Cell permeabilization and molecular transport by laser microirradiation

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ABSTRACT

Highly focused pulsed laser microbeams can be used to precisely dissect, inactivate, or perturb cells and subcellular targets. Here we introduce a new technique which employs pulsed microbeams to transiently permeabilize the plasma cell membrane and affect the delivery of molecules from the extracellular environment into the cell. This optically produced cell permeabilization can be applied using non-specific or specific modalities. In the non-specific modality, which we term 'optoporation', the pulsed microbeam is focused onto the glass coverslip on which the cells are plated. The generation of mechanical transients in connection with irradiation of the glass achieves molecular delivery to a number of cells proximal to the irradiation site. In the specific modality, termed 'optoinjection', the microbeam is focused direcly onto the plasma cell membrane and achieves molecular delivery into that cell alone. To quantify the irradiation geometry involved in these and other microbeam processes, as well as examine the possibility of certain non-linear effects, we have developed a system using photochromic polymer films to characterize microbeam propagation and its effects within microirradiated targets. These photochromic polymers confirm that the laser microbeam are indeed focused to submicron dimensions within the targets in our systems. In addition the behavior of such polymers at higher pulse energies and irradiances indicate that multiphoton absorption and/or plasma formation may mediate some laser microirradiation processes.

Keywords: microirradiation, plasma membrane, cell permeabilization, photochromic dye, multi-photone processes, plasma formation

1. BACKGROUND AND MOTIVATION

Highly focused laser microbeams have been used in biology for over 25 years[1-4]. These pioneering works, and the steady, but rather slim stream of studies which followed, focused primarily on the use of laser microbeams to precisely dissect, inactivate, ablate or transiently perturb cells or subcellular targets [5-9]. The increasing versatility and affordability of laser sources (both pulsed and CW) along with the recent "renaissance" in optical microscopy has produced a new wave of interest in the use of laser microbeams to investigate biological phenomena and the development of multiparametric microscope systems for cell manipulation and monitoring.

Optoporation (or transient cell permeabilization) is a fairly new technique that has been added to optical biology tool chest. It is closely related to optical ablation and inactivation techniques in that we use the thermodynamic transients generated by pulsed microbeams to perturb a cellular structure; in this case the plasma cell membrane. This perturbation serves to transiently permeabilize the cell membrane and affect the delivery of molecules from the extracellular environment. Previous efforts to optoporate cells relied on UV-irradiation[9-12]. Because of the known effects of UV damage to cells[13] we employed visible (VIS) or infrared (IR) laser beams in our studies. Our ultimate goal is to create a reproducible method for the delivery of membrane-impermeable reagents to plated or suspension cells within standard tissue culture growth media. Note, that we are avoiding the use of gel matrices used in high throughput systems or other forms of media, since it is often desired to manipulate the cell or assay cellular activity immediately after introduction of the exogenous reagent. The goal of the present study is to assess the efficacy of laser microbeam methods for molecular delivery and its dependence on both the laser parameters employed and the properties of the molecule to be delivered.

A number of biological applications (investigations of cellular growth, differentiation, development, senescence, cancer) would benefit from a novel technique to introduce reagents into cells *in vivo*. Conventional methods for introducing DNA, RNA, and proteins into cells are mainly chemical or mechanical in nature. Transfection of plasmid DNA in a select number of cell types can be performed by calcium phosphate treatment, liposomal delivery, or electroporation. Recombinant and purified proteins as well as antibodies are routinely microinjected into target cells while electroporation is utilized less typically because of the high concentration of the introducing reagent that is needed. Optoporation may have some distinct advantages relative to these other methods since the population of cells need not be exposed to a chemical reagent and the molecular delivery can be achieved in a closed system without external micromanipulation.

