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Authors

Soughayer, JS
Krasieva, T
Jacobson, SC
[et al.](#)

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Technical Notes

Characterization of Cellular Optoporation with Distance

Joseph S. Soughayer,[†] Tatiana Krasieva,[‡] Stephen C. Jacobson,[§] J. Michael Ramsey,[§] Bruce J. Tromberg,[‡] and Nancy L. Allbritton^{*†}

Department of Physiology and Biophysics and Beckman Laser Institute, University of California, Irvine, California 92697-4560, and Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, Tennessee 37831

We have developed and characterized cellular optoporation with visible wavelengths of light using standard uncoated glass cover slips as the absorptive media. A frequency-doubled Nd:YAG laser pulse was focused at the interface of the glass surface and aqueous buffer, creating a stress wave and transiently permeabilizing nearby cells. Following optoporation of adherent cells, three spatial zones were present which were distinguished by the viability of the cells and the loading efficiency (or number of extracellular molecules loaded). The loading efficiency also depended on the concentration of the extracellular molecules and the molecular weight of the molecules. In the zone farthest from the laser beam (>60 μm under these conditions), nearly all cells were both successfully loaded and viable. To illustrate the wider applicability of this optoporation method, cells were loaded with a substrate for protein kinase C and the cellular contents then analyzed by capillary electrophoresis. In contrast to peptides loaded by microinjection, optoporated peptide showed little proteolytic degradation, suggesting that the cells were minimally perturbed. Also demonstrating the potential for future work, cells were optoporated and loaded with a fluorophore in the enclosed channels of microfluidic devices.

Many biologic and biochemical techniques require the introduction of hydrophilic or membrane-impermeant molecules into cells. A large number of methods have been developed, but typically the plasma membrane is transiently permeabilized and then the exogenous molecule is introduced into the cytoplasm. One method is microinjection, which loads cells with relatively high concentrations of molecules. However, the extent of cellular damage can be unpredictable and is frequently high.^{1–3} Addition-

ally, the speed at which cells can be loaded is very low and the experimental geometry must be able to tolerate the introduction of the large glass microinjection pipet. Lipid-based delivery systems (liposomes) often yield high survival rates, but the percentage of cells loaded is frequently quite low and highly variable among different cell types.^{1,2} Electroporation has been shown to be an effective method for delivery of DNA and RNA into cells.^{3–6} However, the poor loading efficiency of electroporation has largely limited its use to genetic material, for which the cell can synthesize much larger quantities of the protein product (compared to the introduced quantity of RNA/DNA). In addition, the production of gas at the electrode surface can complicate the use of electroporation. Laser-based methods have been shown to permeabilize cells to exogenous molecules but have not become commonly used techniques.^{7–15} Potential reasons are the requirement for special absorptive coatings or membranes with some of the laser-based methods, the frequent use of UV or IR wavelengths, and the difficulty in combining the complicated illumination geometry with subsequent cellular analysis for some of the strategies.^{7,8,10–12,15–17} The development and characterization of laser-based methods utilizing visible wavelengths and simple optical geometries without the need for specialized coatings may

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* Corresponding author. Phone: 949-824-7784. Fax: 949-824-8540. E-mail: nllabri@uci.edu.

[†] Department of Physiology and Biophysics, University of California.

[‡] Beckman Laser Institute, University of California.

[§] Oak Ridge National Laboratory.

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increase the utility of laser-based cell loading.

