

Physiological Monitoring of Optically Trapped Cells: Assessing the Effects of Confinement by 1064-nm Laser Tweezers Using Microfluorometry

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ABSTRACT We report the results of microfluorometric measurements of physiological changes in optically trapped immotile Chinese hamster ovary cells (CHOs) and motile human sperm cells under continuous-wave (CW) and pulsed-mode trapping conditions at 1064 nm. The fluorescence spectra derived from the exogenous fluorescent probes laurdan, acridine orange, propidium iodide, and Snarf are used to assess the effects of optical confinement with respect to temperature, DNA structure, cell viability, and intracellular pH, respectively. In the latter three cases, fluorescence is excited via a two-photon process, using a CW laser trap as the fluorescence excitation source. An average temperature increase of $<1.0 \pm 0.30^\circ\text{C}/100\text{ mW}$ is measured for cells when held stationary with CW optical tweezers at powers of up to 400 mW. The same trapping conditions do not appear to alter DNA structure or cellular pH. In contrast, a pulsed 1064-nm laser trap (100-ns pulses at $40\text{ }\mu\text{J}/\text{pulse}$ and average power of 40 mW) produced significant fluorescence spectral alterations in acridine orange, perhaps because of thermally induced DNA structural changes or laser-induced multiphoton processes. The techniques and results presented herein demonstrate the ability to perform in situ monitoring of cellular physiology during CW and pulsed laser trapping, and should prove useful in studying mechanisms by which optical tweezers and microbeams perturb metabolic function and cellular viability.

INTRODUCTION

Optical laser traps (optical tweezers) use gradient forces, derived from a spatial gradient in the light intensity, to produce stable, three-dimensional traps suitable for the confinement and micromanipulation of dielectric particles and biological specimens (Ashkin and Dziedzic, 1987; Ashkin et al., 1987). To date, laser traps, and the optical forces they produce, have found use in a variety of biological applications including, for example, cell and organelle manipulation (Ashkin and Dziedzic, 1989a,b; Ashkin et al., 1990; Berns et al., 1989; Liang et al., 1991, 1994); the measurement of forces associated with transport and adhesion (Block et al., 1990; Kuo and Sheetz, 1993; Svoboda et al., 1993; Finer et al., 1994); and the study of basic cellular and molecular structure and function (Seeger et al., 1991; Vorobjev et al., 1993). For many of these applications 1064 nm Nd³⁺:YAG lasers have been used, because these sources provide stable radiation in a spectral region that is relatively transparent to cells, including most biological chromophores and water (Ashkin and Dziedzic, 1989b; Block et al., 1990; Svoboda and Block, 1994). Although 1064 nm is generally assumed to be a safe, noninvasive wavelength that minimizes the extent of light-induced cell damage, the exact mechanisms of trap-induced biological effects are largely unknown. Similarly, the question of

whether 1064-nm optical tweezers can cause subtle physiological changes in cells remains unanswered. There are several methods for determining precisely how optical tweezers perturb living specimens under study. One approach is to evaluate cell physiology before and after laser trapping. For example, in the study of sperm cells (Tadir et al., 1989, 1990; Colon et al., 1992) and chromosomes (Vorobjev et al., 1993), specific trapping parameters (e.g., power, exposure time, wavelength) were found to result, respectively, in decreased sperm velocity, altered motility patterns, and the inhibition of mitosis subsequent to trapping. Another approach is to monitor specimen physiology during the trapping process using, for example, fluorescence spectroscopy (Kohen et al., 1987; Kohen and Hirschberg, 1989), in conjunction with endogenous or exogenous probes (Herman and LeMasters, 1993). Advantages of this method include the ability to study specific subcellular regions and physiological processes in real time. Examples of this approach include measurements of cellular autofluorescence (Konig et al., 1995b) and exogenous probe fluorescence (Liu et al., 1995a; Chapman et al., 1995; König et al., 1995a) to assay metabolic function, temperature, and viability in optically trapped Chinese hamster ovary (CHO) cells and human spermatozoa.

We previously provided evidence for localized cell heating (Liu et al., 1995a; Chapman et al., 1995) and for negligible variation in reduced coenzyme autofluorescence (Konig et al., 1995b), induced by infrared (1064 nm) optical tweezers. In this work, we report the results of new experiments that further assess the effects of confinement on immotile CHO and motile human sperm cells. The physiology of optically trapped cells is studied while the cells

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remain confined by the laser tweezers, using exogenous membrane and nucleic acid fluorescent probes, such as laurdan, propidium iodide, acridine orange, and Snarf, that assay cellular temperature, viability, DNA structure, and pH, respectively. Fluorescence spectra are acquired from cells under a variety of continuous-wave (CW) and pulsed-mode trapping conditions, in an attempt to identify critical threshold levels for the onset of physiological changes. Under CW trapping conditions, we observe an average temperature increase of $<1.0^{\circ}\text{C}$ per 100 mW of trapping power, no denaturation of cellular DNA, and no change in cellular pH. For extended exposure times (>2 min), however, some cells lose viability, indicating that mechanisms other than heating may be responsible for cell damage. In comparison, pulsed-mode (Q-switched) trapping at pulse energies of ≥ 40 $\mu\text{J}/\text{pulse}$ are found to produce significant DNA denaturation in sperm cells, a process that may be related to rapid, transient heating ($\Delta T \approx 100^{\circ}\text{C}$) and to multiphoton processes. For three of the exogenous probes used to monitor physiological states, visible fluorescence was excited using the CW infrared trapping beam as the sole fluorescence excitation source. This process can occur under CW conditions by virtue of two-photon absorption effects, derived from the extremely high-power densities that exist in the vicinity of the beam focus (Hanninen et al., 1994; Liu et al., 1995b). This result is important to the physiological study of optically trapped specimens, because infrared optical tweezers can be used not only to confine and manipulate samples, but also to simultaneously generate in situ fluorescence for diagnosis. Thus, with proper fluorophore selection, optical tweezers can be used to simultaneously micromanipulate and monitor vital processes in cells.

