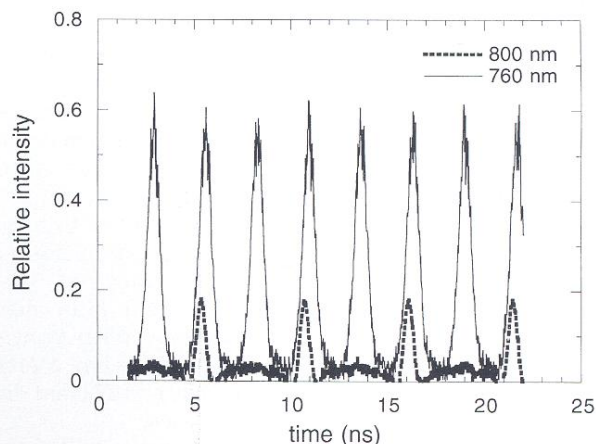


Fig. 2. Oscilloscope recording of Ti:sapphire laser output made with a 1-GHz detector. The presence of variable-amplitude, subnanosecond pulses at approximately 180 and 360 MHz is shown. The traces at 760 and 800 nm are taken with the same gain. Amplitude differences of a factor of 3 result in a factor-of-9 difference in two-photon effects because of the squared dependence.

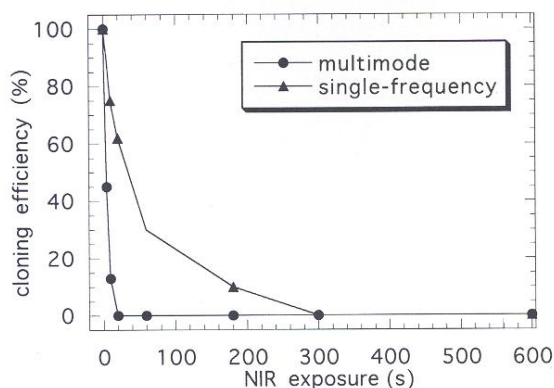


Fig. 3. Cloning efficiency versus exposure time for CHO cells for multimode cw beams and single-frequency beams (760 nm, 88 mW).

a result of two-photon excitation of the intracellular exogenous live-cell fluorophore, we trapped spermatozoa ($n = 20$) without incubation of fluorophores and monitored the stop of flagellar motion as a damage indicator. Loss of motility occurred after 132 ± 50 s in true cw 760-nm traps, compared with 35 ± 20 s in multimode 760-nm traps of the same average power. It should be mentioned that paralyzed cells are damaged but may be still alive. Experiments with the dead-cell indicator propidium iodide alone (no live-cell indicator in the medium) confirmed the obtained results of trap-induced cell death and earlier onset of paralysis.

A final proof of cell damage by highly focused NIR laser beams and damage amplification by multimode operation versus single-frequency operation was obtained when we evaluated the cloning efficiency of Chinese hamster ovary (CHO) cells. Single CHO cells were exposed to highly focused 760-nm (88-mW) multimode beams versus single-frequency beams. There was no incubation with exogenous chromophores. Maintained in an incubator for 5–6 days after exposure, the exposed cell was considered to be unaffected by 760-nm beams if a clone consisting of >50 cells was produced. CHO cells normally have a rapid reproduction time (one cell division) of ~ 12 h. As can be seen from Fig. 3, no cell was able to form a clone at 20-s exposure time in the case of multimode exposure. In contrast, at the same irradiation time 60% of the cells

remained unaffected by true cw exposure. However, no clone formation was found at 5-min exposure.

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