2. OPTOPORATION MATERIALS, METHODS AND RESULTS

For the optoporation experiments we use a unique multi-parametric microscope developed at the Beckman Laser Institute. The Confocal Ablation and Trapping System (CATS) integrates a trapping beam from a (CW Titanium:Sapphire laser) with an ablation beam (Q-switched Nd:YAG with 2nd, 3rd, and 4th harmonic generation) into a laser scanning confocal fluorescence microscope (Zeiss LSM 410). Standard internal confocal microscope optics are replaced with two quartz dichroic beam splitters (260-380 nm, 420-650 nm, and 700-1070 nm). By separating the orthogonal polarization components of the Ti:Sapphire laser, these optical elements allow for up to two independently controlled optical trapping beams to enter the microscope collinearly. The beam of a high power pulsed (visible wavelength) laser used for ablation is brought into the microscope through the standard epifluorescence port using a "two way" coupling device. Switching this mirror allows the microscope to function as a standard (non-confocal) fluorescence system with Hg-arc lamp excitation. All the optics, mirror mounts, and beam splitters used to bring these four laser beams into the microscope are "bread-boarded" on a vibration isolation table. Solenoid-driven mirror mounts provide 0.25 micron movement resolution for all four laser beams and are controlled individually by joysticks. A cooled color CCD camera (Optronics, Inc.) with real-time processing and signal integration capabilities is used for recording true-color images. Confocal laser scanning capabilities with three photomultiplier tube (PMT) channels are available in both trapping and ablation modes using Argon ion or He:Ne laser excitation.

The optoporation experiments used a fundamental (1064 nm) or frequency-doubled (532 nm) Q-sw Nd:YAG laser (SureLite II, Continuum) emitting pulses 4-6 ns in duration. These pulses were focused through a 100x, 1.3 NA, Phase 3 or DIC oil immersion Zeiss Neo-Fluar objective and delivered to either CHO-K1 (Ovary, Chinese hamster, cricefulus griseus, CCL 61), PtK_2 (Kidney, Marsupial, Potorous tridactylis CCL 56) or HeLa (Epitheloid carcinoma, cervix, Human, CCL 2) cells grown on glass coverslips. To assess molecular transport, specific, membrane-impermeable, fluorescent molecules were placed in the cell media prior to irradiation. We investigated the characteristics of this laser-induced transport phenomena with several different probes to examine the effect of size (300-5000 Da), charge, and concentration (1 - 100 μM) of the fluorescent molecules introduced into the surrounding cell media.

The first set of experiments aimed to achieve molecular delivery to a number of cells in a nonspecific manner (optoporation). This was done by delivering a Nd:YAG laser pulse to the microscope glass and relying on the thermodynamic transients generated as a result to affect permeabilization of the plasma membrane in cells proximal to the irradiation site. This is illustrated in Fig. 1a where 10 μM Propidium Iodide (PI) was used as the fluorescent probe in a culture of CHO cells. After irradiation with a single 2 μJ pulse of 532 nm light (Fig. 1b), a subset of cells adjacent to the site of irradiation (arrow) show an intense orange glow which represents the transport of PI into these cells. To remove the PI in the media and assess cell viability, the culture was flushed with a 10 μM solution of a second membrane impermeable fluorescent dye, Oregon Green (OG). Fig. 1c illustrates loss of some damaged cells adjacent to the site of irradiation. The incorporation of OG by some of the remaining cells shown in Fig. 1d indicates those cells suffering permanent membrane damage and loss of viability. The cells identified by the arrows are those which show no OG incorporation but exhibited successful PI transport (Fig. 1b) suggesting that the PI has been delivered to these cells successfully without a loss in cell viability.

