Micromanipulation and Physiological Monitoring of Cells using Two-Photon Excited Fluorescence in CW Laser Tweezers

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ABSTRACT

We report the observation of two-photon fluorescence excitation and cell confinement, simultaneously, 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 cell sample. At the wavelength of 1064 nm, a single focused gaussian laser beam is used to simultaneously confine, and excite visible fluorescence from, a human sperm cell that has been tagged with propidium iodide, a exogenous fluorescent dye that functions as a viability assay 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 sperm 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 has gradually transitioned between a live and dead state. Two-photon excited fluorescence was also observed in chinese hamster ovary cells (CHOs) that were confined by cw laser tweezers and stained with either propidium iodide or Snarf, a pH-sensitive dye probe. 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 induced photodamage, via two-photon processes.

Keywords: Laser trapping, two-photon, multiphoton, fluorescence, cell viability, exogenous, nucleic acid

1. INTRODUCTION

Optical tweezers are an effective tool for the confinement and micromanipulation of dielectric particles[1] and biological cells and organisms[1,2]. By focusing a continuous wave (cw) laser beam to its diffraction limit with a high numerical aperture objective lens, a large gradient in the beam intensity profile can be produced, 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. Although 1064 nm is generally assumed to be a safe, non-invasive wavelength that minimizes the extent of light-induced cell damage, the exact mechanisms of trap-induced biological effects at this wavelength are still largely unknown. Similarly, the question of whether 1064 nm optical tweezers can cause subtle physiological changes in cells remains unanswered. The optical power densities in infrared optical tweezers are sufficiently large, as a result of diffraction-limited focusing, so as to induce localized sample heating[3], and perhaps even two-photon absorption effects[4], under continuous wave conditions.

Recently, it was shown that two-photon fluorescence excitation using a cw laser is possible[5]. This latter result is especially significant, given that two-photon absorption and fluorescence excitation processes have, thusfar, 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]. The ability to monitor specimen physiology during the trapping process might therefore be possible using, for example, fluorescence spectroscopy[10,11], in conjunction with endogenous or exogenous probes. This approach could have several advantages, including the ability to study specific sub-cellular regions and physiological processes in real-time. Here, we report our recent results of experiments that use cw optical tweezers as a source for two-photon excited fluorescence concurrent with the optical trapping process and cell sample confinement.

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2. EXPERIMENTAL METHODS

2.1. Preparation of cell samples with fluorescent probes

Human sperm cells and chinese hamster ovary (CHO) cells were prepared for labeling with nucleic acid (propidium iodide) and pH-sensitive (Snarf) probes. All fluorescent probes were acquired from Molecular Probes Inc. CHO cells were maintained in culture using standard procedures. 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. Cells were first incubated at 37°C for 35 minutes, 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 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 g for 10 minutes. The resulting pellet was then resuspended in HTF-h and maintained at a constant temperature (~20 °C) until the time of experimentation.

The nucleic acid stain propidium iodide (PI) was 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[12]. 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 viable. 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. 5 µl of 1 mg/ml aqueous PI (Molecular Probes, Inc., Cat.No. P-3565) was added to a 1 ml PBS/cell suspension (PI ~5µg/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.

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, fluorescences yellow-orange (~ 580 nm) under acidic conditions, and deep red (~ 630 nm) when the pH is basic[13,14]. 18 µl of 1 mg/ml Snarf-1/DMSO (pH of 7.4) was added to 3 ml of CHO suspension medium to form a Snarf concentration of 6 µg/ml (10 µM). In the case of CHO cells, the intracellular and extracellular pH is balanced with the addition of 20 µM of the potassium ionophore nigericin 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. A fluorescence emission spectrum could then acquired. Since the emission spectrum is pH-sensitive, it could be used to monitor 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.

Prior to cell staining, absorption spectra were acquired for the fluorescent probes using a UV-VIS spectrophotometer. In the case of PI, pure DNA was stained, added to a buffer solution, and then loaded in cuvettes for analysis. For Snarf, ~ 10 ml of the dye, in either an ethanol or buffer solution, was placed in a quartz cuvette and subsequently analyzed. After measuring absorption spectra, cells were tagged, and then the cell suspensions were loaded into a customized sample chamber containing temperature monitoring and control functions.

