

Laser trapping microscopy as a diagnostic technique for the study of cellular response and laser-cell interactions

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ABSTRACT

Optical laser trapping microscopy has emerged as a powerful tool not only for the optical manipulation of cells and macromolecules, but also for the study of cellular physiological responses via force transduction and fluorescence imaging. We describe here the most recent results from our laboratory in the use and application of laser trapping microscopy to a variety of studies at the cellular and molecular levels. Fluorescence spectroscopy and imaging have been successfully combined with optical micromanipulation. A single near-infrared laser beam is used for two-photon fluorescence excitation and micromanipulation of trapped biological specimens. Cell viability is observed and monitored with a Nd:YAG laser ($\lambda=1064$ nm) and an Al:GaAs diode laser ($\lambda=809$ nm). Traps and conventional fluorescence imaging are also used simultaneously to examine T-cell activation dynamics.

Keywords: Optical laser trapping, fluorescence detection, two-photon fluorescence, force transduction, immunoassay, T-cell activation, T-cell receptor, calcium imaging

1. INTRODUCTION

Since Ashkin et al.¹ first described the optical trapping of micrometer-sized dielectric particles in a single beam gradient force trap, optical laser traps (optical tweezers) have been successfully used in a variety of biological applications,² ranging from DNA stretching and the study of cell-signaling mechanisms to microchemistry and materials engineering.

When a laser beam, such as one from an Nd:YAG (1064 nm) or near-infrared diode laser (809 nm), is focused to a diffraction-limited spot using a high-numerical-aperture lens, it creates a single-beam gradient force trap. The trap can be used to confine and manipulate microscopic objects: dielectric particles, such as latex microspheres; and biological specimens, such as bacteria, viruses and cells.

In this paper, we report the most recent results from our laboratory in the use and application of laser trapping microscopy to a variety of studies at the cellular and molecular levels. In the study of cellular responses, fluorescence spectroscopy and imaging have been successfully combined with optical micromanipulation to investigate (1) two-photon excited fluorescence from exogenous probes in optical traps and (2) B lymphocyte-T lymphocyte interactions via site-selective placement and orientation of cells. Here we show how the two-photon fluorescence diagnostic technique can be used to monitor cellular physiology, such as viability of optically trapped cells. We also demonstrate that T-cells are effectively polarized antigen sensors, a result which should further our understanding of cell activation, signal processing and the human immune response.

2. TWO-PHOTON FLUORESCENCE DIAGNOSTIC METHOD

In this section, we report the results of two-photon excited fluorescence from exogenous probes in continuous wave (cw) infrared optical tweezers, and show how this technique can be used to monitor cellular physiology, such as viability of optically trapped cells.

The experimental setup is shown in Fig. 1. The laser beam is first passed through a long-pass filter (>800 nm), deflected by a dichroic beam splitter, and then focused onto the sample by an oil-immersion, 100 \times , 1.3-numerical-aperture (NA) microscope objective lens. Fluorescence 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 ($\sim 1 \mu\text{m}$) and spectral ($\sim 1 \text{ nm}$) measurements in an inverted confocal microscope geometry with good signal-to-noise (S/N). The lasers used are Nd:YAG ($\lambda=1.064 \mu\text{m}$) and Al:GaAs ($\lambda=809 \text{ nm}$).

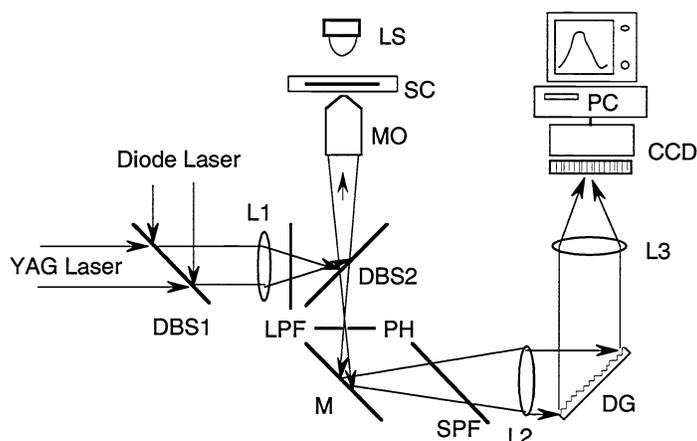


Fig. 1 Experimental system for implementing optical trap and two-photon fluorescence detection.. L1-L3: lenses; DBS1-2: dichroic beam splitter; MO: microscopic objective; SC: sample chamber; LS: light source; PH: pinhole; M: mirror; DG: diffraction grating; CCD: charge coupled detector; and PC: personal computer.