MATERIALS AND METHODS

Preparation of cell samples with fluorescent probes

CHO cells and human sperm cells were prepared for labeling with nucleic acid (acridine orange, propidium iodide), membrane (laurdan), and pH-sensitive (Snarf) probes. All fluorescent probes were acquired from Molecular Probes (Eugene, OR). CHO cells were maintained in culture using standard procedures (Freshney, 1987). In the case of CHO cells, the culture medium consisted of GIBCO's minimum essential medium, supplemented with 10% vol fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (all chemicals from Life Technologies, Grand Island, NY). Cells were first incubated at 37°C for 35 min and then treated with 0.25% trypsin to cause the cells to detach, followed by a fresh medium rinse to deactivate the enzyme. The medium with detached CHO cells was placed in a culture tube and centrifuged for 5 min at $1000 \times g$. The supernatant was removed and the cell pellets were then resuspended in phosphate-buffered saline (PBS) solution. Fresh human spermatozoa, acquired from healthy male donors, were placed in a sterile container and diluted in HEPES buffered human tubal fluid (HTF-h). The samples were centrifuged once at $200 \times g$ for 10 min. The resulting pellet was then resuspended in HTF-h and maintained at a constant temperature ($\sim 20^{\circ}\text{C}$) until the time of experimentation.

For monitoring cell temperature, 25-cm² tissue culture flasks containing CHO cells were incubated with the membrane probe laurdan. One hundred microliters of a 4×10^{-4} M solution of laurdan/ethanol was added to 3 ml

of PBS/CHO cell suspension medium to produce a 12 μM labeled suspension. The dye incubation was complete within ~ 30 min. For incorporation into sperm cells, the same molar concentration of dye, in an ethanol concentration of ~ 0.5 – 2.0% , was added directly to the HTF-h sperm cell suspension. After incubation for 2 h at room temperature, the suspension of living cells was diluted in PBS and then injected into a temperature-controlled microchamber. A period of 72 h was required to complete dye incubation, but few sperm cells survived the process. Hence, the majority of sperm cell temperature measurements were performed on dead samples.

Propidium iodide (PI) was used as a vital stain to monitor the live/dead state of CHO and sperm cells. PI is a nonfluorescent, membrane-impermeant probe that is incorporated into cells upon loss of viability. Once incorporated, PI binds to nucleic acids and exhibits characteristic red fluorescence (Haugland, 1994). Five microliters of 1 mg/ml aqueous PI (Molecular Probes; cat. no. P-3565) was added to a 1-ml PBS/cell suspension (PI ~ 5 $\mu\text{g}/\text{ml}$, 7.5 μM). Identical procedures were used to label CHO and sperm cells. If the cell was initially dead, it required <1 min for the dye to penetrate the membrane and bind to the nucleic acids. Measurements were made immediately after dye labeling.

Acridine orange (AO) was used to monitor DNA fluorescence. AO is an intercalating dye that differentially stains double-stranded versus single-stranded nucleic acids (Darzynkiewicz, 1990). When intercalated into double-stranded DNA, the dye exhibits green fluorescence when excited with blue light. Alternatively, when intercalated into single-stranded nucleic acids, the products derived from condensation and precipitation processes fluoresce red. The AO concentration was chosen to avoid the effects of incomplete RNA denaturation or complete DNA denaturation within the cell (Darzynkiewicz, 1990). For optical calibration purposes, the AO dye (Molecular Probes; cat. no. A-3568) was first used to label pure DNA obtained from herring sperm (Sigma, St. Louis, MO; cat. no. D-3159). Purified solid DNA was dissolved in deionized water. The condensed solution was then stirred with a glass needle for 1 h so as to break up the DNA fragments into smaller lengths (~ 500 base pairs). The solution was further diluted in water in the amount of 10 mg/ml of DNA to dye, sonicated until the solution became clear (~ 1 h), and stored at 4°C . For the living CHO and sperm cells, AO was first dissolved in distilled water at a concentration of 10 mg/ml. Fifty microliters of AO solution was mixed with 1 ml of buffer solution. Ten microliters of this solution was then mixed with 1 ml of PBS solution (AO ~ 5 $\mu\text{g}/\text{ml}$, 16.5 μM) containing the living sperm cells. Fluorescence from live cells was measured within 5–30 min of dye incubation.

Cellular pH was studied using a pH-sensitive membrane probe, Snarf (Molecular Probes; cat. no. C-1271). Eighteen microliters of 1 mg/ml Snarf-1/dimethyl sulfoxide (pH of 7.4) was added to 3 ml of CHO suspension medium to form a Snarf concentration of 6 $\mu\text{g}/\text{ml}$ (10 μM). The intracellular pH was adjusted by adding 20 μM potassium ionophore nigericin (Molecular Probes; cat. no. N-1495) (Haugland, 1994) to the suspension buffer and altering the extracellular pH to desired levels. Incubation was performed at 37°C for 20 min so that the pH could equilibrate inside and outside the cell.

Before cell staining, absorption spectra were acquired for all fluorescent probes using a UV-VIS spectrophotometer (Beckman Instruments, Fullerton, CA; model DU-7). For laurdan and Snarf, ~ 10 ml of each dye, in either an ethanol or a buffer solution, was placed in a quartz cuvette and subsequently analyzed. In the case of AO and PI, pure DNA was stained, added to a buffer solution, and then loaded into cuvettes for analysis. After absorption spectra were measured, cells were tagged, and then the cell suspensions were loaded into a customized sample chamber containing temperature monitoring and control functions (Liu et al., 1995a). The sample temperature could be varied from 20°C to 60°C , with an accuracy of $\pm 0.03^{\circ}\text{C}$.

Infrared laser tweezers and microfluorometry system

An experimental system (Fig. 1) for the simultaneous implementation of infrared optical trapping and fluorescence spectroscopy has previously

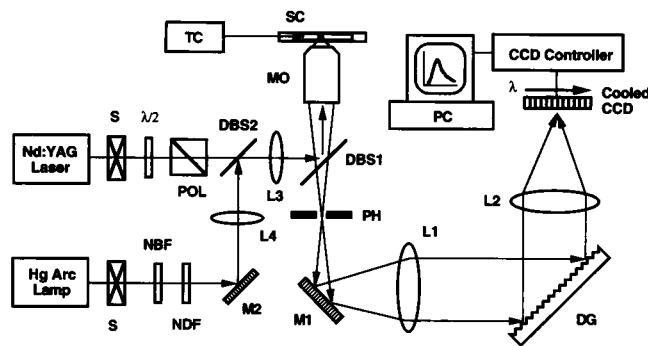


FIGURE 1 Schematic of the experimental system used for optical laser trapping and physiological monitoring of biological specimens. A 1064 nm Nd:YAG laser is used to create an optical trap and excite sample fluorescence via two-photon absorption processes. A mercury arc lamp is used as an alternative UV fluorescence excitation source. Fluorescence is collected by a microscope objective (MO), passed through a pinhole (PH), and then spectrally analyzed using a diffraction grating-CCD detector array combination. Other components include a shutter (S), half-wave-plate ($\lambda/2$), polarizer (POL), dichroic beam splitter (DBS), and optical filter set (NBF, NDF).