2.2. Optical tweezers and spectroscopy system

The experimental apparatus used in the present set of experiments is identical to that previously described[3] and is depicted in Fig. 1. A linearly polarized TEM_{00} gaussian beam, derived from a cw Nd:YAG laser at 1064 nm, is first passed through a longpass filter ($\lambda > 800$ nm), deflected by a dichroic beamsplitter, and then focused to its diffraction-limited spot-size ($2\omega_0 \sim 0.8$ µm) onto a sample using an oil-immersion, 100 X, 1.3 N.A. microscope objective (Zeiss Neofluar). The fluorescence, which is emitted by a small volume of the sample, is collected by this same objective lens, passed through a pinhole aperture, collimated with beam expansion optics, and directed onto a 300 g/mm diffraction grating which disperses the optical signal. This

signal is then focused onto a computer-controlled CCD array. In this configuration, the optical system could acquire micron-resolved fluorescence with good signal-to-noise (S/N) ratios with a CCD array having 578 pixels and 16-bit sensitivity. The spectral response of the system was measured using a calibrated radiance source 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. The laser power incident on the sample was obtained by first measuring the power at the entrance aperture to the microscope objective, and then applying a transmission correction factor that was independently determined.

2.3. Measurement Techniques

Following 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 - 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 prior to spectral measurements. For cells labeled with the PI 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. This phenomenon is sensitive to trapping powers in the range of ~ 20 - 400 mW, and can yield fluorescent photon rates of ~ 10^4 - 10^8 photon s^{-1}, signal levels that can be easily detected in the present system.

3. EXPERIMENTAL RESULTS

Two-photon absorption, provided by the cw near-infrared laser trap was used to excite fluorescence from sperm and CHO cells labeled with the vital stain propidium iodide (PI) and the pH indicator snarf. This process requires that the specific stain has a significant absorption band at the two-photon fluorescence excitation wavelength (1064 nm/2 = 532 nm), and that a sufficient number of fluorescent photons (~ 10^4 - 10^8 photon s^{-1}) can be produced for photodetection. As an example, single-photon absorption spectra for the PI dye (Fig. 2) 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 fluorescence spectrum (Fig. 2), 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 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 ($I_{Fluor} \sim P^2$) fashion with applied laser power (Fig. 3). The departure from a pure square-law relationship is attributed to fluctuations in laser power, and to photobleaching effects that were observed to occur at higher laser powers. There is also the possiblility that multiphoton processes are contributing to the fluorescence excitation process. For laser powers in the 30 to 200 mW range, a detector integration time of 5 s was used to acquire the fluorescent signals. In the case of two-photon fluorescence excitation, it is estimated that a fluorescent photon rate of ~ 3.0 x 10^5 - 10^7 photon s^{-1} is produced, based on a trapping power of 100 mW at 1064 nm, a focused spot size of ~1 μm^2, a two-photon cross-section of 10^{-50} cm^4 s-1 $molecule^{-1}$ $photon^{-1}$, and a fluorescence quantum yield of 1%. Experimentally, an IR laser power of 100 mW was found to produce fluorescence signals of ~ 10^6 - 10^7 photons s^{-1}, as measured by the CCD array. We note that these numbers are only estimates, since precise values for the two-photon cross-section and quantum efficiency of these dyes are, to date, undetermined.

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 μm/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. 4) show a gradual increase in the fluorescence intensity with trap confinement time. Specifically, emission is observed only after a two 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 loses viability and transitions between a live and dead state. Similar results were obtained for PI-labeled immotile CHO cells.

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 μM 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 simultaneously excited. The resulting spectra are distinct for each of the four different pH values (Fig. 5), 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.

4. DISCUSSION

The fluorescence spectra derived from PI-stained dead sperm cells provide evidence that physiological monitoring can proceed simultaneously with laser trapping, and provide valuable 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. 4). 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[15]. However, the results are contrary to those of preliminary measurements made with laurdan and AO dyes 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 motile" cells represent a sub-group of the entire populaton which are particularly susceptible to photodamage. It is likely that other yet unknown photochemical or photobiological processes, including multiphoton effects, are responsible for the observed degradation in cell viability. In such a case, other cell viability assays, capable of monitoring various metabolic processes (e.g. enzymatic activity, mitochrondrial action, ATP production), might be needed to determine the origin of this physiological change.

While the present set of experiments have focused on the use of two 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. For example, the use of fluorescent probes such as Indo-1 and Rhodamine 123 might be useful for monitoring metabolic function, such as calcium-ion activity, or cell viability, including mitochrondrial activity[8,10-12]. Alternatively, more site-selective probes may be required to monitor specific intracellular receptors, proteins, and reduced coenzymes (e.g. NADH, NADPH) that dictate cellular physiology at the molecular level. Furthermore, 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 tweezers and laser microbeams.