A single focused Gaussian laser beam is used to simultaneously confine and excite visible fluorescence from biological samples labeled with specific fluorescent dyes. In previous work,³ we considered a human sperm cell that has been tagged with propidium iodide (PI), a fluorescent dye that functions as an assay of cellular physiological state. The intensity at the dye peak emission wavelength ($\lambda=620 \text{ nm}$) was found to exhibit a near-square-law dependence on incident trapping beam power, a behavior consistent with a two-photon absorption process. Fig. 2 shows the dependence of fluorescence intensity on pump laser power for the fluorophore PI bound to the DNA of the dead cells using $\lambda_{\text{trap}}=1064 \text{ nm}$ or $\lambda_{\text{trap}}=809 \text{ nm}$.

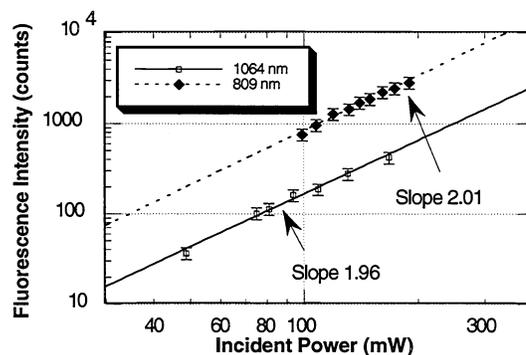


Fig. 2 Dependence of PI fluorescence intensity from cells on trapping laser power. The intensity varies with nearly a square-law dependence on incident laser power. The CCD integration time was 5 seconds for Nd:YAG ($\lambda=1.064 \mu\text{m}$) and 2 seconds for Al:GaAs ($\lambda=809 \text{ nm}$).

To assess viability in an 809 nm trap, two-photon fluorescence is recorded from T cells loaded with Live-Dead probes (Molecular Probes, Inc.). The Live-Dead assay kit is comprised of two molecules: SYTO 10, a fluoregenic substrate that is cleaved only in viable cells to form a green fluorescence membrane-impermeant product; and DEAD Red, a high-affinity red fluorescent DNA stain that is only able to pass through the compromised membranes of dead cells. For live cells, only two-photon fluorescence peaked at 525 nm could be observed; for dead cells, only fluorescence around 612 nm could be detected. This is shown in Fig. 3. With addition of ethanol, the 525 nm fluorescence decreases and the 612 nm from DEAD Red signal increases. Ultimately, only red fluorescence is observed indicating that the cell is totally dead.

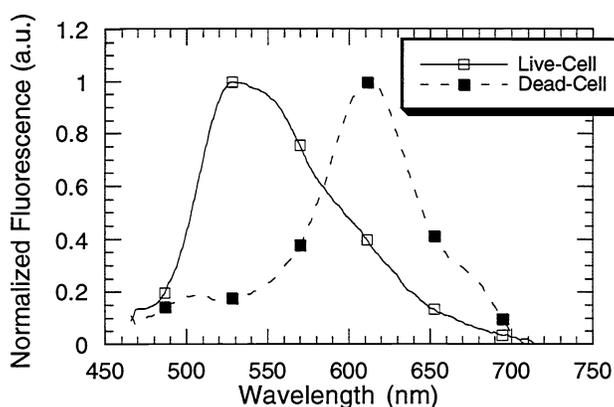


Fig. 3 Two-photon fluorescence spectra of live and dead T-cells under the trapping and exciting diode laser source.

In conclusion, we have observed two photon fluorescence excitation in cw Nd:YAG laser and diode laser optical tweezers and have demonstrated its application in assessing cell physiology during sample confinement. The techniques presented herein should find broad applications in physiological studies of optically confined samples.

3. POLARIZED T-CELL DETECTION ABILITY TO ANTIGEN REVEALED BY AN OPTICAL TRAP

In the second part of this paper, we report the latest results of B lymphocyte-T lymphocyte interaction study with an optical trap.

T-cell contact with antigen-presenting B cells initiates an activation cascade which includes an increase in T-cell intracellular calcium and leads to T-cell differentiation and proliferation.^{4,5} We studied cell-cell contact requirements for T-cell activation using an optical tweezers to control the orientation of T-cell/B-cell pairs and fluorescence microscopy to measure subsequent T-cell $[Ca^{2+}]_i$ response.^{6,7} B cells or beads coated with antibodies to the T-cell receptor are trapped with a titanium-sapphire laser and placed at different locations along the T-cell, which has a polarized appearance defined by the shape and direction of crawling. T-cell $[Ca^{2+}]_i$ is detected as an emission shift from the combination of fura-red and oregon-green, two cytoplasmic $[Ca^{2+}]$ indicators. T cells which are presented antigen at the leading edge have a higher probability of responding and a shorter latency of response than those contacting B-cells or beads with their trailing end.