Optoinjection and optoporation are laser-based methods capable of loading exogenous molecules into selected cells.^{7–14} With both methods, a pulsed laser beam is used to transiently permeabilize cells. Mechanisms by which laser irradiation (photothermolysis) and laser-generated stress transients perturb cell membranes are still under investigation.^{14,16–19} For optoinjection, a single cell is directly irradiated by the focused laser beam and this cell alone is loaded with exogenous molecules.^{7–13,20} Under appropriate conditions, cells can be loaded with little visible damage and with high survival rates.^{13,20} However, the throughput is low, the concentrations loaded are limited, and the cell must be directly aligned with the laser beam. For optoporation, the laser beam does not usually interact with the cell but rather interacts with an absorptive medium, frequently polyimide.^{13–16,18} A mechanical transient or stress wave is then produced as a result of optical breakdown, ablation, or rapid heating of the absorbing medium.^{14,21} This shock wave or mechanical transient interacts with nearby cells, producing temporary alterations in the plasma membrane. The exact nature by which shock waves transiently permeabilize membranes remains unknown but is an active area of investigation.^{14,16,18,21} Advantages of optoporation over optoinjection are that more than one cell can be loaded simultaneously and that the cells and laser beam do not need to interact directly or be precisely coaligned. Recently Krasieva et al. reported the successful optoporation of cells with a visible wavelength of light and without specialized absorptive coatings.¹³ However, the cellular mortality rate was as high as 50% making the technique of limited utility for many biologic and biochemical applications.¹³

In this work, we characterize the parameters affecting the cellular delivery of molecules by optoporation with a visible wavelength and without specialized absorbing surfaces. Molecular size and concentration and distance from the focal point of the pulsed laser source are examined. A recently described capillary electrophoresis-based tool, the laser-micropipet, is used to quantify cell loading by optoporation.²² Most importantly for biologic/biochemical experiments, a region of cells is identified in which the cells are both loaded with exogenous molecules and nearly 100% viable. Additionally, optoporation has the potential to load cells that are located in tightly confined spaces or sealed systems such as microfluidic devices.²³ To further illustrate the utility of this optoporation method, we also demonstrate the loading of cells within the channels of a microfluidic device.

EXPERIMENTAL SECTION

Reagents. All fluorescent reagents were obtained from Molecular Probes (Eugene, OR). The PKC peptide substrate (RFARKG.SLRQKNV) is derived from the pseudosubstrate region of PKC²⁴ and was obtained from the Beckman Peptide and Nucleic

Acid Facility at Stanford University. The C-terminus was amidated, and the peptide was labeled on the N-terminus with fluorescein (abbreviated F-PKC) as previously described.²⁵ All other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Conjugation of Texas Red to Glycine. Texas Red succinimidyl ester was reacted overnight (21 °C) with a 20-fold molar excess of glycine in an aqueous buffer containing HEPES (50 mM, pH 8.0) and dimethyl formamide (15%). The reaction product was analyzed by HPLC on a reverse-phase C18 column (Altech, Deerfield, IL) with a gradient of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. No free Texas Red or Texas Red succinimidyl ester was observable.

Optoporation of Cells. The microscopy system used for optoporation cells was similar to that described previously.^{13,22} Cells were cultured in chambers composed of a Teflon O-ring attached to a no. 1 glass cover slip. The cell chambers were mounted on the stage of an inverted fluorescence microscope (Nikon). The cells were placed in buffer A (135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM MgCl₂, 2 mM CaCl₂, pH 7.4) containing Texas Red–dextran, Texas Red–glycine, Oregon Green, or F-PKC and maintained at 37 °C with an objective heater (Bioprotechs, Butler, PA). A frequency-doubled Q-switched Nd:YAG laser (New Wave, Sunnyvale, CA) was used to generate a single laser pulse (10 μJ, 5 ns pulse width, 532 nm) which was directed into the microscope. The pulse was focused (~0.3–0.4 μm at its waist) with a microscope objective (100×, 1.3 n.a., Olympus) within the cover slip adjacent to its interface with the buffer. After delivery of the single laser pulse, cells were incubated for 3 min. The cells were washed by flowing buffer A containing 10 mM glucose (3–5 mL/min, 37 °C) over them for 20 min. Oregon Green diacetate (10 μM) in buffer A was then added for 1 min to the cells. The cells were washed and placed in buffer A containing 10 mM glucose. In some instances, the optoporation cells were placed in Dulbecco's modified eagle media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) and incubated overnight at 37 °C in 5% CO₂, after which they were examined for viability.