5. CONCLUSION

Optically trapped CHO and human sperm cells were studied, using specific exogenous probes to assess changes in cellular physiology (viability and pH) as a result of optical confinement with 1064 nm laser tweezers. A cw near-IR laser source was used to excite visible fluorescence in cells via a two-photon absorption mechanism, thereby making tweezers 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.

6. ACKNOWLEDGMENTS

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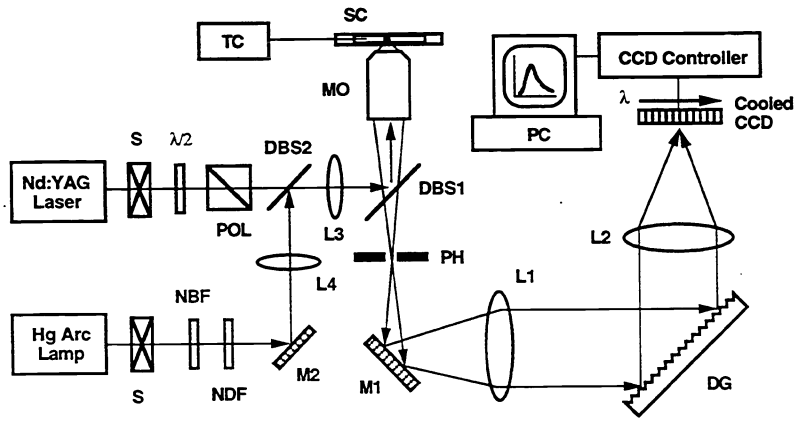

Fig. 1. Experimental system for implementing optical trapping with fluorescence spectroscopy for physiological monitoring.

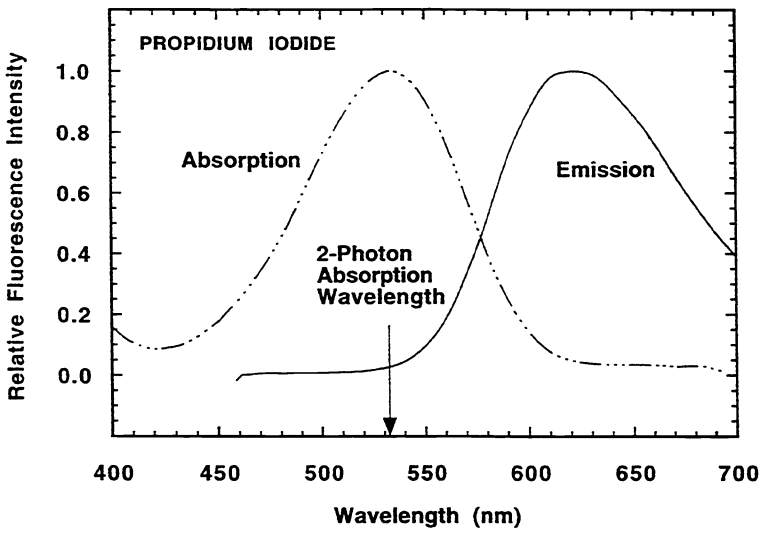

Fig. 2. Absorption and emission spectra for the vital stain propidium iodide. The absorption spectrum shifts by ~ 50 nm once the dye becomes bound to DNA. The peak in fluorescence emission occurs at 620 nm, when excited at the two-photon excitation wavelength of 532 nm.

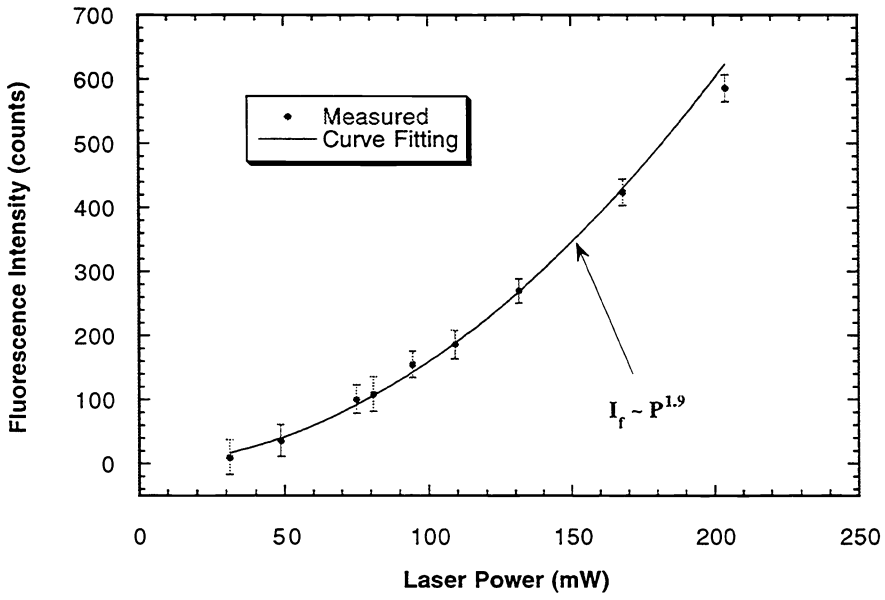

Fig. 3. Dependence of fluorescence intensity on pump (trapping) laser power for the fluorophores propidium iodide (PI) and snarf. The intensities vary with nearly a square-law dependence on laser power for both dyes, a behavior consistent with a two-photon absorption process.

