*J. Biochem.* **134**, 219–224 (2003)
DOI: 10.1093/jb/mvg132

# Elevation of Plasma Membrane Permeability on Laser Irradiation of Extracellular Latex Particles

**Yukihiro Umebayashi, Yuuichi Miyamoto, Masayoshi Wakita, Akiko Kobayashi and Tsuyoshi Nishisaka***

*School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Tatsunokuchi, Nomi-gun, Ishikawa 923-1292*

Received February 10, 2003; accepted May 14, 2003

**In this report, we describe a laser-latex combination system that enables membrane-impermeable molecules to penetrate cell membranes. Laser light (Q-switched Nd:YAG laser, 532.5 nm) was used to irradiate a mixture of commercial latex particles (blue dyed, 1 μm in diameter) and mouse fibrosarcoma (Meth-A) cells. After irradiation, membrane permeability was evaluated by flow cytometric assaying using propidium iodide (PI) and fluorescein diacetate (FDA). The proportion of permeabilized-resealed cells was affected by changes in the light intensity (~780 mW/cm$^2$), the irradiation time (~240 s), and/or the particle concentration (~10$^9$ particles/ml). The permeability persisted up to 20 min after light irradiation. Near the sites of individual particles, the permeability of the cell membrane is modified, probably due to localized temperature changes. These results suggest that this laser-induced permeabilization strategy constitutes a new means of delivering exogenous materials into living cells.**

**Key words: flow cytometry, laser, latex particles, membrane permeabilization, propidium iodide.**

Abbreviations: FBS, fetal bovine serum; FDA, fluorescein diacetate; LSW, laser-generated stress waves; Nd:YAG laser, neodymium:ytrium aluminum garnet laser; PBS, phosphate-buffered saline; PI, propidium iodide.

The introduction of exogenous materials into the cytoplasm of living cells is often useful in medicine, cellular biology and molecular biology. A number of methods involving chemical, viral or physical approaches has been developed to transfer DNA and other macromolecules into mammalian cells, although each has limitations (*1–4*).

Some investigators have reported laser-based systems for obtaining membrane permeability (*5–8*). Kurata *et al.* demonstrated that foreign DNA can be introduced into a target cell using a laser microbeam (*5*). According to them, when the laser beam was precisely focused on the cell membrane, the membrane at the site of the beam impact was modified through a local thermal effect. Since such "microbeam pricking" can be performed through the walls of culture dishes, it reduces the possibility of contamination accompanying many other *in vitro* methods. However, this technique has several shortcomings, including the impossibility of injecting molecules into non-adherent cells; the number of cells that can be injected is also a limiting factor (*5*, *6*, *8*).

The aim of this study is to develop a new method for permeabilizing cell membranes using visible laser light that is not focused on the cell surface. It was expected that the goal of membrane permeabilization could be achieved by the addition of prestained particles combined with exposure of the cell suspension to visible light. Namely, when light enters medium containing both dyed particles and cells, the light is absorbed by the particles, producing local photothermal and photomechanical stresses (*e.g.* temperature increases, cavitation, and pressure waves) (*9–12*). The localized stresses alter the plasma membrane permeability of the cells without affecting their viability. Because these stresses dissipate within a few tens of nanoseconds, the damage is only very local. And, since this method does not require focusing light on the cell surface, it is possible to treat many cells at once.

Latex particles have been used in numerous applications in the biomedical and biotechnology fields, such as for phagocytosis assaying (*13–15*), immunoagglutination tests (*16*, *17*), biological cell labeling (*18–20*), and drug-delivery systems (*21*, *22*). The polystyrene latex adopted in our investigation sticks non-specifically to many surfaces and molecules because of its hydrophobic surface (*18*, *23*).

In this study, we examined the effects of various protocol parameters such as power density, laser light irradiation time, latex concentration, and incubation time after light irradiation on the percentage of membrane-permeabilized cells. The degree of membrane permeabilization was determined by flow cytometry based on propidium iodide (PI) uptake. Exposure of the cells to fluorescein diacetate (FDA) confirmed plasma membrane resealing.