Microinjection of Cells. Cells were microinjected using a commercial microinjection system (Transjector 5246, Eppendorf, Westbury, NY). Injection pipets were commercially prepared (Eppendorf). The concentration of F-PKC was 50 μM in the pipet.

Fluorescence Microscopy. Cells were illuminated with light centered around 545 nm (HQ545/30 filter, Chroma Technology, Brattleboro, VT) for Texas Red excitation or 485 nm (485DF22 filter, Omega Optical, Brattleboro, VT) for Oregon Green excitation. Light from a single cell was spatially and spectrally filtered (an HQ610/75 filter (Chroma Technology) for Texas Red emission or a D530/30 filter (Omega Optical) for Oregon Green emission) and collected with a photomultiplier tube (PMT). The current from the PMT was processed and digitized as previously described and displayed with custom software written in Viewdac (Keithley-Metrabyte, Taunton, MA).^{22,26} The background fluorescence of nonoptoporation cells (cells greater than 500 μm from the laser pulse) was subtracted from fluorescence values recorded for

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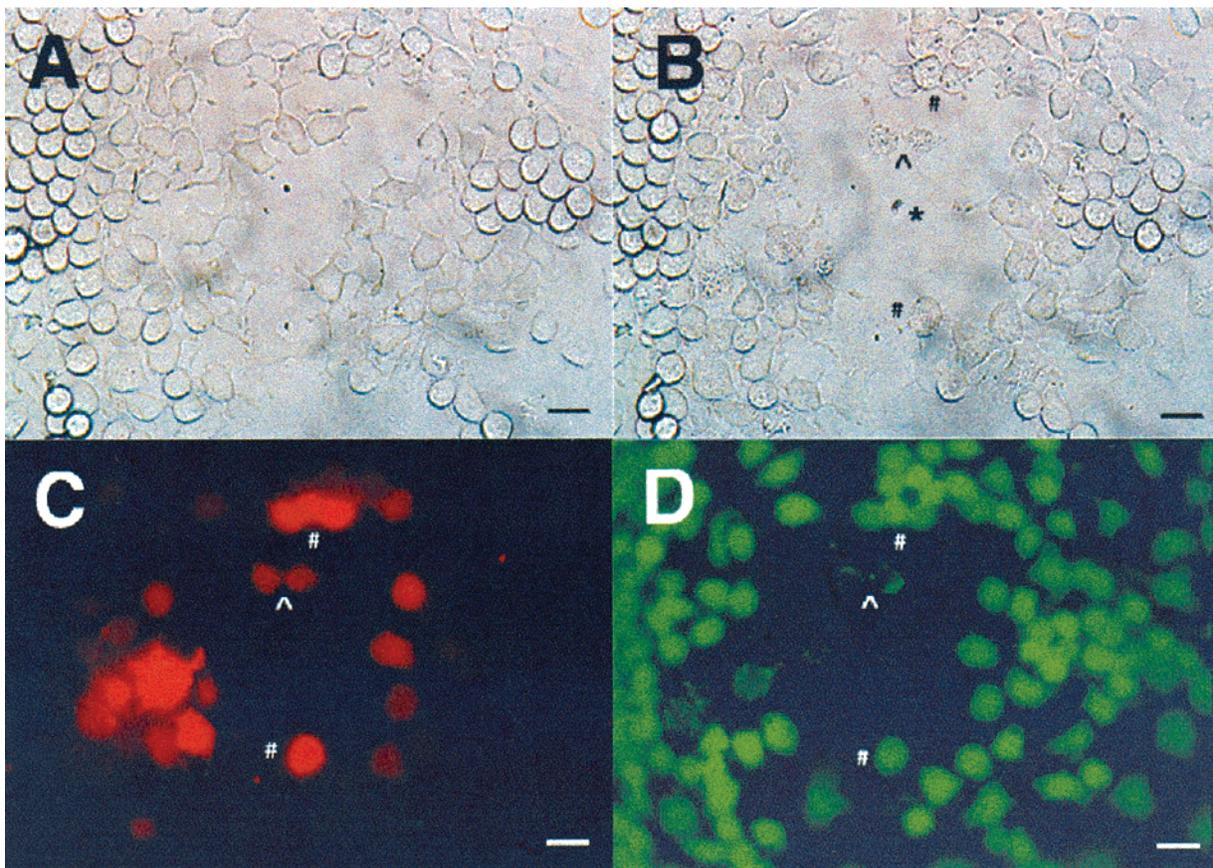