been reported (Liu et al., 1994, 1995a; Chapman et al., 1995). Briefly, the output from a 1064-nm Nd:YAG laser (Quantronix, Smithtown, NY; model 114) is first conditioned (polarized, spatially filtered, and collimated) and then expanded so as to fill the back aperture of a high numerical aperture, oil immersion microscope objective (MO) (Zeiss Neofluar 100 \times , 1.3 NA). This produces a diffraction-limited beam ($2w_0 \approx 0.8 \mu\text{m}$ in water) in the focal plane with the requisite gradient forces for optical trapping. When operated in CW mode, up to 500 mW of laser power could be transmitted to a trapped sample. The laser trapping power that reaches a specimen in the focal plane is first calibrated for the specific high-NA objective (Svoboda and Block, 1994; Liu et al., 1995a) by measuring the amount of power incident on the MO back aperture, and the transmittance of the MO, under the coupling conditions encountered during trapping experiments. For the Zeiss Neofluar 100 \times , a transmittance of ~ 0.60 was derived at 1064 nm. In Q-switched (pulse) mode, the laser could produce pulses having widths of ~ 100 ns, repetition rates that could be varied from single shot to 2 kHz, and pulse energies as high as 500 $\mu\text{J}/\text{pulse}$. To confine motile cells, the Q-switched laser trap was operated with pulse energies up to 100 $\mu\text{J}/\text{pulse}$ (without sample ablation) and repetition rates of ≥ 10 Hz. A $\lambda/2$ waveplate (Newport, Irvine, CA; model 05RP12) and an analyzer (Newport; model 05BC16-PC.9), in conjunction with a photodetector (Coherent, Palo Alto, CA; model 212) was used to precisely control the laser output power or energy.

Using the minimum amount of laser power required to confine an immotile CHO or a motile sperm cell, an individual cell was grabbed with optical tweezers and held stationary at a height of $\sim 5 \mu\text{m}$ above the bottom chamber coverslip. To excite fluorescence from optically trapped samples that were labeled with exogenous fluorescent probes, two different optical sources were used. For samples tagged with the laurdan dye, ultraviolet light (365 nm) from a 200-W mercury arc lamp (Oriental, Stamford, CT; model 66006) was introduced into the optical system (Fig. 1), made coaxial with the primary trapping beam, and brought to focus on a specimen by the Zeiss MO. A sample was exposed to ultraviolet light at a power density of $\sim 10 \mu\text{W}/\text{cm}^2$ for 0.1 s, and exposure parameters were chosen to prevent photobleaching of the fluorescence signal. For cells labeled with the PI, AO, or Snarf dyes, which have absorption bands in the 400–600 nm wavelength range, the CW infrared trapping beam was itself used to excite fluorescence, via a two-photon absorption process (Liu et al., 1995b). This phenomenon is sensitive to trapping powers in the range of ~ 20 –400 mW and can yield fluorescent photon rates of $\sim 10^4$ – 10^8 photon s^{-1} , signal levels that can easily be detected in the present system. After excitation, the fluorescence emission from the sample is collected by the same MO,

passed through a dichroic beam splitter, transmitted through a pinhole aperture located in the confocal plane, dispersed by a 300 g/mm diffraction grating (Instruments SA, Edison, NJ; model 510-18-050), and then focused onto a thermoelectrically cooled and computer-controlled CCD array (Princeton Instruments, Trenton, NJ; model TE/CCD-576E&EM and ST-130 camera controller). In this configuration, the optical system could acquire micron-resolved fluorescence with a CCD array with 578 pixels and 16-bit sensitivity. The spectral response of the system was measured using a calibrated radiance source (Optronics Labs, Orlando, FL; model 550GC) and used to correct the acquired spectra. Fluorescence emission spectra were acquired using integration times that varied from milliseconds to seconds, depending on the incident power level.

Monitoring cell temperature with the membrane probe laurdan

The membrane-specific probe laurdan was used to monitor changes in cell temperature incurred during optical trapping. We previously reported a technique for assessing the extent of localized heating induced by infrared optical tweezers in liposomes and CHO cells (Liu et al., 1994, 1995a; Chapman et al., 1995). In the present study, we applied the same optical measurement technique to the study of human sperm cells. Laurdan, an environmentally and temperature-sensitive dye probe with an absorption maximum at 365 nm, incubates into the cell membrane and readily responds to changes in membrane phase and fluidity. With a change in temperature and a corresponding change in membrane fluidity and dipole relaxation, the fluorescence emission of laurdan undergoes a Stokes shift of nearly ~ 40 –50 nm in the 440–490-nm wavelength range. By calibrating this fluorescence shift with changes in cell temperature, the extent of cell heating induced by the absorption of an infrared trapping beam can be quantified. Fluorescence emission spectra from a single, untrapped cell were first acquired at different temperatures, using a temperature-controlled microchamber. A calibration curve, derived from the generalized polarization (GP) ratio (Parasassi et al., 1990, 1991; Liu et al., 1995a), is then generated to relate shifts in fluorescence emission maxima (or change in GP) with temperature. GP is defined as $(I_{\lambda_1} - I_{\lambda_2}) / (I_{\lambda_1} + I_{\lambda_2})$, where I_{λ_1} and I_{λ_2} are the wavelengths of maximum laurdan fluorescence in polar ($\lambda = 440$ nm) and nonpolar ($\lambda = 490$ nm) media. After calibration measurements have been made, the specimen temperature is initially preset using the microchamber controls, and the optical trapping beam is applied. The observed fluorescence spectral shift is converted to specific values of ΔGP and temperature change (ΔT) using the calibration curve. The change in temperature is then quantified as a function of laser trapping power and trapping time. The fluorescence emission is most sensitive to temperature when laurdan has been completely incubated into the cell membrane.