The second aim was to achieve molecular delivery specific to a single cell (optoinjection) (Fig. 2). A 33-residue polypeptide conjugated to Texas Red (TR) was used as the fluorescent probe and added to a culture of HeLa cells at 50 μM (Fig. 2a). Molecular transport into a single cell was achieved through direct exposure of a single 0.5 μJ pulse of 532 nm radiation. Fig. 2b shows a slight swelling of the irradiated cell. Fig. 2c is the fluorescent image after the culture has been flushed with saline and selective transport of the TR-labeled peptide into the cell is confirmed. Ten minutes after irradiation, the polypeptide diffuses into neighboring cells. In the future, an assay to definitively assess cell viability will be performed. We should note that the concentration of the reagent in the media is 10-20 times higher for optoinjection vs. optoporation (50-100 μM versus 5-20 μM). However, optoporation usually results in the death of 20-50% of the affected cells following microirradiation while optoinjection produces no visible signs of cell damage. Future viability assays as well as modification of optoporation protocol (lower cell density, greater distance to target site of a microbeam) may serve to improve the method.
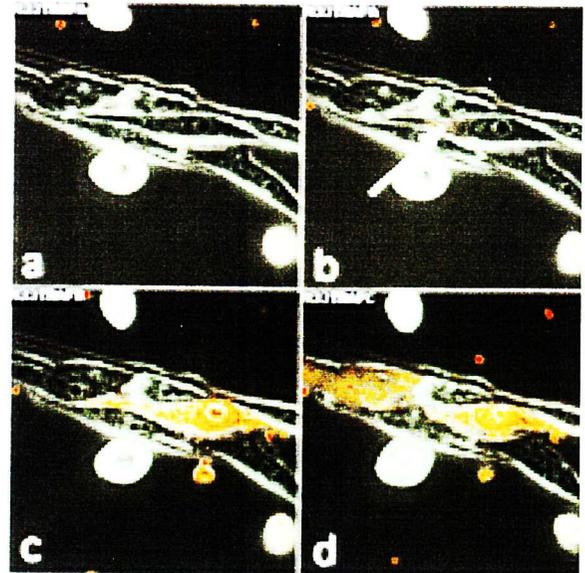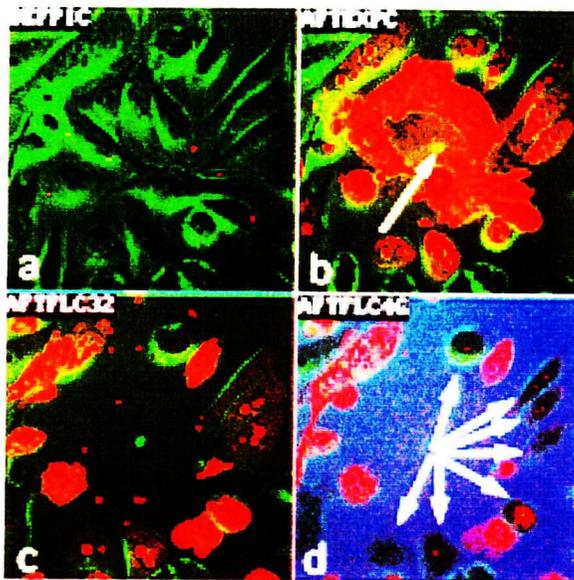
Fig. 1: Molecular delivery of Propidium iodide to a subset of cells (optoporation). Image (a) cells prior to poration, (b) cells immediately after poration (arrow indicates an indentation on the glass surface at the site of microbeam delivery), (c) after washing PI away, and (d) after addition of Oregon Green. **Fig. 2:** Molecular delivery to a single cell (optoinjection). Image (a) cells prior to poration, (b) arrow indicating site of microbeam irradiation, (c) porated cell with Texas Red conjugated peptide, chamber flushed with PBS and, (d) 10 min. post irradiation, showing polypeptide diffusion into neighboring cell.

To date, we have demonstrated specific and non-specific molecular transport through microirradiation (532 nm laser beam) in CHO, PtK$_2$, and HeLa cells using membrane impermeable compounds including PI, YO-PRO, and Texas Red-labeled dextrans and polypeptides. These probes have sizes between 300 and 5000 Da and are either cationic or neutral. We have not been able to deliver fluorescein-conjugated dextran (anionic compound, M.W. 3000). However, we have been able to transport negatively charged non-conjugated OG into PtK2 cells. The required pulse energy (up to 2 μJ) and number of pulses for optoinjection of OG were significantly higher than for zwitterionic or positively charged compounds. Curiously, the negatively charged dyes are green in color, and the positively charged and neutral dyes are red. Thus in all cases where molecular delivery was successful, the fluorophore displayed absorption of the 532 nm radiation while delivery of fluorophores transparent to 532 nm proved unsuccessful. To investigate this further, we introduced a fundamental (1064 nm) harmonic of a Nd-YAG laser to our microscopy system. Initial results shows the ability both to optoporate and optoinject PI into different cell lines utilizing a 1064 nm beam (Figs. 3 and 4). Thus optical absorption by the fluorophore is not a prerequisite for its delivery into cells, however, the pulse energies appear to be 25-50 times higher energy per pulse than those used for 532 nm irradiation.