This study was partly supported by a Grant-in-Aid for Exploratory Research (No. 12878167) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

*To whom correspondence should be addressed. Tel: +81-761-51-1685, Fax: +81-761-51-1685, E-mail: saka@jaist.ac.jp

 

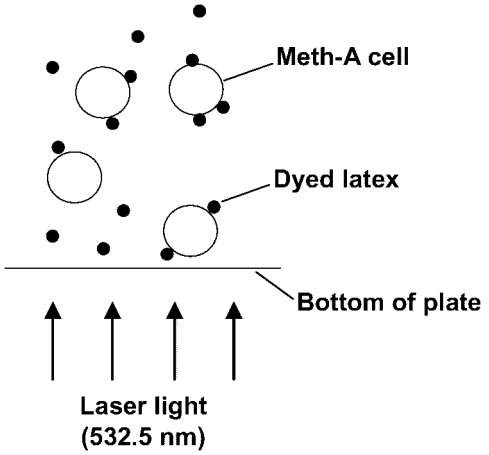


Fig. 1. **Schematic representation of the experimental setup.** Laser light was directed on the cell-latex mixture through the bottom of the 96-well plates.

## MATERIALS AND METHODS

*Materials*—Latex particles (blue dyed, made of styrene, 1 μm in diameter) were purchased from Polysciences (PA, USA). PI was from Molecular Probes (OR, USA). FDA was from Nacalai Tesque (Kyoto). RPMI 1640 medium and phosphate-buffered saline (PBS) were from Invitrogen (Tokyo,). Fetal bovine serum (FBS) was from Nipro (Osaka). Penicillin and streptomycin were from Meiji Seika (Tokyo).

*Cell Preparation*—Murine fibrosarcoma (Meth-A) cells were obtained from the Riken Cell Bank. The cells were grown in suspension culture in RPMI 1640 medium supplemented with FBS (10%), penicillin (50 units/ml), and streptomycin (100 μg/ml) in a 37°C humidified incubator (5% $CO_2$, 95% air). For the experiments, cells at the logarithmic growth phase were spun down, washed three times with PBS, and then resuspended in PBS ($2 \times 10^6$ cells/ml). Latex particles were then added to the cell solution. Subsequently, an aliquot of the mixture of cells and microspheres was added to each well (100 μl/well) of a transparent 96-well plate (Wako Pure Chemicals, Osaka). The plates were placed on ice up to the time of light irradiation.

*Light Source and Light Exposure System*—A Q-switched Nd:YAG laser (Spectra Physics, CA, USA) tuned to 532 nm was used. The repetition rate and pulse width were 10 Hz and 7–9 ns, respectively. The light was directed on an area equal to that of the bottom of the well, namely 6 mm in diameter, and was applied to each suspension of cells and microspheres at room temperature. The light irradiation scheme is presented in Fig. 1.

*Staining with PI and FDA*—The cells were stained with PI and FDA by a modification of the protocol described previously (*24*). Immediately after light exposure, 10 μl of PI (1 mg/ml) was added to each well, and then the cells were stained at room temperature in the dark for 2 min. 100 μl of resealing buffer, which consisted of RPMI medium containing 10% FBS, was added to each well. Subsequently, FDA was added to the cell suspension to a final concentration of 20 μg/ml, and then the cells were incubated in a 37°C humidified incubator for 10 min. The cells were then centrifuged, the supernatant containing PI and FDA removed, and the cells finally resuspended in 0.5 ml PBS. The cell suspensions were placed in test tubes and kept ice-cold during analyses.

*Flow Cytometry Analysis*—The fluorescence signal of individual cells was assessed with a flow cytometer (EPICS ELITE, Beckman Coulter, USA) as described previously (*24*). The fluorescence was excited with an argon-ion laser emitting at 488 nm. Green fluorescence (for FDA) and red fluorescence (for PI) were collected using 525 ± 5 nm band-pass and 600 ± 5 nm long-pass filters, respectively. Fluorescence signals were stored and processed with software program Multigraph (Beckman Coulter, USA). A total of 10,000 cells was examined per sample.