Figure 1. Optoporation of cells. Shown are transmitted light (A, B) and fluorescence (C, D) images of cells before (A) and after (B–D) optoporation. An * marks the divot formed in the cover slip by the laser beam (B). The fluorescence of Texas Red and Oregon Green is displayed in parts C and D, respectively. Two cells (marked by a ^) are loaded with Texas Red but are nonviable since they possess minimal Oregon Green and have a very altered morphology compared to that of other cells (see part B). Several cells that were loaded with Texas Red and that were viable on the basis of their Oregon Green content and morphology are marked with a #. The scale bar is 20 μm .

optoporated cells. An analog camera (Nikon) was used for the photography of cells and microfluidic devices.

Capillary Electrophoresis. Capillary electrophoresis and fluorescence detection were performed as described previously with the following exceptions.²² The capillary length was 65 cm, with an optical window created 50 cm from the inlet. The outlet reservoir was held at a positive potential of 8–11 kV. Samples were loaded into the capillary by gravitational fluid flow, and the volume loaded was calculated from Poiseuille's equation.²⁷ Typically, buffer solutions were loaded into the capillary by placing the capillary inlet into the sample solution and elevating the level of the inlet solution 3 cm above the level of the outlet solution for 10 s. The contents of cells were loaded into capillaries using the laser-micropipet method described previously.²² Analytes were detected by laser-induced fluorescence (LIF) as described previously.²²

Optoporation on Microfluidic Devices. Glass microfluidic devices were fabricated as described previously.²³ These devices each possessed two rectangular channels (12 μm depth, 50 μm width) in a "cross" shape with channel lengths of 2.1 and 1.3 cm from inlet to outlet. The shorter channel intersected the longer channel 0.7 cm from its inlet. Cells were loaded into the channels and incubated overnight at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere. The

channels were rinsed with buffer A and then loaded by pressurized fluid flow with buffer A containing Texas Red–dextran. Cells were optoporated as described above with a single laser pulse delivered to the channel lumen and focused at the glass/water interface. After a 3 min incubation, the channels were filled with buffer A containing 10 mM glucose.

RESULTS AND DISCUSSION

Optoporation of Cells. Rat basophilic leukemia (RBL) cells were placed in a solution of Texas Red-conjugated dextran (3 kD) and optoporated with a single pulse from a Nd:YAG laser (532 nm). The beam was focused at the cover slip/buffer interface. After optoporation, the cells were washed with buffer and living cells were identified by incubation with Oregon Green diacetate (a nonfluorescent, membrane-permeant molecule). Only viable cells metabolize the diacetate to its fluorescent analogue, Oregon Green, and retain it intracellularly.²⁸ Three zones of cells were visible following optoporation (Figure 1). In the first region, less than 30 μm from the laser beam, virtually all of the cells died. Many were physically detached from the cover slip by the shock wave, and the remainder failed to retain the Oregon Green. Very few cells in this zone were loaded with Texas Red. In the second

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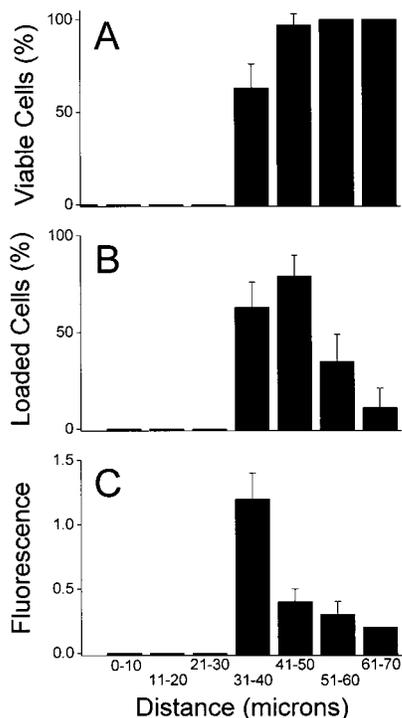