Monitoring intracellular pH with Snarf

To monitor the intracellular pH level during trapping, the fluorescent pH indicator carboxy Snarf was used. Snarf, which has an absorption band in the green-yellow portion of the visible spectrum, fluoresces yellow-orange (~ 580 nm) under acidic conditions and deep red (~ 630 nm) when the pH is basic (Bassnett et al., 1990; Whitaker et al., 1991). In the case of CHO cells, the intracellular and extracellular pH is balanced with the addition of the potassium ionophore nigericin. Snarf was first incorporated into a CHO cell, and the cellular pH level was set at one of several possible values. A fluorescence emission spectrum, excited by either a UV or two-photon infrared trapping beam excitation source, was then acquired. Because the emission spectrum is pH-sensitive, it could be used to monitor the cellular pH level during the trapping process. As long as the intracellular and extracellular pH levels are precisely balanced, no change in fluorescence emission is observed, regardless of the laser trapping powers and exposure times.

Monitoring cell viability and cellular DNA with nucleic acid stains

The nucleic acid stains propidium iodide (PI) and acridine orange (AO) were used to assess cell viability and to monitor changes to the cellular DNA during CW and pulsed laser trapping. PI, a dead cell probe with an absorption maximum at 536 nm, penetrates the cell membrane and binds to the cellular DNA only when the cell is dead or dying (Haugland, 1994). Red fluorescence is then observed in the 550–700-nm range, with a maximum fluorescence intensity that occurs at ~ 620 nm. No fluorescence is observed as long as the PI dye remains in solution or the cell is alive. Red fluorescence from PI, excited by two-photon absorption of the 1064-nm trapping beam, is an indicator of decreased cell viability and the onset of cell death. We previously employed two-photon excited PI fluorescence to study the effects of 760-nm and 800-nm laser tweezers on human spermatozoa (Konig et al., 1995a). In this work, PI fluorescence spectra were acquired, using 1064-nm excitation, under conditions of increasing laser power (0–200 mW), and fixed laser power but increasing trap exposure time (0–7 min). Different laser powers were used to evaluate the relationship between fluorescence intensity and pump power, to confirm the occurrence of two-photon absorption processes.

Acridine orange (AO), which has been extensively used to study the molecular structure of nucleic acids (Darzynkiwicz et al., 1975; Darzynkiwicz, 1990; Darzynkiwicz and Kapuscinski, 1990), was used to monitor the DNA denaturation process in living sperm cells. After permeating the cell membrane and binding to DNA, AO possesses absorption and emission maxima at 502 nm and 526 nm, respectively. These maxima shift to 460 nm and 650 nm, respectively, when double-stranded DNA is denatured, either by thermal or chemical means (Darzynkiwicz, 1990; Thomas, 1993). While the bulk of chromatin DNA is packed in a way that does not allow for AO intercalation in most living cells (Delic et al., 1991), the DNA inside sperm cells are unpacked and can therefore be effectively stained with the AO dye. After AO labeling of CHO and sperm cells, trapping experiments and fluorescence spectral measurements were performed under both CW and pulsed-mode conditions. For CW trapping, conditions were kept identical to those used for cells tagged with the laurdan membrane probe. Here, laser powers ranged from 0 to 400 mW, and trapping times were kept below 10 min per sample. In pulsed (Q-switched) mode, 100-ns pulses with energies of up to 110 μ J/pulse were applied to cells, at repetition rates varying from 100 Hz to 1 kHz. Here, confinement times were < 5 min.

RESULTS

After dye incubation, labeled cells in suspension were loaded into a Rose microchamber and placed in the optical tweezers microscope. Immotile CHO and sperm cells were brought into the field of view by translating the microscope stage and then trapped using a laser power of ~ 10 mW. Motile sperm cells were trapped in the same manner, but required laser powers that varied from ~ 20 mW to 200 mW, depending on cell motility. In all cases, the lowest power level required for trapping was initially used, so as not to effect physiological changes before spectral measurements. During trapping, sample fluorescence was excited using either conventional UV (365 nm) or near-infrared two-photon ($1064 \text{ nm}/2 = 532 \text{ nm}$) sources.

Cell temperature measurements

Fluorescence emission spectra for an untrapped and immotile sperm cell tagged with laurdan and maintained at 25°C and 40°C, exhibit a temperature dependence and a charac-

teristic emission peak at the wavelength of 440 nm (Fig. 2). When the temperature of the cell is increased, the fluorescence intensity decreases at 440 nm and increases at 490 nm, a behavior consistent with the change in cell membrane fluidity at elevated temperatures. However, unlike the spectra from liposomes, sperm cell spectra do not exhibit a dramatic fluorescence red shift with temperature, a behavior normally attributed to an abrupt transition in membrane phase state, as sensed by the laurdan probe. Nonetheless, the sperm spectra can be used to generate a GP calibration curve (Parasassi et al., 1990, 1991). A GP curve for sperm cells with heterogeneous membranes tends to be less sensitive to small temperature variations but displays a greater linear dynamic range that facilitates cell monitoring over a broader range of temperatures. The results of measurements made on at least 330 cells (five cells per temperature setting) (Fig. 3) reveal that the variation in GP with temperature is nearly linear (regression coefficient > 0.98) over the range from 25°C to 40°C, with a rate of change $\Delta \text{GP}/\Delta T \sim 1.10 \pm 0.20 \times 10^{-2}/^\circ\text{C}$. Nearly identical results are obtained for the cells when they are cooled down from a higher temperature, indicating that the change in membrane permeability is reversible, and that the initial sample temperature can be recovered. After performing the above calibration procedure, the microchamber temperature was preset to 26.5°C, the optical tweezers were turned on, and an immotile cell was confined. The laser trapping power was then varied from 50 mW to 400 mW, in 50-mW intervals. Fluorescence spectra were acquired after each sample had been trapped for at least 10 s, so that a steady-state equilibrium condition for cell heating could be established. After each spectrum is converted to an effective temperature, the relationship between induced ΔT and applied trapping beam laser power, P , can be established (Fig. 4). It is found that sperm cell temperatures increase on the average by $\sim 0.75 \pm 0.30^\circ\text{C}/100 \text{ mW}$ at 1064 nm. In similar experiments, live, motile sperm cells (~ 100 cells) incubated with

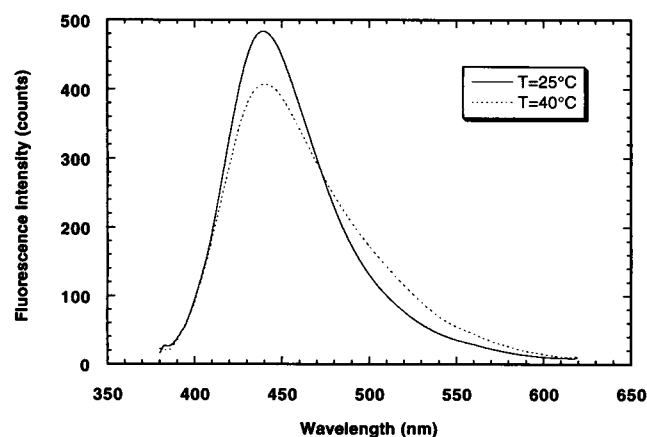


FIGURE 2 Fluorescence emission spectra from an immotile human sperm cell tagged with the laurdan fluorescent membrane probe. The change in fluorescence intensity with temperature is used to quantify the heating effects induced by optical trapping at the wavelength of 1064 nm.