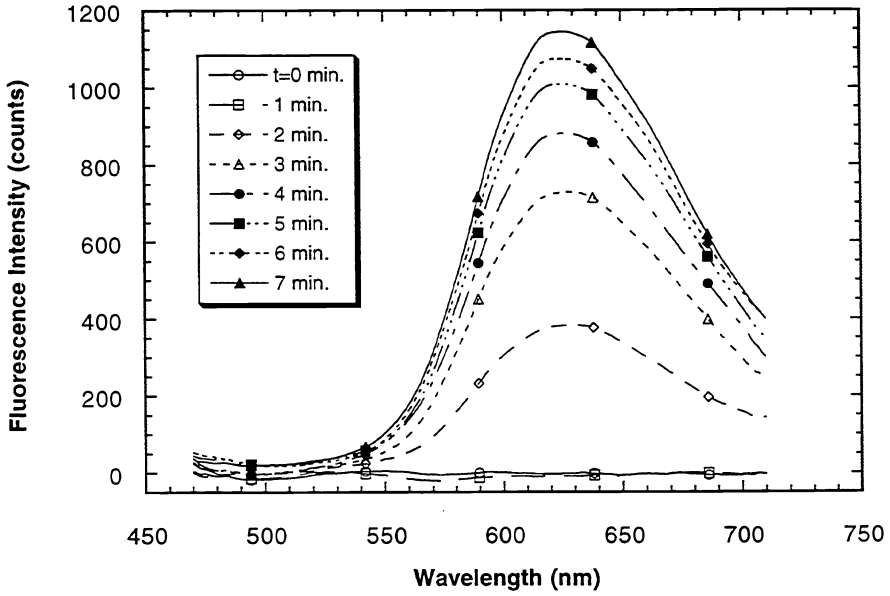

Fig. 4. Time evolution of the fluorescence spectrum from a PI-labeled sperm cell.

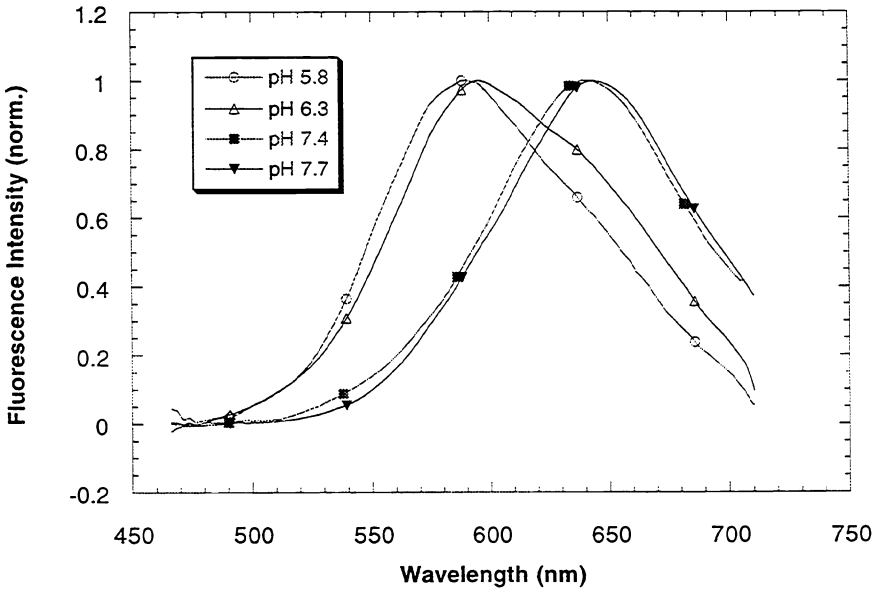

Fig. 5. Two-photon excited fluorescence spectra for CHO cells labeled with the pH-indicator Snarf. For cells with pH levels set initially at 5.8, 6.3, 7.4, and 7.7, no change in pH was observed following cw laser trapping at 200 mW.