Figure 2. Distance dependence of cell viability and loading. Cells were optoprotected in the presence of Texas Red-conjugated dextran (3 kD, 167 μ M). Shown are the percentage of viable cells (A), the percentage of Texas Red-loaded cells (B), and the measured fluorescence of those cells that were visibly loaded (C) at varying distances from the laser beam. The percentages of viable and Texas Red-loaded cells were calculated on the basis of the total number of cells present prior to optoprotection. Each data point is the average of 20 or more cells from a total of five experiments. The error bars represent a single standard deviation of the data points.

region, \sim 31–60 μ m from the laser beam, most of the cells contained Texas Red and many were also able to retain the Oregon Green. This zone possessed a mixture of live cells successfully loaded with the Texas Red-conjugated molecule and dead cells. In the third region, $>$ 60 μ m from the laser beam, few cells were visibly loaded with Texas Red; however, virtually all of the cells were viable. These data suggested that there may exist an optimal distance between the laser beam and the cells to achieve a high degree of loading and a good survival rate.

Characterization of the Three Zones of Optoprotection. To further characterize the different cellular regions occurring after optoprotection, the percentage of living cells, the percentage of loaded cells, and the amount of loaded fluorophore were measured at varying distances from the laser pulse (Figure 2). Loaded cells were defined as those with visible Texas Red fluorescence, while living cells were those retaining Oregon Green. The amount of loaded fluorophore was measured only in viable cells and was the relative fluorescence quantified with a photomultiplier tube. Within the first region, all cells died. In the second zone, 30–80% of the cells were visibly loaded with a 60–90% survival rate. Few cells greater than 60 μ m from the focused beam possessed visible Texas Red fluorescence, but all were viable. Cells exposed to the Texas Red-conjugated dextran but not optoprotected displayed no fluorescence with the spectral properties of Texas Red. In some instances, the cells were examined for viability 24 h after optoprotection. No cells were present in the first region less than

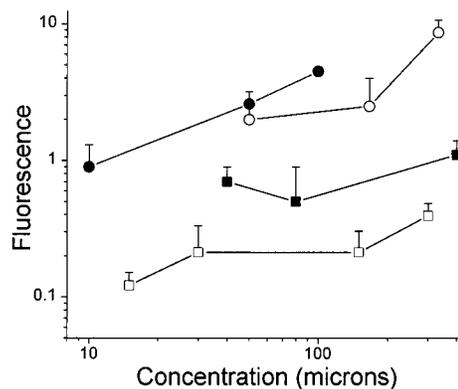


Figure 3. Influence of molecular weight on loading efficiency. Cells were optoprotected in the presence of varying concentrations of Texas Red conjugated to glycine (800 D (closed circles)) or dextran (3 kD (open circles), 10 kD (closed squares), or 40 kD (open squares)). Shown are the measured fluorescences of viable cells at distances of 40–50 μ m from the laser beam. Each data point is the average of 20 or more cells from a total of five experiments. The error bars represent single standard deviations of the data points.

30 μ m from the laser beam. In the second and third regions (31–60 and $>$ 60 μ m, respectively, from the laser beam), the number of cells present was substantially greater than that present during optoprotection. This increase in cell number is due to division of the surviving cells. While none of the cells in the third zone contained a visible fluorophore, most of the cells in the second zone contained Texas Red, although at a lower concentration than was present immediately following optoprotection. The decreased concentration is most likely due to the dilution of the fluorophore during cell division and subsequent growth in cell size.

