Two-photon fluorescence excitation in continuous-wave infrared optical tweezers

Y. Liu and G. J. Sonek

Department of Electrical and Computer Engineering and Beckman Laser Institute and Medical Clinic, University of California, Irvine, Irvine, California 92717-0001

M. W. Berns, K. Konig, and B. J. Tromberg

Department of Physiology and Biophysics and Beckman Laser Institute and Medical Clinic, University of California, Irvine, Irvine, California 92717-0001

Received June 28, 1995

We report the observation of two-photon fluorescence excitation in a continuous-wave (cw) single-beam gradient force optical trap and demonstrate its use as an *in situ* probe to study the physiological state of an optically confined sample. In particular, a cw Nd:YAG (1064-nm) laser is used simultaneously to confine, and excite visible fluorescence from submicrometer regions of, cell specimens. Two-photon fluorescence emission spectra are presented for motile human sperm cells and immotile Chinese hamster ovary cells that have been labeled with nucleic acid (Propidium Iodide) and pH-sensitive (Snarf) fluorescent probes. The resulting spectra are correlated to light-induced changes in the physiological state experienced by the trapped cells. This spectral technique should prove extremely useful for monitoring cellular activity and the effects of confinement by optical tweezers. © 1995 Optical Society of America

Optical laser traps, or optical tweezers, are an effective tool for the confinement and micromanipulation of dielectric particles[1] and biological cells and organisms.[1,2] Focusing a continuous-wave (cw) laser beam to its diffraction limit with a high-numerical-aperture objective lens can produce a large gradient in the beam intensity profile, whereby gradient forces dominate over scattering forces and an optical laser trap is created. A sample becomes spatially confined in the vicinity of the beam focus where optical power densities can routinely exceed 1–100 MW/cm^2. To minimize photothermal and photobiological effects that might occur at such high power densities, the laser wavelength is often chosen to be at 1064 nm,[2] or at other near-infrared (700–900-nm) wavelengths, respectively. Still, the optical power densities in infrared optical tweezers are sufficiently large, as a result of diffraction-limited focusing, to induce localized sample heating,[3] and perhaps even two-photon absorption effects,[4] under cw conditions. Recently it was shown that two-photon fluorescence excitation is possible with a cw laser.[5] The latter result is especially significant, given that two-photon absorption and fluorescence excitation processes have thus far been possible only with pulsed lasers[6–8] and furthermore that they have been extensively used as physiological probes to monitor and assess cellular activity and viability.[7–9]

In this Letter we report the observation of two-photon-excited fluorescence from exogenous probes in cw infrared optical tweezers. At the wavelength of 1064 nm, a single focused Gaussian laser beam is used simultaneously to confine, and excite visible fluorescence from, a human sperm cell that has been tagged with Propidium Iodide, a fluorescent dye that functions as an assay (live/dead monitor) of cellular physiological state. The intensity at the dye peak emission wavelength of 620 nm exhibits a near-square-law dependence on incident trapping-beam photon laser power, a behavior consistent with a two-photon absorption process. In addition, for a cell held stationary in the optical tweezers for a period of several minutes at a constant trapping power, red fluorescence emission was observed to increase with time, indicating that the cell had gradually transitioned from a live to a dead state. These results suggest that, for samples suitably tagged with fluorescent probes and vital stains, optical tweezers can be used to generate their own *in situ* diagnostic optical probes of cellular viability or activity through two-photon processes. This capability should facilitate the monitoring of spectral changes related to the physiology of trapped specimens.

The experimental apparatus used in the present experiments is identical to that previously described.[3] A linearly polarized TEM$_{00}$ Gaussian beam, derived from a cw Nd:YAG laser at 1064 nm, is first passed through a long-pass filter ($\lambda > 800$ nm), deflected by a dichroic beam splitter, and then focused to its diffraction-limited spot size ($2\omega_0 \sim 0.8$ μm) onto a sample by an oil-immersion, 100×, 1.3-N.A. microscope objective (Zeiss Neofluar). The fluorescence, which is emitted by a small volume of the sample, is collected by the same objective lens, passed through a pinhole aperture, collimated with beam expansion optics, and directed onto a 300-groove/mm diffraction grating, which disperses the optical signal. This signal is then focused onto a computer-controlled CCD array. The optical system provides high-resolution spatial ($\sim$1-μm) and spectral (1-nm) measurements in an inverted confocal microscope geometry with good signal-to-noise ratios. The laser power incident upon the sample was obtained first by measurement of the power at the entrance aperture to the microscope objective and then by application of a transmission correction factor that was independently

