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CELL VIABILITY AND DNA DENATURATION MEASUREMENTS BY TWO-PHOTON FLUORESCENCE EXCITATION IN CW Al:GaAs DIODE LASER OPTICAL TRAPS

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ABSTRACT

Cell viability and DNA denaturation are measured through two-photon fluorescence excitation using a single diode laser beam as the trapping and exciting source simultaneously. Two-photon fluorescence emission spectra are presented for CHO cells and T lymphocytes loaded with various fluorescent probes. This single beam method is demonstrated to be a safe tool to monitor the viability of optically trapped cells, even under intense 809 nm diode laser illumination ($\sim 10^6$ W/cm²). The dynamics of cellular necrosis is monitored by adding ethanol to the cell suspension during trapping. Thermal damage to heat-treated samples is assessed by recording shifts in the emission spectra from trapped cells loaded with the nucleic acid probe, acridine orange. © 1999 Society of Photo-Optical Instrumentation Engineers. [S1083-3668(99)00802-3]

Keywords two-photon absorption; fluorescence; diode laser; optical traps; cells.

1 INTRODUCTION

Optical traps and multiphoton fluorescence are promising new tools for micromanipulating and monitoring single cells.^{1–6} Optical traps are typically formed using continuous wave (CW) near-infrared (NIR) lasers. Because of their low cost, broad wavelength availability, excellent power, and outstanding reliability, diode lasers have become standard tools for trapping. These same advantages have also driven the development of CW sources (both semiconductor and solid-state) for two-photon excitation and imaging.^{7–11} In fact, depending on the precise source wavelength, power, and mode structure, it is not unreasonable for multiphoton processes to occur, perhaps inadvertently, in CW optical tweezers.¹² Thus, the intentional combination of these methods in a single focused laser beam can be a particularly powerful and convenient approach to monitoring and manipulating physiological processes in single cells.^{13–15}

In this study we describe key parameters for optimizing the performance of a CW diode laser microscope for simultaneous trapping and multiphoton excitation. In previous work, we employed relatively large, expensive Nd:YAG or Ti:sapphire lasers for combined trapping/fluorescence experi-

ments. In practical terms, compact and inexpensive diode lasers would be the ideal choice for micromanipulation/monitoring systems. As a result, we demonstrate in this paper cell viability and DNA denaturation monitoring using two-photon-excited fluorescence from dye-tagged cells in 809 nm Al:GaAs diode laser optical traps.

2 MATERIALS AND METHODS

In the preparation of the living cells, the murine HEL-restricted CD4⁺ T cell (IE5)¹⁶ was grown in RPMI 1640 containing 10% fetal bovine serum (RPMI/FBS) 10 mM HEPES and 1% NEAA, glutamine, and Na⁺ pyruvate. Cells were maintained in a humidified incubator at 37 °C with 5% CO₂, 95% air. The live–dead assay kit (Molecular Probes, Inc.) was selected to be the indicator of cellular viability. This kit is composed of two probes: a membrane-permeant nucleic acid stain, SYBR 14, along with the conventional dead-cell stain, propidium iodide (PI). When cells are incubated briefly with these two stains, live cells with intact membranes fluoresce bright green, whereas cells with damaged membranes fluoresce red. Both red and green fluorescence are primarily derived from the nucleus. Since the nucleus has a limited dye bind-

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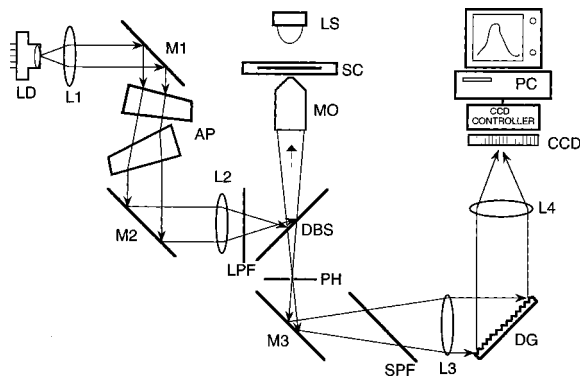
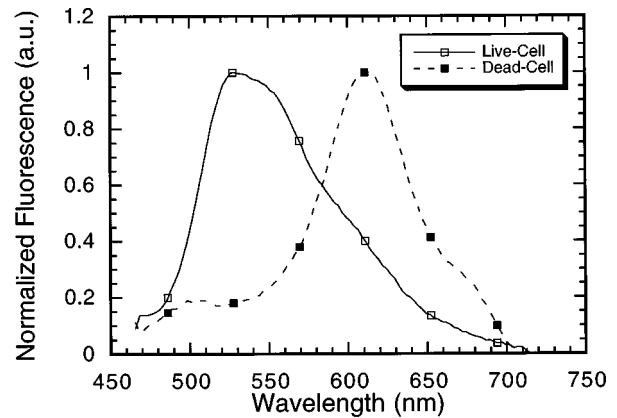