The delivery of the fluorophore to the cells was inversely correlated with the distance of the cells from the optoprotecting laser pulse. At distances closest to the pulse, cells were more likely to be damaged irreversibly. However, there is a region in which cells load substantial amounts of fluorophore but receive only minimal damage. When the distance between the cells of interest and the laser beam is optimized, optoprotection can be performed with good loading efficiency and high cellular survival rates. The exact location of this optimal zone will most likely depend on multiple factors, including the cell type and density, the energy of the laser pulse, and the light-absorbing molecules in the glass substrate and aqueous buffer.

Influence of Molecular Weight on Loading Efficiency. To determine whether molecular size influenced the efficiency of loading, cells were optoprotected in the presence of Texas Red conjugated to glycine (800 D) or dextran (3, 10, or 40 kD). In all cases, three zones of cells identical to those described above were present. The sizes of the zones and their distances from the laser pulse were similar for the different molecular weight molecules. At all extracellular concentrations, the amount of Texas Red–glycine or Texas Red–dextran loaded into cells was inversely related to its molecular weight (Figure 3). When the concentration of Texas Red–glycine or Texas Red–dextran was plotted against the measured cellular fluorescence, the cellular fluorescence was adjusted to take into account the number of Texas Red molecules per dextran or glycine molecule. The degree of substitution ranged from 0.3 to 3.3 Texas Red molecules per carrier molecule. At an extracellular concentration of \sim 50 μ M, a 10-fold greater quantity

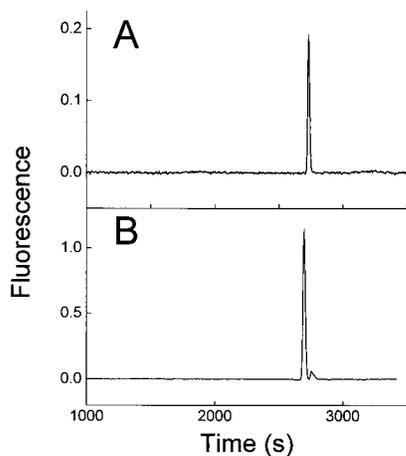


Figure 4. Electropherograms of the contents of a cell 75 μm from the laser beam. (A) Oregon Green dissolved in buffer A was loaded (5 amol) into a capillary, electrophoresed, and detected by LIF. (B) A cell was optoporated in the presence of Oregon Green (100 μM). The cell's contents were then loaded into a capillary and electrophoresed.

of glycine compared to dextran (40 kD) was loaded into the cells. In addition to optoporation, a variety of other laser-based techniques employing mechanical transients (or waves) can be used to load cells with exogenous molecules.^{14,15,17} Several different mechanisms have been proposed to account for the increased cellular permeability following interaction with the mechanical transient. These include the forcible injection of extracellular solution into the cells, the formation of plasma membrane pores through which molecules can diffuse, and the involvement of plasma membrane proteins such as water channels.^{13,14,16,18} The finding that the efficiency of loading depends on the molecular weight of the introduced molecule suggests that passive diffusion of the molecules through plasma membrane pores (of variable size) may play a role in optoporation-induced increases in plasma membrane permeability.

Laser-Micropipet Analysis of Optoporated Cells. Most cells greater than $\sim 70 \mu\text{m}$ from the optoporating laser beam did not contain visible Texas Red fluorescence. However, it is possible that these cells did load with Texas Red but the concentration was below the detection limits of conventional fluorescence microscopy. To determine whether these distant cells were loaded with the fluorophore, cells were optoporated in the presence of Oregon Green (100 μM , 368 D molecular mass). Cells 75 μm from the laser pulse were then lysed and loaded into a capillary using the laser-micropipet.²² The cellular contents were separated by electrophoresis through the capillary and detected by laser-induced fluorescence. For each of the nine cells examined, a single peak with a migration time similar to that of Oregon Green was present on the electropherogram (Figure 4). When cells were exposed to Oregon Green but not optoporated, no peaks were present on the electropherogram (data not shown). Thus, there is a region at a distance from the laser beam with virtually all cells loaded and alive. Under the appropriate conditions, optoporation can be performed with 100% cell viability and with 100% of the cells loaded.