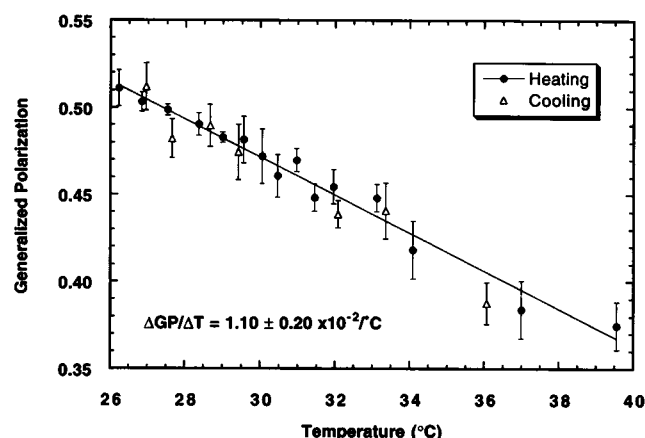


FIGURE 3 A calibration curve of generalized polarization (GP) versus temperature. GP is defined as the ratio $(I_{41} - I_{42})/(I_{41} + I_{42})$, where I_{41} and I_{42} are the fluorescence intensities at 440 and 490 nm, respectively. Experimental data are presented for cells that are heated from 26°C to 40°C (●) and cooled back down (Δ). The process, in terms of GP, is reversible.

laundan were initially trapped with 100 mW of laser power, and fluorescence spectra were measured at different cell temperatures. These measurements produced a calibration factor of $\Delta GP/\Delta T \approx 3.4 \pm 2.0 \times 10^{-3}/^{\circ}\text{C}$ for the live cells. An increase in laser trapping power from 100 mW to 500 mW was found to produce a $\Delta T = 3.7^{\circ}\text{C}$, corresponding to a heating rate of $\sim 0.92 \pm 0.70^{\circ}\text{C}/100 \text{ mW}$.

Cell viability studies

Two-photon absorption, provided by the CW near-infrared laser trap (Visscher, 1993; König et al., 1995b; Liu et al., 1995b), was used to excite fluorescence from CHO and sperm cells labeled with the vital stain propidium iodide (PI), the nucleic acid stain acridine orange (AO), and the pH indicator Snarf. Here, the specific dye probes should have a

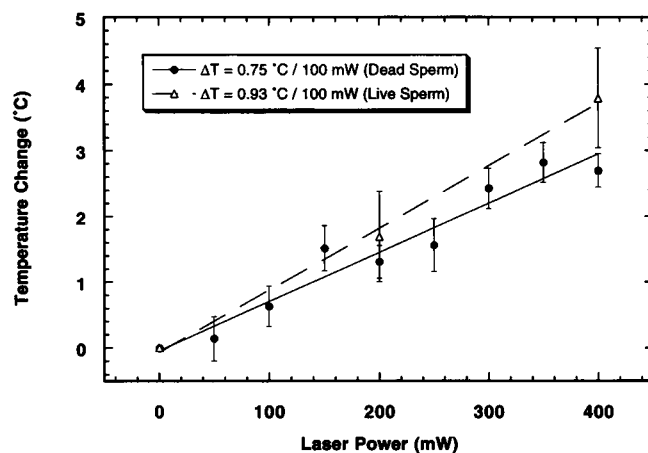


FIGURE 4 Relationship between induced temperature change and applied laser power for sperm cells that are dead (●) and alive (Δ). A regression analysis yields an average temperature change of 0.73°C and 0.93°C per 100 mW for dead and living cells, respectively.

significant two-photon absorption cross section (Bighouse, 1991; Xu et al., 1995) at the two-photon fluorescence excitation wavelength ($1064 \text{ nm}/2 = 532 \text{ nm}$), and a sufficient number of fluorescent photons should be produced for photodetection. Although a two-photon absorption spectrum cannot be derived from a one-photon absorption spectrum based on parity conditions and transition selection rules, it is nonetheless useful to examine the single-photon absorption spectrum in light of the fluorescence emission spectrum derived from two-photon processes. As an example, the single-photon absorption spectra for the PI dye (Fig. 5) reveal that the wavelength of maximum absorption shifts from 490 nm, when PI exists in pure water, to 536 nm, when the dye becomes bound to pure DNA. The corresponding two-photon fluorescence spectrum (Fig. 5), derived from a dead sperm cell incubated with PI and excited by a 200-mW laser trapping beam, exhibits strong emission with a maximum at 620 nm (red fluorescence) and a bandwidth exceeding 150 nm. For laser powers in the 30 to 200 mW range, a detector integration time of 5 s was used to acquire the fluorescent signals. Experimentally, an IR laser power of 100 mW focused to a spot size of $\sim 1 \mu\text{m}^2$ was found to produce fluorescence signals of $\sim 10^6$ – 10^7 photons s^{-1} , as measured by the CCD array. For the PI and Snarf exogenous probes used herein, the peak fluorescence intensity, excited via the two-photon process, was found to vary in nearly a quadratic ($I_{\text{Fluor}} \approx P^2$) fashion with applied laser power (Fig. 6). The departure from a pure square-law relationship is attributed to fluctuations in laser power, to detector noise, and to photobleaching effects that were observed to occur at higher laser powers. For Snarf, an observed power-law exponent slightly larger than 2 may also indicate that there are contributions to the fluorescence from multiphoton (e.g., three-photon) processes. A measurement of the polarization anisotropy in the fluorescence emission spectra should be able to confirm whether this deviation is, in fact, derived from three-photon effects.