Fig. 3: Molecular delivery of Propidium iodide (10 μM) to a of CHO cells using 1064 nm, 35 μJ/pulse.
**Fig. 4:** Molecular delivery of Propidium iodide to a single PtK$_2$ cell using 1064 nm, 25 μJ/pulse

4. PHOTOCHROMIC FILM MEASUREMENTS

To further develop and optimize cell microirradiation techniques, it would be helpful to have accurate characterizations of the optical dosimetry used to produce the effects we observe. The small irradiation volume and high divergence of light emanating from the microscope objective present formidable challenges for accurate measurement of microbeam spot size and pulse energy at the target site. To our knowledge, no cellular microirradiation study has had solid measures of either of these parameters. However, experience in our laboratory indicates that the desired microirradiation effect is critically dependent on careful adjustment of both parameters. Thus to examine the physical mechanisms involved in pulsed microirradiation processes and quantify their effects, an accurate determination of microbeam spot size and pulse energy is essential.

To approach one component of this problem, we have developed a novel technique which can potentially image and reconstruct the transverse irradiance profile of the focused laser beam at any axial position along the path of beam propagation. Our recording media is a thin (20-100 μm) polystyrene or polymethylmethacrylate film embedded with a photochromic compound, for example, 5-Cloro-1',3'-dihydro-1',3',3'-trimethyl-6nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole] (spiropyran).

Spiropyran (SP) is stable in its closed form (A). As shown in Fig. 5, form A is transparent to VIS and IR light but absorbs UV radiation which leads to a heterolytic break of the C-O bond resulting in a transformation to an open form. The open form is known as merocyanine (B) which has a long chain of conjugation and can absorb visible light (λ_{max}=560 nm). If excited, form B will emit a quantum of red light or undergo photocyclization and revert to form A. The quantum yield of fluorescence is significantly higher than the quantum yield of photoclosure. However, SP has one disadvantage which is occurrence of the back thermal reaction B $\rightarrow$ A; nonetheless the decay kinetics of the open form of the SP dispersed in the polymer deviate considerably from first order behavior and exhibits a lifetime on the order of 1 hr[14].
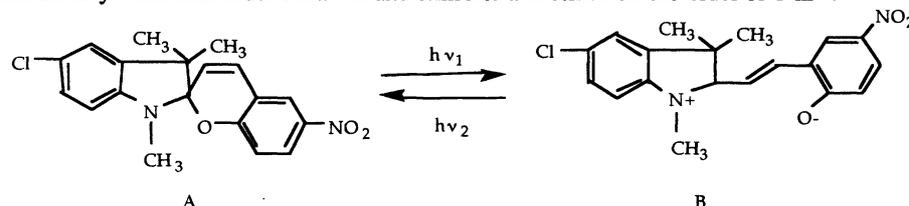
5-Cloro-1',3'-dihydro-1',3',3'-trimethyl-6nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole]
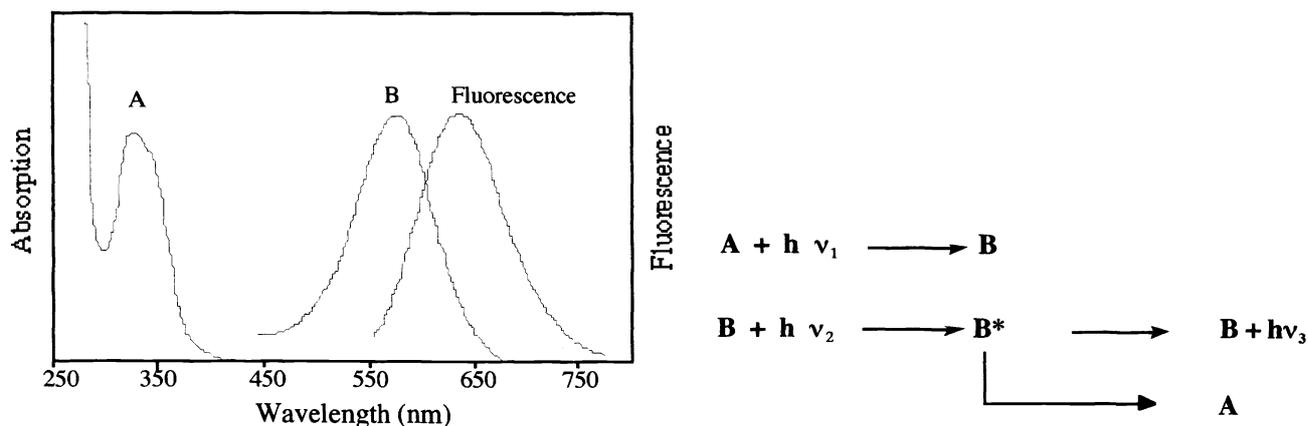
Fig. 5: Absorption and emission spectra of SP closed (A) and open (B) forms.