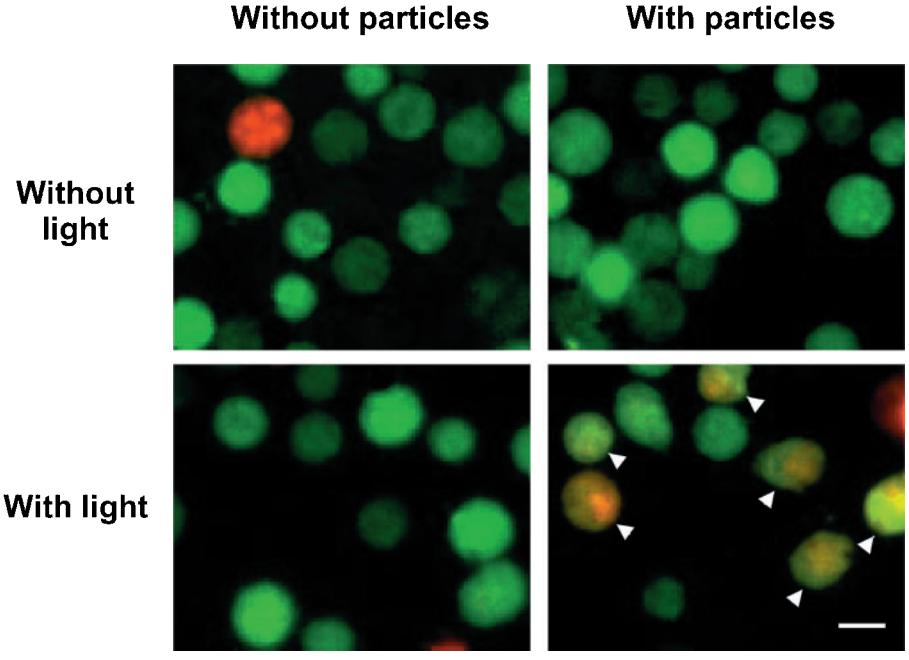


Fig. 2. **Fluorescent microscopic images of typical PI-permeabilized Meth-A cells.** The cells were mixed with latex particles ($4.6 \times 10^8$ particles/ml) and then exposed to 780 mW/cm$^2$ laser light for 30 s. Subsequently, the cells were stained with PI and FDA (see "MATERIALS AND METHODS"). The images show unpermeabilized cells (green), permeabilized-resealed cells (green + red, arrowhead), and dead cells (red). The scale bar represents 10 μm.