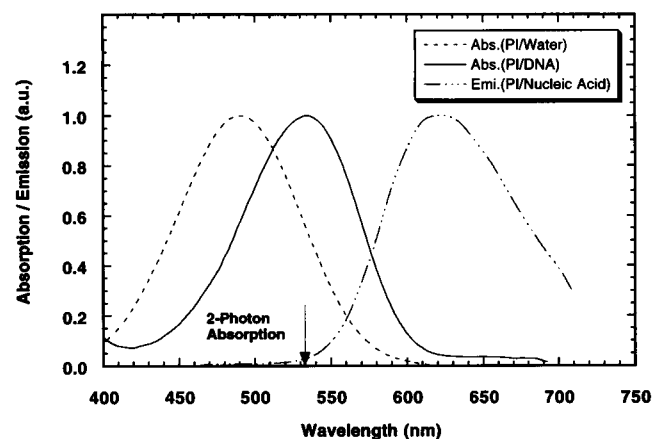


FIGURE 5 Absorption and emission spectra for the vital stain propidium iodide. The absorption spectrum shifts by $\sim 50 \text{ nm}$ once the dye becomes bound to DNA. The peak in fluorescence emission occurs at 620 nm, when excited at the two-photon excitation wavelength of 532 nm.

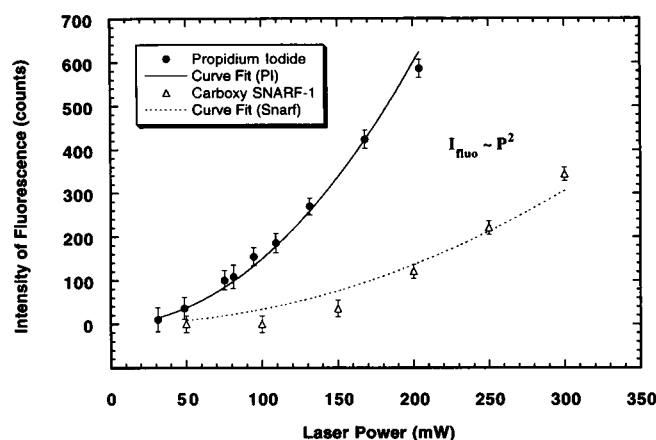


FIGURE 6 Dependence of fluorescence intensity on pump (trapping) laser power for the fluorophores propidium iodide (PI) and Snarf. The intensities vary with nearly a square-law dependence on laser power for both dyes, a behavior consistent with a two-photon absorption process. The measurements were made using a 5-s signal integration time.

To illustrate the process of physiological monitoring using the excitation of a vital stain by the optical trap itself, a sperm cell that was initially swimming within the sample chamber at an estimated velocity of $<10 \mu\text{m/s}$ (low motility) was trapped with 300 mW of laser power and held stationary by the optical tweezers. PI fluorescence emission was simultaneously recorded in 1-min intervals over a period of 7 min. The spectra (Fig. 7) show a gradual increase in the fluorescence intensity with trap confinement time. Specifically, emission is observed only after a 2-min period has elapsed. Subsequent to this time, the emission spectrum continues to get stronger, until it reaches its maximum intensity at the end of the 7-min observation period. These results indicate that it is possible to spectrally monitor changes in the physiological state of the optically trapped sperm cell, as it transitions between a live and dead state.

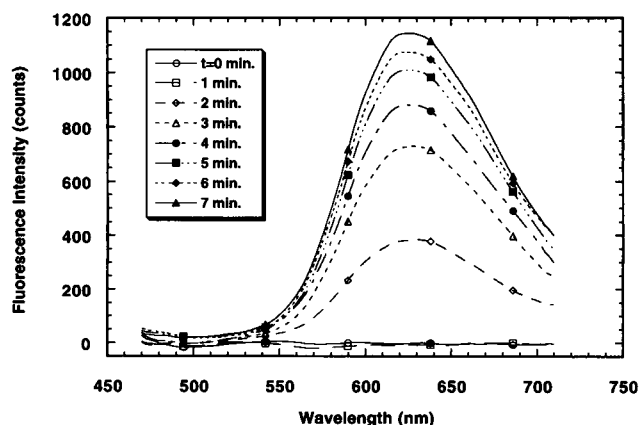


FIGURE 7 Time evolution of the fluorescence spectrum from a PI-labeled sperm cell. The increase in fluorescence intensity with increasing time provides evidence for the real-time monitoring of the physiological state of an optically trapped cell, in this case indicating the onset of cell death.

Similar results were obtained for PI-labeled immotile CHO cells.

The fluorescence emission from acridine orange provides a means to assay, at the molecular level, potential changes in the genetic material of a cell. Individual AO-stained living sperm cells were initially confined by optical tweezers operating at 100 mW, while an AO fluorescence spectrum was acquired. The laser power was then increased to 400 mW, and another spectrum was measured. Under continuous wave conditions, the fluorescence spectra (Fig. 8) show features characteristic of double-stranded DNA, with a wavelength maximum at 525 nm (green fluorescence), and an emission bandwidth that ranges from ~ 480 nm to 700 nm. In this case, no spectral shift was observed, indicating that no obvious DNA denaturation has occurred. However, more dramatic effects were observed when the trapping laser was operated in pulsed (Q-switched) mode. In this case, a single sperm cell could be effectively confined with a train of 100-ns pulses at repetition rates between 100 Hz and 1 kHz. Pulse energies could be varied from $10 \mu\text{J}$ to $\sim 110 \mu\text{J/pulse}$. After ~ 10 s of confinement at a pulse energy of $40 \mu\text{J/pulse}$ and an average power of 40 mW, red fluorescence emission was observed at ~ 640 -650 nm. With increased confinement time, the fluorescence spectra showed that a decrease in green fluorescence (525 nm) was accompanied by an increase in red fluorescence (650 nm) (Fig. 8). The threshold for this effect was found to occur at a pulse energy of $\sim 30 \mu\text{J/pulse}$.