Fig. 6 demonstrates the use of the proposed photochromic system for characterizing microbeam propagation within a sample. Immediately prior to exposing the film to the microbeam, the film is "charged" by illumination with near UV or blue light to convert the spiropyran in the polymer to the open (B) form. We subsequently irradiate the "charged" polymer with visible light from a laser microbeam (either 488 nm CW or 532 nm pulsed microbeam) which leads to either fluorescent

emission or to photoclosure of the open form in the exposed film volume. We then image the film using the confocal laser microscope (Zeiss LSM 410) which can rapidly produce line scans in XZ or XY planes without inducing significant thermal cyclization. This results in both axial and transverse views of the photochromic polymer and provides the microbeam dimensions at any location within the polymer sample. Since photocyclization is linear with absorbed energy density, the spot diameter can be accurately determined for any focusing condition. Image analysis of both XY and XZ views revealed the sub-micron dimensions of the 532 nm microbeam and its Gaussian intensity profile.
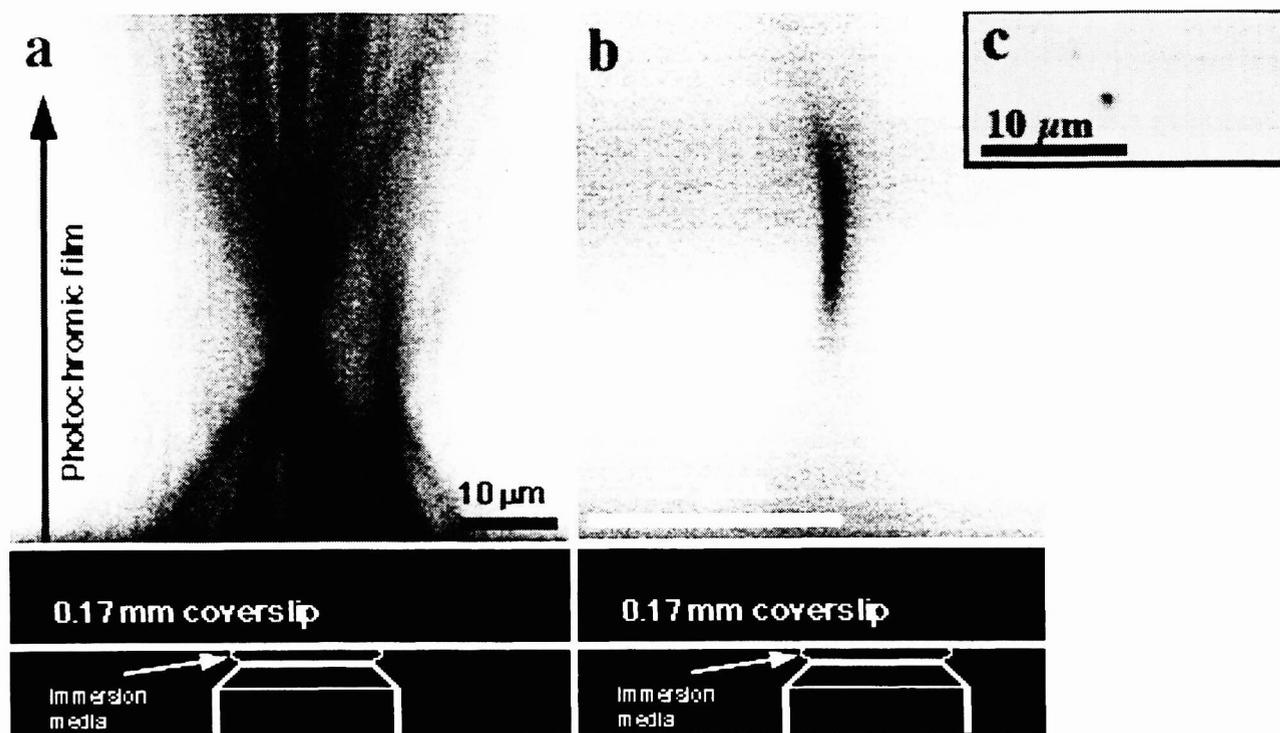
Fig. 6: Microbeam profiles of irradiation sites as measured by the photoclosure of merocyanine form and confocal fluorescence imaging of a photochromic polymer film: (a) XZ confocal scan of two 488 nm CW exposures with different exposure times and focal plane locations, (b), (c) confocal scans of pulsed 532 nm exposure with multiple pulses, (b) XZ scan, (c) XY scan.