3.1. Methods

Cell Culture. The murine hen egg lysozyme (HEL)-restricted, CD4⁺ T cell (IE5) and MHC II-restricted B cell (2PK3) hybridomas were grown in RPMI 1640 containing 10% fetal bovine serum (RPMI/FBS) 10 mM HEPES and 1% NEAA, glutamine, and sodium pyruvate. Cells were maintained in a humidified incubator at 37°C with 5% CO₂/95% air. IE5 cells were moderately adherent to plastic flasks at 37°C and were resuspended for collection by gentle shaking at room temperature. Antigen presenting 2PK3 cells were incubated with 10 µg/ml HEL for between 3 and 12 hrs. This protocol produced a maximal response from IE5 T cells as judged by a contact-dependent [Ca²⁺]_i response about 70% of cells. T cells were also probed with antibody-coated latex microspheres. We used 6 µm diameter polystyrene microspheres stabilized with sulfate charges (IDC, Portland Or). 100 µg/ml goat-α hamster IgG in 10% PBS was adsorbed to beads for 8 hrs at room temperature, centrifuged and washed twice with 10% PBS and then conjugated with 50 µg/ml hamster α-mouse CD3ε for 3 hrs. Beads were centrifuged and washed twice before use.

Optical Trapping. The geometry of T cell-B cell contact was manipulated using a tunable, near infrared titanium:sapphire laser producing a trapping beam at about 760 nm (Berns et al., 1992). The trapping laser was introduced via the TV port of a Zeiss Laser Scanning Confocal microscope (LSM 410). A short-pass (720 nm) dichroic reflector was used to separate trapping and fluorescence excitation beams. A 100X 1.3 NA Neofluor objective and focused the near infrared and visible beams, resulting in 60 mW trapping power at the focal plane. This arrangement allowed trapping and fluorescence-based [Ca²⁺]_i measurements on the same cells.

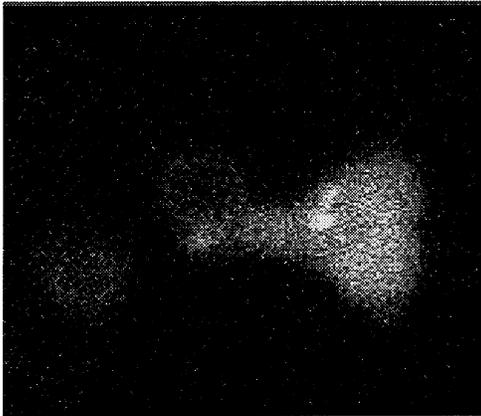
[Ca²⁺]_i imaging. To measure T-cell [Ca²⁺]_i on the LSM, IE5 cells were co-loaded with a combination of fura-red/AM (5 µM) and oregon-green/AM (2 µM), two long-wavelength Ca²⁺ indicators which respond to the 488-nm excitation line of the argon laser. Cells loaded for 1.5 hr at 37°C produced a red to green shift when [Ca²⁺]_i was elevated. This shift was quantified by scanning cells with the argon laser and dividing the fluorescence intensity signals from two photomultipliers with emission bands of 520-570 nm (green) and >610 nm (red). In these experiments a single, 2PK3 cell or antibody-coated bead was held in the trap on a heated stage and positioned so that it made contact with a particular region of a dye-loaded T cell. Once the cells were positioned, the trapping beam was cut off and 488 nm laser excitation were performed. A third photomultiplier collected a Ca²⁺-insensitive blue emission band (400-480) from incandescent illumination which was used to produce a brightfield image. 30-40 scans at 10 s intervals were made to determine whether a [Ca²⁺]_i increase occurred in the T cell following contact with APC. T cells not responding within 400 s were scored as unresponsive.