The laser-micropipet technique loads close to 100% of the cell's contents into the capillary.²² Therefore, to estimate the concentration of the intracellular fluorophore in the cells 70 μm from the

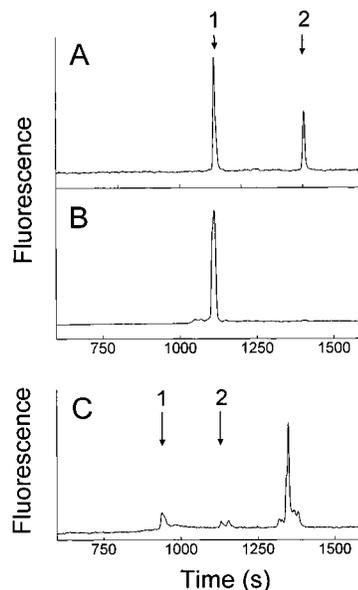


Figure 5. Electropherograms of F-PKC standards and the contents of cells loaded with F-PKC. (A) Nonphosphorylated (peak 1) and phosphorylated (peak 2) F-PKC dissolved in buffer A were loaded (60 amol total peptide) into a capillary, electrophoresed, and detected by LIF. (B) A cell was optoporated in the presence of F-PKC (30 μM), and its contents were loaded into a capillary and electrophoresed. The electropherograms in parts A and B were obtained on the same day under identical electrophoretic conditions. (C) A cell was microinjected with F-PKC, and its contents were electrophoresed. The arrows labeled 1 and 2 mark the migration times of nonphosphorylated and phosphorylated F-PKC, respectively, under identical electrophoretic conditions. Multiple peaks are present on the electropherogram. The major peak is most likely a proteolytic product of F-PKC.

laser beam, the areas of the Oregon Green peaks on the electropherograms were compared to those of electrophoresed standards.²² The quantity of Oregon Green loaded into the cells was variable (3 ± 6 amol ($n = 9$)). For a typical cell volume of 1 pL, this represents an average concentration of 3 μM , 3% of the extracellular Oregon Green concentration. This loading efficiency is similar to that achieved by electroporation and microinjection.^{1,2,4}

Optoporation of a Kinase Substrate into Cells. To demonstrate the utility of the technique, cells were optoporated and loaded with F-PKC, a fluorescent substrate for protein kinase C (PKC).²⁴ This substrate has been used to monitor the intracellular activation of PKC.²⁵ The contents of single cells (75 μm from the optoporation beam) were then analyzed by capillary electrophoresis as described above. For each cell ($n = 6$), a single major peak with a migration time similar to that of F-PKC was present on the electropherogram (Figure 5A,B). In contrast to F-PKC loaded into cells by microinjection, F-PKC loaded by optoporation was not substantially proteolyzed (Figure 5B,C). When cells were exposed to a pharmacologic activator of PKC, phorbol myristic acid (PMA), and then analyzed by capillary electrophoresis, a single major peak was again present on each electropherogram ($n = 2$). However, the migration time now corresponded to that of the phosphorylated form of F-PKC (data not shown). Thus the cells were still capable of substrate phosphorylation following optoporation. These data suggested that cells can be optoporated and loaded with exogenous molecules with minimal cellular perturbation.

Optoporation on Glass Microfluidic Devices. To test the feasibility of optoporating within tightly confined spaces, cells were