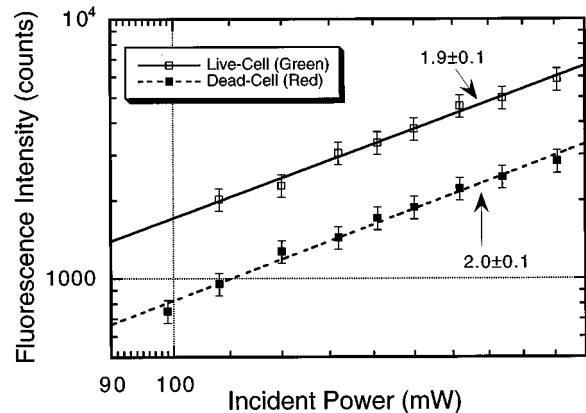
Fig. 1 Experimental system for implementing optical trap and fluorescence detection. LD: laser diode; AP: anamorphic prism pair; M1–M3: mirrors; L1–L3: lenses; LPF: long pass filter; DBS: dichroic beamsplitter; MO: microscopic objective; SC: sample chamber; LS: light source; PH: pinhole; SPF: short pass filter; DG: diffraction grating; CCD: charge coupled detector; and PC: personal computer.

ing capacity, once these binding sites are saturated by fluorescent probe, the fluorescence intensity does not increase with the increasing dye concentration in the medium. 20 μL live-cell stain (SYBR14) with a concentration of 1 mM and another 20 μL dead-cell stain (PI) with a concentration of 1 mM were added to a 2-mL room-temperature T-cell suspension. The entire cell suspension was then loaded into a microscope viewing sample chamber. CHO (Chinese hamster ovary) cells were maintained in culture using standard procedures,¹⁷ and subjected to heat treatments.¹⁸ Tubes with 1 ml aliquots of cells were heated for 5 min in a water bath at appropriate temperatures. The tubes were then transferred to an ice-cold bath and 4 ml of an ice-cold solution of AO in SMT medium was added to each tube. The tubes were then equilibrated to room temperature for measurements. The fluorescent probe, AO (acridine orange), an indicator of the DNA denaturation, was used to compare differences between nuclei in normal versus heat-treated cells. 10 μL of AO with a concentration of 1 mM was mixed with 1 mL PBS/cell suspensions ($\sim 10^5$ cells/mL) for normal and heat-treated CHO cells.

Our experimental setup is shown in Figure 1. An optical trapping beam, derived from an AlGaAs laser diode (SDL 2352-H1) with maximum optical output power of 500 mW at 809 nm, was collimated by a cylindrical collimating lens (06GLC001, Melles Griot, NA=0.615), and circularized by a pair of anamorphic prisms. After passing through a second lens (biconvex lens with $f=20$ cm) and a long pass ($\lambda > 780$ nm) filter, the beam was deflected by a dichroic beamsplitter, and focused to a spot size ($2\omega_0 \sim 0.8 \mu\text{m}$) in the specimen plane of an inverted microscope using an oil immersion, 100 \times , 1.3 NA objective (Zeiss Neofluar). Typical sample powers ranged from 80 to 190 mW. The fluorescence excited by this trapping beam was collected by the



(a)



(b)

Fig. 2 (a) Two-photon-excited fluorescence spectra of live and dead cells loaded with the live–dead assay (see text for detail). Fluorescence was excited by a two-photon absorption process while the samples were held in 160-mW, 809-nm cw optical traps. (b) Log–log power dependence of the fluorescence intensity vs the incident power for live and dead cells. The slopes for the live and dead cells were both near 2.0. The integration time of the CCD was 20 s.

same objective lens, passed through a dichroic mirror and a pinhole aperture, collimated with beam-expansion optics, passed through a short pass filter ($\lambda < 750$ nm), and directed onto a 300-groove/mm diffraction grating, which dispersed the optical signal. The signal was then focused onto a thermoelectrically cooled CCD array (Princeton Instruments, Inc.). Spectral data, collected over a 300 nm bandwidth, were acquired and analyzed using a personal computer.

3 RESULTS AND DISCUSSION

Figure 2(a) shows the two-photon-excited fluorescence spectrum detected from live and dead trapped T-cells. Both dead and live probes were in the sample solution. For live cells, only fluorescence spectra with emission maxima at 525 nm are observed. In contrast, dead cells only exhibited fluorescence spectra which peaked at 612 nm. During the measurements of the fluorescence, the integra-

tion time of the CCD was typically 20 s. If only the solution was excited and there was no cell in the optical trap, we could not observe fluorescence from either SYBR 14 or PI even using the highest output power of the diode laser (190 mW measured after objective) and 35 s detector integration time. This is due to the fact that fluorescence can only be excited when SYBR 14 penetrates the live cell membrane and binds to DNA. In contrast, PI does not penetrate live cell membranes. Under normal conditions, PI is nonfluorescent. However, when the cell is damaged, the "dead-cell" probe traverses the plasma membrane, binds to DNA, and exhibits red fluorescence. This feature is especially advantageous for optical trapping studies because the trapping beam cannot excite the two-photon fluorescence in the dye solution surrounding the trapped sample, but only from the cell sample held at the focal plane.