3.2. Results and discussion

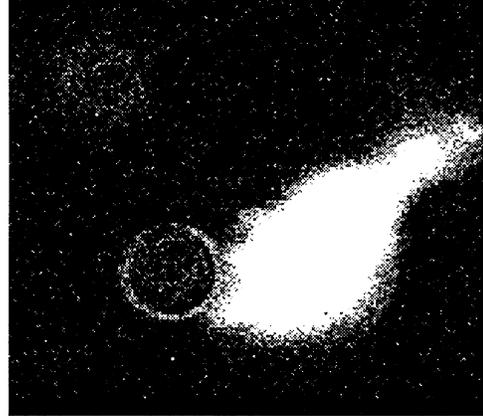
T cell sensitivity to antigen localized by optical trapping. T-cell sensitivity to B cells was polarized by using a laser-based optical trap to control the point of initial cell-cell contact while measuring T-cell [Ca²⁺]_i. With the B cell placed at the T-cell tail, no response occurred, and the B cell detached from the T cell within two minutes. Trapping the loose B cell and placing it at the leading edge of the same T cell rapidly elicited a T-cell [Ca²⁺]_i increase. To investigate whether polarity could be observed via TCR engagement alone, we used beads coated with antibodies to the CD3 subunit of the TCR complex to mimic TCR engagement in the absence of any coreceptors and got similar results (Fig. 4).

A

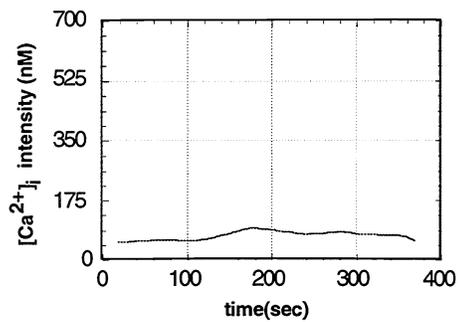
a. rear contact



b. front contact

**B**

a



b

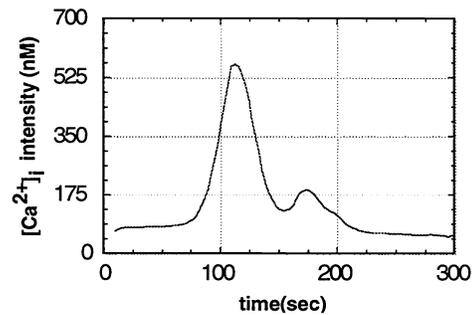
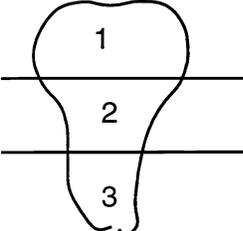


Figure 4. Optical trapping of antibody-coated bead reveals functional polarity of T cell.

(A) Bright field, fluorescence intensity overlays of T-cell-bead pairs with antibody-coated bead (round shape) trapped at either the tail (a) or leading edge (b) of the T cell. T cell intensity ratio generated from oregon-green and fura-red co-loaded into IE5 cells (dark, red=low $[Ca^{2+}]_i$; bright, green=high $[Ca^{2+}]_i$;--see Methods). (B) Time course of $[Ca^{2+}]_i$ for cells shown in (A).

The results of 29 different T-B cell pairs and 109 bead-T cell are summarized in Table 1. T cells which were presented either with antigen or anti-CD3 mAb at the leading edge(contact zone 1) had a higher probability of response and shorter latency of response than those contacting with their tail(contact zone 3).

Table 1. Polarized T-cell Response to TCR Stimulation

contact zone (on T cell)	cells responding (%)		latency (sec)	
	B cell	anti-CD3 bead	B cell	anti-CD3 bead
	82 (14/17)	87 (77/87)	42 ± 16	52 ± 15
	80 (4/5)	82 (14/17)	60 ± 22	78 ± 39
	31 (4/13)	6 (1/15)	146 ± 29	340

T cells were stimulated by either antigen-presenting B cells or anti-CD3-coated beads at the region indicated. Region 1 is defined as the leading edge. Latency indicates the delay between contact and a detectable $[Ca^{2+}]_i$ increase in the responding population.

These findings show not only that T-cell is a polarized antigen sensor, but also that receptor stimulation can be directly correlated to a functional response. Such studies are expected to aid in the design of new therapies for promoting or inhibiting immune response within the human body.

4. SUMMARY

Optical laser trapping microscopy has emerged as a powerful tool not only for the optical manipulation of cells and macromolecules, but also for the study of cellular physiological responses via force transduction and fluorescence imaging. We describe here the most recent results from our laboratory in the use and application of laser trapping microscopy to a variety of studies at the cellular and molecular levels. These include: 1) The use of a single infrared laser beam for two-photon fluorescence excitation and micromanipulation of trapped biological specimens. and 2) Activation of T cells using receptor-specific microspheres delivered to different cellular regions via an optical trap.

5. ACKNOWLEDGMENTS

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