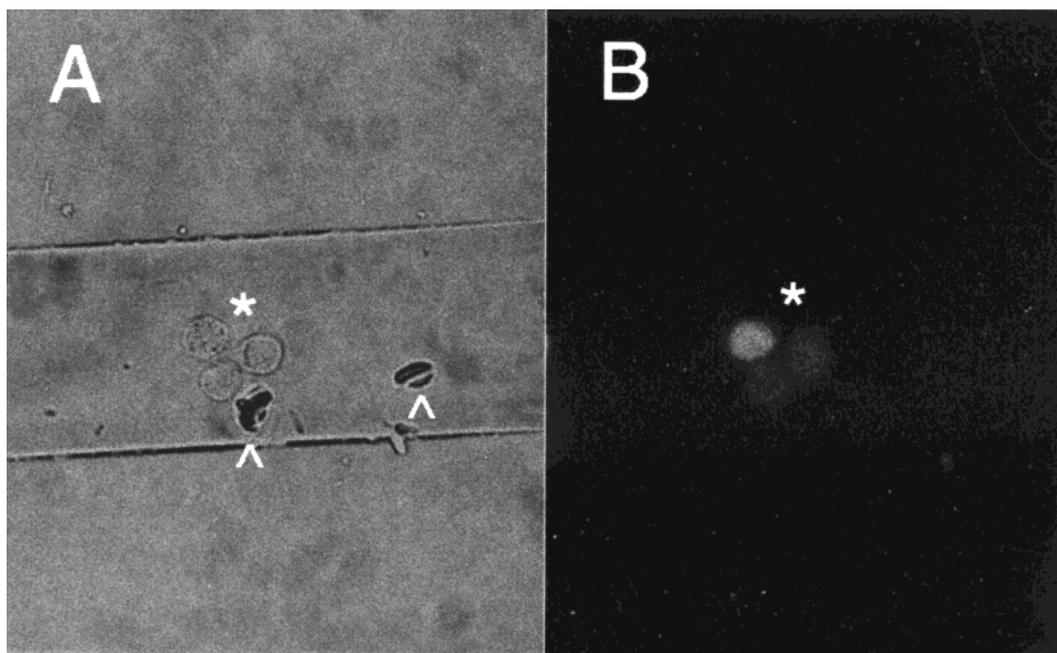


Figure 6. Optoporation on a microfluidic device. Cells placed in the channel of a glass microfluidic device were optoporated in the presence of Texas Red-conjugated dextran (3 kD, 300 μ M). (A) Shown is a transmitted light image of a portion of the channel and walls after optoporation. Within the lumen are three viable cells (marked by an *) and two nonviable cells (marked by a ^). (B) Shown is a fluorescence image of the same channel region in part A. The three viable cells are fluorescent and loaded with Texas Red. The two dead cells exhibit no fluorescence.

cultured within a channel (50 μ m width) on a glass microfluidic device. Texas Red–dextran (3 kD) was loaded into the channel, and a single laser pulse focused at the glass/water interface was delivered to the channel lumen. Most cells within a distance of \sim 50 μ m from the laser beam appeared irreversibly damaged. At greater distances, a mixture of dead cells and successfully loaded cells was present (Figure 6). Many cells as far as \sim 200 μ m from the beam possessed visible Texas Red fluorescence. These data suggested that the effect of the shock wave on the cells in microchannels was intensified compared to that on cells in a nonenclosed space. Since the acoustic impedances for water and glass differ by nearly an order of magnitude, the walls of the channels most likely reflect the shock wave, focusing the acoustic energy along the channel path.^{29,30} On the microfluidic devices, the optimal distance between the laser beam and the cells will depend on the microchannel geometry and the composition of the walls, as well as the cell type, the cell density, and the energy of the laser pulse.

CONCLUSIONS

Introduction of membrane-impermeant molecules into cells is required for many chemical and biological assays. Optoporation offers the advantage of being remotely operated, i.e., allowing

permeabilization of cells without direct manipulation. This feature is desirable for some applications, such as the loading of molecules into cells contained in enclosed glass microfluidic devices. To make optoporation more practical for the general scientific community, we have characterized some of the parameters that define optimal cell loading and viability. Specifically, we have demonstrated that at a certain distance from the beam (in this case \sim 70 μ m) nearly all cells were loaded with excellent survival rates and the amount of loaded molecule was similar to that achievable with microinjection. Furthermore, cellular damage from permeabilization was substantially less with optoporation under these conditions than with microinjection. At shorter distances from the laser beam ($<$ 60 μ m), higher concentrations of exogenous molecules could be delivered to cells but at a cost of reduced viability. Finally, we showed that optoporation is attainable on glass microfluidic devices, opening the door for further work in this field.

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