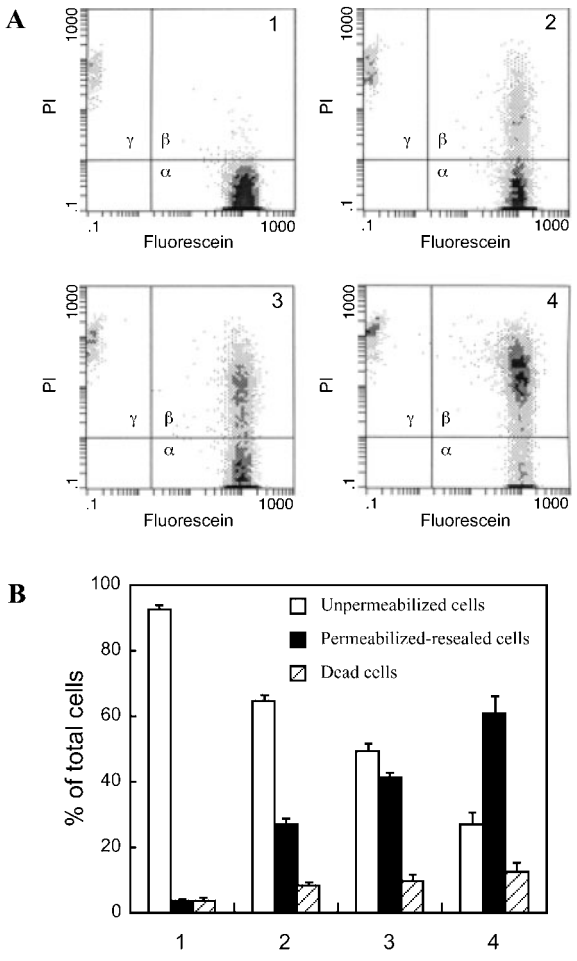


Fig. 3. **Flow cytometric measurement of Meth-A cells staining PI and FDA.** The cells were mixed with latex particles and then exposed to light (see Fig. 2). (1) No light, (2) 390 mW/cm$^2$, (3) 520 mW/cm$^2$, (4) 780 mW/cm$^2$. (A) Typical two-dimensional flow cytometry histograms of PI against fluorescein. Regions α, β, and γ denote the subpopulations of unpermeabilized cells, permeabilized-resealed cells, and dead cells, respectively. (B) Results obtained with the histograms.

*Fluorescence Microscopy*—Microscopy was performed with an inverted fluorescence microscope (BX 50, Olympus) equipped with a 100 W Hg-lamp, using a ×20 objective and an appropriate block filter. Images were recorded with an AxioVision (Carl Zeiss) coupled to an AxioCam camera, and digitally processed with image software Adobe Photoshop 5.5® (Adobe Systems, USA).

*Data Analysis and Statistics*—Each individual experiment was independently carried out at least in quadruplicate. Data are represented as mean values with standard error of the mean. Differences were examined for statistical significance with the two-sided *t*-test.

## RESULTS

*Fluorescence Microscopy*—The ability of this method to introduce extracellular materials into the cytoplasm of living cells was investigated by double staining with propidium iodide and fluorescein diacetate. Propidium iodide readily enters cells with injured membranes but is excluded from intact cells; hence, it serves as a marker of permeabilized cells. The efficiency of plasma membrane resealing was determined by exposing the cells to FDA. This non-fluorescent compound readily enters intact cells and then undergoes hydrolysis by endogenous esterase, which releases free fluorescein, which can be detected as its green fluorescence. Fluorescein is unable to escape from intact cells but is not retained by cells with damaged membranes and can therefore be used as a marker for cells with intact membranes. Figure 2 shows digitally enhanced images of examples of the four populations of experimentally manipulated cells resulting from these procedures. No difference was observed between cells that were pretreated with particles or light only and untreated cells. Only the cells treated with both particles and light exposure were effectively permeabilized and resealed.

*Effect of Light Intensity on Membrane Permeabilization*—The numbers of dead, unpermeabilized and successfully permeabilized-resealed cells were determined by flow cytometric analysis. Figure 3A shows typical two-dimensional PI-FDA histograms of cells. The histograms revealed three subpopulations of cells, defined by the rectangular windows denoted as regions α, β, and γ. The three subpopulations of cells are as follows: (i) intact viable cells containing fluorescein, but no PI (region α); (ii) transiently permeabilized, resealed cells characterized by an unchanged fluorescein-pool and a high-level of PI (region β); and (iii) irreversibly permeabilized, dead cells exhibiting no fluorescein, but very high PI-fluorescence (region γ). Particles showing neither green nor red fluorescence were classified as debris and thus excluded from the analysis. The percentage of cells showing dual fluorescence increased significantly on light irradiation (Fig. 3B). Note that as the power of the light increased, the number of unpermeabilized cells decreased, whereas that of permeabilized-resealed cells increased without significant cytotoxicity.

*Effects of the Irradiation Time and Latex Content on Membrane Permeabilization*—Data describing the effect of the irradiation time on the enhancement of permeabilized-resealed cells are shown in Fig. 4. As the irradiation time increased, the percentage of dead cells increased and that of unpermeabilized cells decreased. Light irradiation increased the percentage of successfully permeabilized-resealed cells in a bell-shaped manner, with a peak at 45 s of irradiation. After this time, there was a decrease in the percentage of successfully permeabilized-resealed cells, as cell death became more prominent.