0146-9592/95/212246-03$6.00/0

determined.[3] We prepared sperm cells, acquired from human donors, by diluting the seminal fluid in human tubal fluid (HTF-h), centrifuging the suspension, and then resuspending the resulting pellets in phosphate buffered saline. The dead-cell stain Propidium Iodide (PI; Molecular Probes, Inc.), in a 2.5-μM concentration, was then added to a 3-mL sample solution, and the entire cell suspension was loaded into a Rose chamber. During trapping studies, individual motile sperm cells were first grabbed and held stationary by the optical tweezers and then examined under different conditions of incident laser power and trapping time.

Figure 1 shows a typical absorption and emission spectrum for the PI dye bound to DNA. The absorption spectrum of a 2.5-μM dye solution was measured with a spectrophotometer (Beckman DU-7), and the emission spectrum was acquired with the experimental system described above from an optically trapped dead sperm cell at a laser power of 200 mW. The vital stain, which is DNA specific, is observed to have its maximum absorption at 536 nm, with an absorption band in the 450–600-nm visible wavelength range that is directly accessible by two-photon absorption. The corresponding emission spectrum, measured for the excitation (and trapping) wavelength of 1064 nm, exhibits a maximum at 620 nm and an emission bandwidth of ~100 nm. This spectrum is virtually identical to that obtained when the dye is excited with UV light or by a single-photon (532-nm) absorption process. As a dead-cell assay, PI fluorescence emission is observed only when the cell is dead or dying,[10] i.e., when the dye can permeate the cell membrane and bind to the cellular DNA. Hence, before cell death, PI red fluorescence cannot be measured as long as the dye remains in solution. This feature is especially advantageous for optical trapping studies because the trapping beam cannot excite two-photon fluorescence in the dye solution surrounding the trapped sample (background) but only from within the sample at the focal plane and only after cell death has been initiated. This has the effect of greatly enhancing the signal-to-noise ratio during fluorescence detection. As an alternative method, a live cell assay could also have been used to monitor cell health and viability during the trapping process.

To confirm further the existence of a two-photon excitation process within the cw optical tweezers, we measured the fluorescence intensity at the peak emission wavelength (620 nm) as a function of laser trapping power at the specimen. We acquired the two-photon signal by using an integration time of 5 s. The results (Fig. 2) indicate a near-square-law dependence of fluorescence intensity on laser power. A curve fit through the data yields an actual dependence of $I_{\mathrm{FLUOR}} \sim P^{1.9}$, with a regression coefficient of >0.998. The departure from a pure square-law behavior is attributed to photobleaching effects that could be observed at very high powers. The number of fluorescent photons generated by the two-photon absorption process were estimated from the expression $dp/dt = QN\delta F^2$, where dp/dt is the fluorescent photon rate, Q is the dye quantum yield, N is the number of dye molecules, δ is the two-photon cross section, and F is the photon flux density. Theoretically, for a laser trapping power of 100 mW at 1064 nm and a focused spot size of ~1 μm^2, corresponding to $F \sim 5.3 \times 10^{25}$ photons cm^{-2} s^{-1}, and assuming values of $\delta \sim 10^{-50}$ cm^4 s atom^{-1} photon^{-1}, $Q \sim 0.01$, and $N \sim 10^6$–10^8,[7] a fluorescent photon rate of $\sim 2.9 \times 10^5$–10^7 photons s^{-1} is predicted. Experimentally, an electronic counting rate of 10^2–10^3 counts s^{-1} pixel^{-1} is obtained (Fig. 2), corresponding to a photon-counting rate of ~10^6–~10^7 photons s^{-1} once the CCD efficiency (16 photons/count) and the pixel number (578 pixels) are accounted for. Error bars on the data account for laser intensity fluctuations ($\leq$10%) and detector noise. By further reducing detector noise (by cooling) and increasing the integration time, one could, in fact, acquire useful fluorescence signals over the range of trapping powers from ~20 to ~400 mW in the present system.