When the photochromic film is exposed to 532 nm irradiation at higher pulse energies which are comparable to those used in the optoporation experiments, the resulting confocal image had an appearance quite different from that shown in Fig. 6. Fig. 7 is a confocal XZ section of photochromic film after such an exposure at two different sites. These exposures are quite odd in that while there is generally an inactivation of fluorescence along the microbeam propagation path, the fluorescence is not fully inactivated in the focal region and indicates that significant amounts of SP remain in the open (B) form at that location (Fig. 7). Exposure using 1064 nm light at similar pulse energies, as shown in Figure 8, often resulted in material removal and a dimming of the fluorescent emission in the regions proximal to the ablation crater. Both these observations are unexpected and may be evidence of significant two photon absorption at these higher pulse energies/irradiances.

In the case of 532 nm irradiation, single photon absorption by the open (B) form of SP should lead to the generation of fluorescence or the conversion of the SP molecule to the closed (A) and non-fluorescent form. If these were the only reaction pathways present, we would expect a darkening of the film in the irradiated volume. However, if the irradiances were sufficient to allow for two photon absorption, the closed form of SP which absorbs in the UV can also be promoted to the singlet state through the 'simultaneous' absorption of two 532 nm photons. This would convert the SP back to the open (B) form which would fluoresce during scanning of the confocal image. In the case of 1064 nm irradiation, we expect the photochromic film to be unable to record the propagation characteristics of the microbeam since the open form of SP does not absorb at this wavelength (see Fig. 6). The fact that conversion of the SP back to the closed (A) is observed only in the region of microbeam irradiation seems to indicate that the open form of the SP molecule was promoted to the singlet state

through 'simultaneous' absorption of two 1064 nm photons which would have the capacity to photoclose the SP molecule. Another possibility is that the irradiances were sufficient enough to induce optical breeakdown and the initiation of a plasma at the irradiation site. The visible radiative emission of the plasma could be responsible for the photoclosure of the SP.
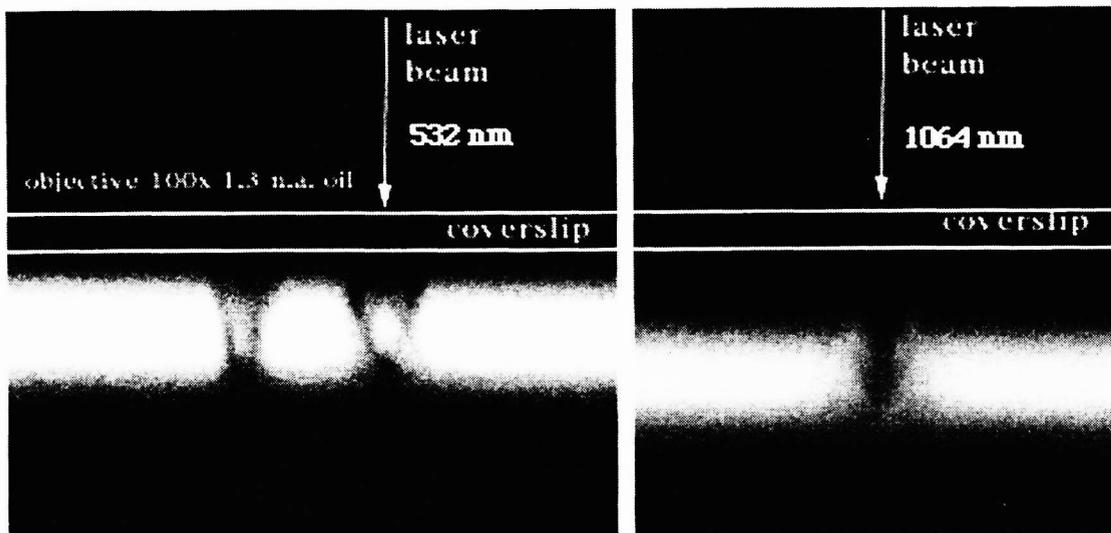