To further highlight the process of cellular spectral monitoring during laser trapping, the fluorescence from CHO cells, incubated with the pH-sensitive indicator Snarf, was measured. The cells were suspended in different pH buffers, with the final pH level adjusted via the addition of $20 \mu\text{M}$

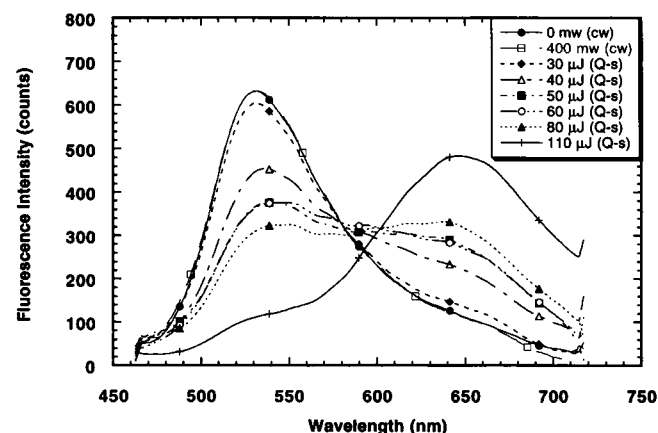


FIGURE 8 Fluorescence emission spectra from the DNA of living human sperm cells stained with acridine orange. Cells trapped with a CW laser exhibit green fluorescence (525 nm) and show no evidence of structural changes or photodamage. When trapped with pulses having an energy of $40 \mu\text{J/pulse}$ at a repetition rate of 100 Hz, the emission exhibits a strong red fluorescence (~ 645 nm), indicating that partial denaturation of the DNA has taken place. The threshold for DNA damage was found to be $\sim 40 \mu\text{J/pulse}$ at 1 kHz.

nigericin. This produced intracellular pH levels of 5.8, 6.3, 7.4, and 7.7, respectively. These values were confirmed by independent microelectrode-based pH measurements. A given cell sample was then trapped by a 200-mW trapping beam, and two-photon fluorescence was simultaneously induced. The resulting spectra are distinct for each of the four different pH values (Fig. 9) and correlate well with the data available from the manufacturer for a given pH value. Otherwise, the spectra show no change in cellular pH level that might result, for example, from the stress of the optical confinement process.

DISCUSSION

Under normal CW trapping conditions, the results derived from membrane and nucleic acid probe fluorescence spectra are well correlated and generally support the conclusion that infrared laser tweezers do not induce significant physiological changes in the trapped specimen. Over the range of trapping powers from ~ 100 mW to 400 mW, average temperature increases of 0.75°C and $0.93^\circ\text{C}/100$ mW were measured in dead and live sperm cells, respectively. Under the same conditions, no denaturation of the cellular DNA and no change in cellular pH level were observed. However, for extended exposure times ($t > 2$ min) at 300 mW, it was possible to observe a loss of viability for low-motility sperm cells, the origin of which is still uncertain. In comparison, Q-switched laser trapping at energies above ~ 40 $\mu\text{J}/\text{pulse}$ (for pulse widths of 100 ns), corresponding to peak powers of 400 mW and producing a transient temperature increase of $\sim 100^\circ\text{C}$, was found to facilitate the conversion of double-stranded DNA to single-stranded DNA, a denaturation process that may either be thermally initiated or derived from the effects of multiphoton processes on the proteins (chromatin), DNA, and the fluorescent dye probes.

The temperature changes measured in both live and dead sperm cells are consistent with those values previously

reported for trapped liposomes ($1.45^\circ\text{C}/100$ mW) and CHO cells ($1.1^\circ\text{C}/100$ mW) (Liu et al., 1995a). For sperm cells, a lower average induced ΔT and a larger standard deviation in ΔT (versus CHO cells) can be attributed to our observations that sperm cells require longer dye incubation times, have greater intrasample variability, and undergo less dramatic membrane phase changes. Thus, measurements of sperm temperature with laurdan are intrinsically less sensitive than laurdan-based CHO temperature measurements. Nevertheless, our results (Fig. 4) strongly suggest that temperature increases of ~ 0.75 – 3.0°C can be expected under normal motile sperm cell trapping and micromanipulation conditions (< 300 mW).

The observation that denaturation of AO-stained sperm cell DNA does not occur under CW trapping conditions (Fig. 8) is consistent with the fact that the laurdan measurements did not show significant steady-state heating effects. With CW laser trapping, the temperature of a trapped cell was found to change by only a few $^\circ\text{C}$. Thermal denaturation of DNA is known to occur at $\sim 70^\circ\text{C}$ (Darzynkiewicz et al., 1975) and would therefore require a trap-induced ΔT of nearly ~ 45 – 50°C (above room temperature) to be observed. Such a temperature change can, however, be realized by rapidly heating the cell under transient, Q-switched trapping conditions. For pulsed-laser trapping, the heating process can be understood in terms of the laser pulse duration (t_p), the pulse repeat frequency, and the sample thermal relaxation time. The temperature rise ΔT , due to the dissipation of heat from a light pulse absorbed by a sample, is given by the expression $\Delta T = Q/\rho m$, where Q is the absorbed energy, ρ is specific heat, and m is the mass of volume being heated (Carslaw and Jaeger, 1959). The time-dependent temperature, after absorption of a pulse by a thin slab, can be approximated by $T = T_0 \exp(-t/\tau)$, where τ is a thermal decay time. The thermal relaxation time, defined as the average time required to achieve a maximum temperature (T_0) at the surface of the absorbing object, is given by the expression $t_r \approx d^2/4\kappa$, where d is the cell diameter and κ is the thermal conductivity of water (10^{-7} m^2/s). Heat is primarily confined to the sample when $t_p \ll t_r$, i.e., transient heating occurs when $1/f \gg t_r$. For a 10- μm -diameter cell, the thermal relaxation time is ~ 250 μs . Assuming a sample absorption coefficient of ~ 0.1 cm^{-1} , a pulse duration (t_p) of 100 ns, and a pulse energy of 40 $\mu\text{J}/\text{pulse}$, a ΔT of $\sim 100^\circ\text{C}$ is predicted. Because $t_p \ll t_r$, only transient heating of the sample occurs. The pulsed trapping conditions employed during the measurement of AO fluorescence (Fig. 8) might, therefore, be sufficient to induce thermal denaturation. We note, however, that processes other than photothermal effects can also result in DNA denaturation, including the use of improper acridine orange dye concentrations (Darzynkiewicz, 1990), exposure to UV radiation (Thomas, 1993), photochemical effects derived from the absorption of visible and near-infrared radiation by cell chromophores (Block et al., 1990), and multiphoton processes (Calmettes and Berns, 1983). For example, photochemical damage induced by two-photon