To confirm that the SYBR 14 and PI fluorescence signals were both excited by two-photon absorption during 809 nm laser trapping, we examined the dependence of the fluorescence intensity on incident laser power. Figure 2(b) shows a log-log plot of fluorescence intensity versus the incident power for 525 and 612 nm live-dead cell signals. Slopes of 1.9 ± 0.1 and 2.0 ± 0.1 for live cell and dead cell, respectively, indicate that the fluorescence was excited by two-photon absorption.

Even for the maximum output power of the diode laser, we did not observe obvious viability changes for the trapped live cell, and the ratio of green/red (525/612 nm) fluorescence did not change during 1000 s trapping time (50 cells were measured). Under these conditions, 190 mW was launched onto the sample (measured after the objective). In order to show that our system can monitor the cell's viability dynamically, 100 μ L of 70% ethanol was added to the sample solution. Ethanol-induced damage to the plasma membrane facilitates transport of PI (dead-cell stain) to the nucleus. After adding "drug" into the suspension of live cells, the green (525 nm) fluorescence decreases and the red fluorescence (612 nm) increases. Figure 3 shows the dynamic process of the trapped T-cell after the ethanol was added into the solution. Prior to ethanol injection, the green signal was dominant. With time, the red/green ration increased until, at 5 min, the 612 nm peak became the dominant spectral feature. The precise amount of time required for a live-dead cell transformation depends on the amount of ethanol added and the individual characteristics of the trapped cell. Since there is no change in viability during laser trapping, the damage is induced by the ethanol alone.

Figure 4 shows two-photon-excited fluorescence spectra derived from normal and heat-treated CHO cells in the diode laser trap. The fluorescence from the acridine orange is green when bound to the double-stranded DNA and red when associated with single-stranded nucleic acids. Our results

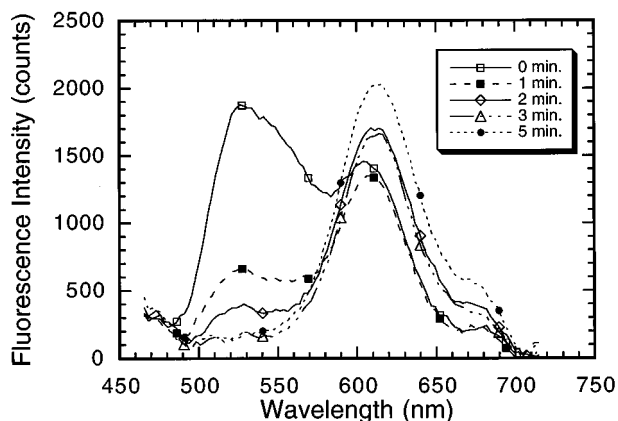


Fig. 3 Cell viability monitored via two-photon fluorescence excitation for a T-cell that was held stationary for 5 min in a 160-mW optical trap after adding ethanol to the sample solution.

show that when held in the 809 nm trap, normal CHO cellular fluorescence peaks around 530 nm, while heat-treated cellular fluorescence peaks around 610 nm. The change in the two-photon excited fluorescence spectra suggests alterations consistent with the conversion of DNA from double-stranded to single-stranded via the heat treatment process.

4 CONCLUSION

In conclusion, we have observed two-photon fluorescence excitation from live and dead cells in near infrared diode laser optical traps and have demonstrated the utility of this technique in monitoring cell viability and DNA denaturation. This suggests that relatively benign two-photon excitation processes can be coupled with inexpensive CW diode lasers to develop safe, micromanipulation/monitoring tools. Although the "live-dead" assay is not ideal for monitoring subtle changes and long-term damage, with appropriate vital dyes, this

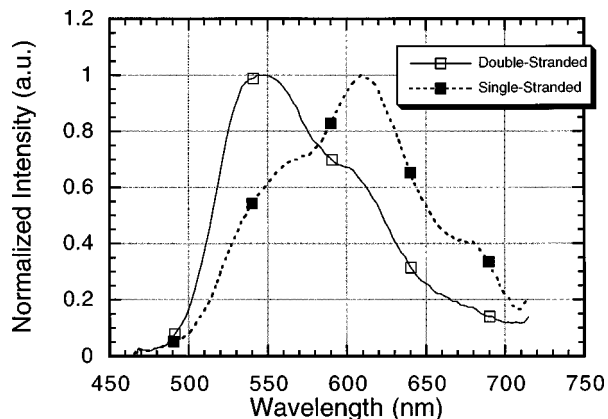


Fig. 4 DNA denaturation monitoring through two-photon fluorescence excitation for CHO cells loaded with the fluorescent probe acridine orange. The trapping and exciting laser power was 160 mW; the integration time of the CCD array was 20 s.

single beam method may find applications in a variety of studies designed to evaluate the dynamic effects of perturbations (e.g., chemicals, heat, light, etc.) on cellular processes.

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