Fig. 7: Microbeam profiles of pulsed-laser irradiation sites; near- or above ablation threshold.

We also produced confocal image and three-dimensional reconstructions of an ablated photochromic film sample as shown in Fig. 8. The mechanical defect produced by such an event is remarkably similar to those produced by laser-induced photodisruption used to dissect membranes in ocular surgery[15]. It has been established that the photodisruptive effect is due to the collapse of a cavitation bubble produced as a result of plasma formation and expansion generated due to the high irradiances achieved in the focal region of the laser beam. A comparison of the laser parameters used in this microbeam application with those used for ocular photodisruption reveals that the irradiances achieved in the focal volume are comparable and thus sufficient for the formation of a plasma. It is also interesting to note that the spatial area over which film deformation is observed correlates roughly with the region in which cell viability is lost in the optoporation experiments (Fig. 1b). Similar images were obtained for ablation of the photochromic film using 532 nm irradiation as well.
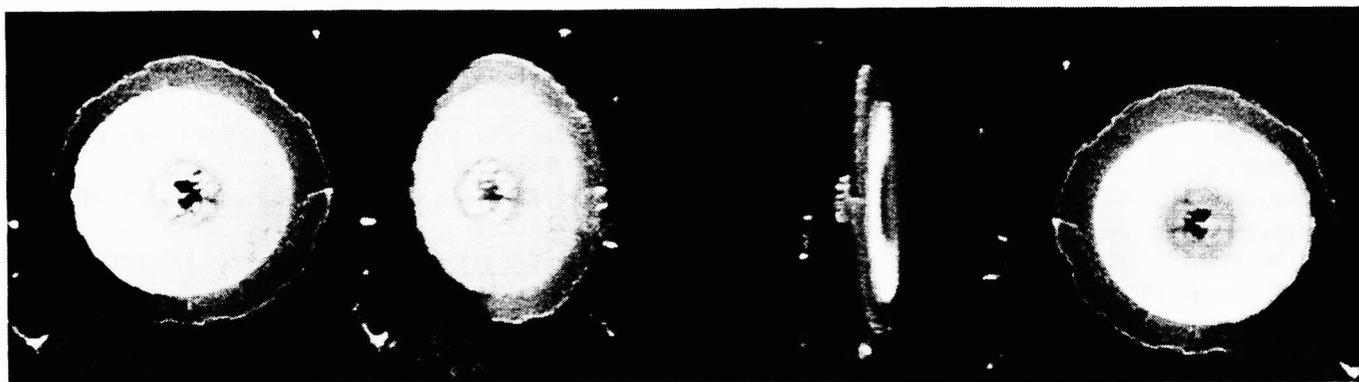

Fig. 8: 3-D reconstruction of the mechanical defect produced by 1064 nm ablation of the photochromic polymer film. Images from four viewing angles (0, 45, 90 and 180) are shown

5. CONCLUSIONS

We have presented results which demonstrate the use of pulsed laser microbeams to achieve molecular transport of membrane impermeable molecules into cells. The technique can be implemented such that molecular transport can be achieved in a number of cells simultaneously or specifically to a single cell. By using photochromic films we are also able to gain insight into the dosimetry of the microbeam within our samples and may also serve as a useful probe for non-linear (i.e., multi-photon or ablative) effects. We hope that the obtained information will facilitate specific experimental and theoretical approaches to gain a mechanistic understanding of processes leading to membrane permeabilization and intracellular probe delivery. Such an understanding is vital for the continued success and optimization of existing laser microirradiation techniques and is essential to spur the development of the next generation of laser-based cellular microirradiation tools.

6. ACKNOWLEDGMENTS

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