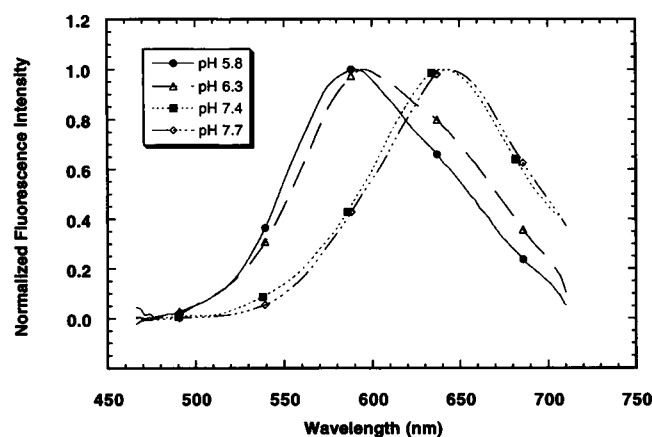


FIGURE 9 Two-photon excited fluorescence spectra for CHO cells labeled with the pH indicator Snarf. For cells with pH levels set initially at 5.8, 6.3, 7.4, and 7.7, no change in pH was observed after CW laser trapping at 200 mW.

absorption processes with 760-nm optical tweezers has recently been reported (Konig et al., 1995a). Given the high peak powers achieved in the present pulsed-mode trapping experiments, it is likely that two- and three-photon effects are also substantial contributors to the denaturation process. The deviation from the square-power law in the case of Snarf (Fig. 6) may provide one indication of this. Clearly, further studies are needed to determine exactly which mechanisms are involved in DNA damage during pulsed-laser trapping.

The fluorescence spectra derived from PI-stained dead sperm cells provide additional information about the status of optically confined cells. A small portion (<10%) of the optically confined sperm cells that were initially alive did, in fact, become immobile and exhibit red fluorescence. The PI fluorescence intensity results infer the onset of cell death with increasing CW trap exposure time at a power of 300 mW (Fig. 7). These results agree, at least qualitatively, with visual observations that, during this 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 a gradual decrease in sperm velocity occurs at high laser powers and exposure times that exceed ~45 s (Tadir et al., 1989, 1990). However, the results are contrary to those of the laurdan and AO spectral measurements that show that minimal photothermal and genetic denaturation effects are induced under the same trapping conditions. For spermatozoa, there exists a distribution of cells having different motilities and metabolic activity levels. We selected low-motility cells for study. Perhaps these "low-motility" cells represent a subgroup of the entire population that is particularly susceptible to photodamage. It is likely that other yet unknown photochemical or photobiological processes are responsible for the observed degradation in cell viability. In this case, other cell viability assays, capable of monitoring various metabolic processes (e.g., enzymatic activity, mitochondrial action, ATP production), are needed to determine the origin of this physiological change.

For three of the exogenous fluorophores used in the present study, the CW infrared optical trap was the sole source of visible fluorescence excitation. This is made possible by a two-photon absorption process (Jiang, 1989; Denk et al., 1990; Visscher and Brakenhoff, 1991; Ridsdale and Webb, 1993; Piston et al., 1994; Berland et al., 1995) that occurs in the trapping zone, where the laser beam is focused to its near-diffraction-limited spot size, the intensity gradient is largest, and infrared optical power densities can exceed hundreds of MW/cm². Although two-photon absorption cross sections are typically very small for most dye molecules (~10⁻⁵⁰ cm⁴ s photon⁻¹) (Hermann and Ducing, 1972; Lin et al., 1984; Jiang, 1989), a sufficient number of fluorescent photons can be produced to facilitate spectral measurements with a simple photodetection system. Experimentally, we find that a 100-mW, 1064-nm laser beam can produce ~10⁶-10⁷ fluorescent photons per second, corresponding to an electronic counting rate of ~10¹-10³ counts

s⁻¹, as measured by our CCD detector array (Fig. 6-8). Although two-photon cross-sectional data have been reported for some dyes (Bighouse, 1991; Xu et al., 1995), the two-photon action cross sections for many other vital stains (Piston et al., 1994), including the fluorophores used in this study, are currently unknown.

Through the above process, optical tweezers effectively become an *in situ* diagnostic probe of the physiological state of the optically confined specimen, having several important advantages over conventional fluorescence microscopy techniques. These include 1) the elimination of additional UV or visible excitation sources that must be introduced into the optical system; 2) the excitation and collection of fluorescence from a small localized region of the trapped sample; 3) the corresponding reduction in background signals derived from out-of-focus fluorescence and photobleaching; 4) the significant reduction in spectral overlap between the wavelengths of the source and signal wavelengths; and 5) the reduction in undesirable photodamage that can occur throughout the cell when using conventional radiation sources. The technique is limited, however, by relatively low signal levels and the possibility that the two-photon process itself can lead to deleterious photochemical and photobiological processes (e.g., thermal/oxidative stress, photodynamic damage) in both dye-labeled and unlabeled cells. In addition, two-photon excited fluorescence could only be acquired for laser powers higher than 30 mW, when a 5-s CCD integration time is used (Fig. 6). The sensitivity of the technique can be further improved, especially at lower trapping powers (<30 mW) by reducing detector noise and increasing the signal integration times.

Although the present set of experiments has focused on the use of four specific fluorescent probes to assess the effects of confinement by 1064-nm laser tweezers, it should be possible to extend the techniques and methodologies described herein to other dye probes, cell specimen types, and two-photon excitation trapping wavelengths. With the development of diode laser traps and the growing interest in the use of optical tweezers at other near-infrared wavelengths (700-900 nm), it is likely that the combination of trapping with conventional, and two-photon excited, fluorescence will provide a practical approach, in future studies, to monitoring cell physiology and assessing the biological effects of near-infrared optical tweezers.

CONCLUSION

Optically trapped CHO and human sperm cells were studied, using specific exogenous probes to assess changes in cellular physiology (temperature, DNA structure, viability, and pH) as a result of optical confinement with 1064-nm laser tweezers. CW and pulsed microirradiation sources were used to excite visible fluorescence in cells via a two-photon absorption mechanism, thereby making twee-

zers a tool for simultaneous specimen manipulation and physiological monitoring. This work should provide a general basis for further study into the processes and mechanisms affecting cellular physiology during the interaction of optical tweezers and microbeams with biological specimens.

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