With the ability to excite visible-absorbing exogenous fluorophores with a single infrared trapping beam and achieve reasonable photon-counting rates with a simple photodetection system, optical tweezers can, therefore, be used to produce their own internal sensing mechanisms that function as *in situ* diagnostic probes of the physiological state of the optically confined

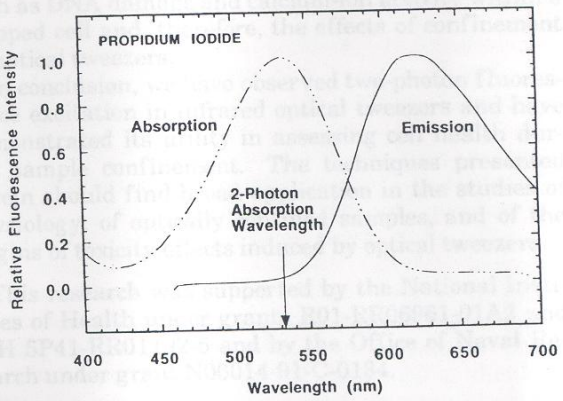

Fig. 1. Measured absorption and emission spectra for the fluorescent dye PI bound to DNA. Fluorescence emission is excited in a dye-tagged human sperm cell by a two-photon absorption process while the sample is held in 300-mW, 1064-nm cw optical tweezers.

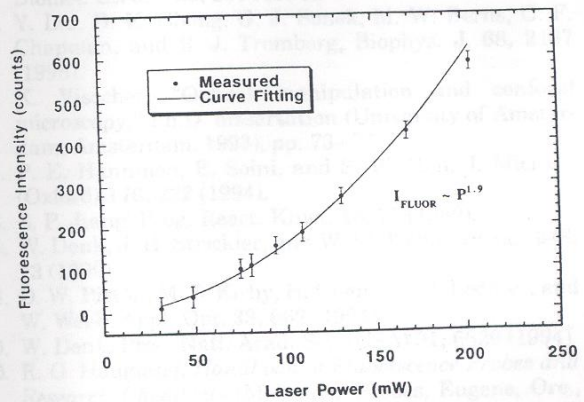

Fig. 2. Dependence of PI fluorescence intensity (I_{FLUOR}) on trapping (pump) laser power. The intensity varies with nearly a square-law dependence on incident laser power ($I_{\mathrm{FLUOR}} \sim P^{1.9}$). The CCD integration time was 5 s.

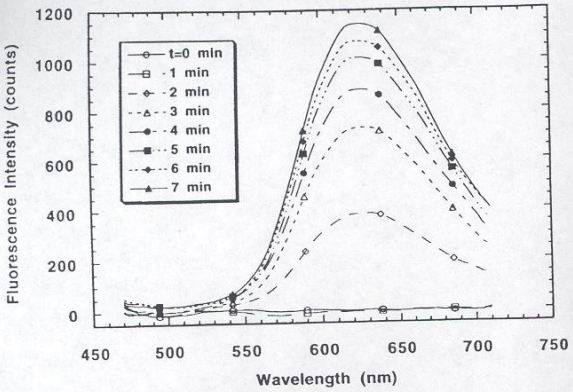

Fig. 3. Time dependence of fluorescence intensity, measured for an optically trapped human sperm cell that is held stationary for 7 min in a 300-mW optical trap.

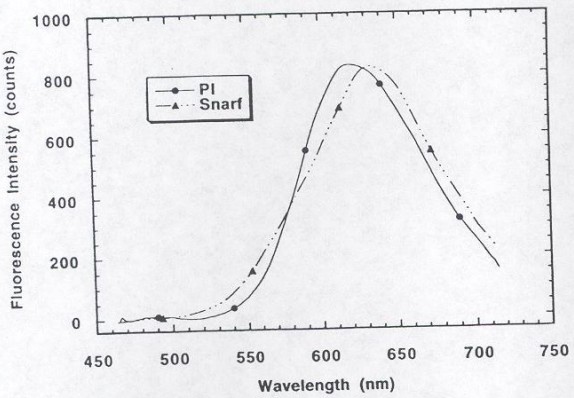

Fig. 4. Two-photon fluorescence emission spectra for two different CHO's that have been tagged with PI and Snarf.

specimen. To demonstrate this process, we trapped a sperm cell that was initially swimming within the sample chamber at an estimated velocity of ~30 μm/s (medium motility) with 300 mW of laser power and held it stationary with the optical tweezers. PI fluorescence emission was simultaneously recorded in 1-min intervals over a period of 7 min. The results (Fig. 3) show a gradual increase in the fluorescence intensity (I_{FLUOR}) with trap confinement time. Specifically, emission is observed only after a 2-min period has elapsed. Subsequent to this time, the emission continues to get stronger, until it reaches its maximum intensity at the end of the 7-min observation period. Here the evolution of the fluorescence spectrum with time has provided a means for spectral monitoring of the cell as it transitions between a live and dead state, with the increase in I_{FLUOR} indicative of the onset of cell death. Qualitatively, these results are found to correlate well with the visual observations that, during the same period of time, the beat frequency of the sperm cell flagellum gradually decreased and eventually ceased to exist at the end of the trapping period. They are also consistent with previous reports that, for exposure times longer than ~45 s, a gradual decrease in sperm velocity occurs.[11] We do not know whether the two-photon processes described herein result in any photodynamic effects.

Although the present experiments focused on the use of PI as the two-photon excitable fluorophore in the sperm cell system, the above results should be applicable to a much broader class of dye probes and cell specimen types. To illustrate this fact, and to highlight the capability of spectral monitoring, we show (Fig. 4) the two-photon-excited fluorescence spectra derived from the dyes PI and Snarf for optically trapped Chinese hamster ovary (CHO) cells. The PI spectrum in the CHO cell (Fig. 4), derived from a trap operating at 200 mW, is identical to that of the sperm cell (Fig. 1), indicating that the cell is already in the process of dying. Alternatively, Snarf is a pH-sensitive dye, having pH-dependent absorption and emission spectra. At a cellular pH of ~7.4, the absorption and emission spectra derived from the Snarf-stained CHO cell have maxima at 580 and 630 nm, respectively (Fig. 4). If, for example, the optical tweezers were to induce, in some manner, a cellular pH change, the Snarf emission spectrum would change with pH level and could thereby serve as a suitable probe of the interaction between the trapping beam and the cell itself. Other types of fluorescent probes, such as Acridine Orange and Indo-1, might also be useful in monitoring, through two-photon excitation, processes such as DNA damage and calcium-ion activity within a trapped cell and, therefore, the effects of confinement by optical tweezers.

In conclusion, we have observed two-photon fluorescence excitation in infrared optical tweezers and have demonstrated its utility in assessing cell health during sample confinement. The techniques presented herein should find broad application in the studies of physiology, of optically confined samples, and of the origins of toxicity effects induced by optical tweezers.

This research was supported by the National Institutes of Health under grants R01-RR06961-01A2 and NIH 5P41-RR01192-5 and by the Office of Naval Research under grant N00014-91-C-0134.

References

1. A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and S. Chu, Opt. Lett. **11,** 288 (1986).
2. K. Svoboda and S. M. Block, Annu. Rev. Biophys. Biomol. Struct. **23,** 247 (1994).
3. Y. Liu, D. K. Cheng, G. J. Sonek, M. W. Berns, C. F. Chapman, and B. J. Tromberg, Biophys. J. **68,** 2137 (1995).
4. K. Visscher, "Optical manipulation and confocal microscopy," Ph.D. dissertation (University of Amsterdam, Amsterdam, 1993), pp. 73–77.
5. P. E. Hanninen, E. Soini, and S. W. Hell, J. Microsc. (Oxford) **176,** 222 (1994).
6. S. P. Jiang, Prog. React. Kinet. **15,** 77 (1989).
7. W. Denk, J. H. Strickler, and W. W. Webb, Science **248,** 73 (1990).
8. D. W. Piston, M. S. Kirby, H. Cheng, W. J. Lederer, and W. Webb, Appl. Opt. **33,** 662 (1994).
9. W. Denk, Proc. Natl. Acad. Sci. (USA) **91,** 6629 (1994).
10. R. G. Haugland, *Handbook of Fluorescence Probes and Research Chemicals* (Molecular Probes, Eugene, Ore., 1995).
11. Y. Tadir, W. H. Wright, O. Vafa, T. Ord, R. H. Asch, and M. W. Berns, Fertil. Steril. **53,** 944 (1990).