The relationship between the concentration of latex particles and the percentage of each kind of cells (*i.e.* unpermeabilized cells, dead cells, or successfully permeabilized-resealed cells) is shown in Fig. 5. The optimal concentration of microspheres was about $10^9$ particles/ml. This concentration permeabilized approximately 50% of the living cells.

*Time Course of PI uptake*—The time course of resealing of permeabilized membranes was examined at a fixed light dose and one latex concentration (Fig. 6). PI was added to the cells at various times after laser irradiation. It is clear from Fig. 6 that an increase in the post-irradiation time (from 0 to 40 min) resulted in a corresponding decrease in the number of PI-injected viable cells (*i.e.* per-

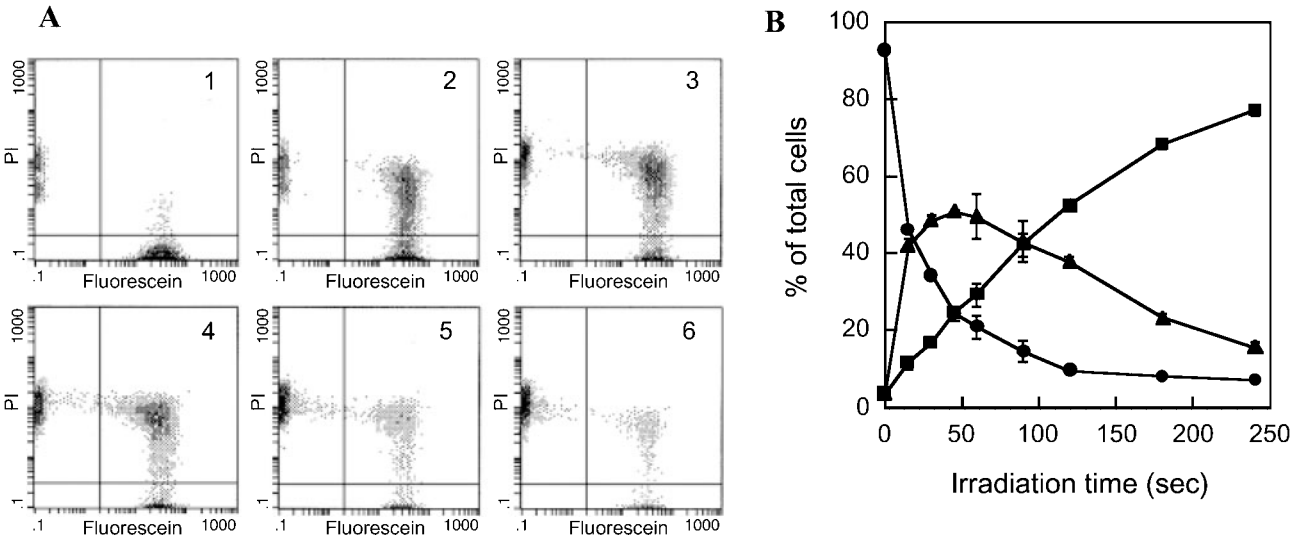


Fig. 4. **The time dependence of permeabilization efficiency, following different irradiation times with 780 mW/cm² light and a fixed particle concentration (4.6 × 10⁸ particles/ml).** (A) Typical two-dimensional flow cytometry histograms of PI against fluorescein (see Fig. 3). (1) No light, (2) 30 s, (3) 60 s, (4) 90 s, (5) 180 s, (6) 240 s. (B) The relative percentages of unpermeabilized cells (circles), permeabilized-resealed cells (triangles), and dead cells (squares) obtained with the histograms.

meabilized-resealed cells). The data show that maximal recovery is achieved within 10–20 min.

## DISCUSSION

The present study showed that a new method involving the combination of prestained latex particles and laser light irradiation can be used to increase the membrane permeability of mouse fibrosarcoma cells without any loss of their viability. This technique should be applicable for the incorporation of biologically active molecules into cells to study their effects on cell function. However, there is one important restriction of its use at the moment. The present study showed that it is only possible to introduce relatively small molecules (PI; $M_T$ 668.4) into mouse fibrosarcoma cells using this technique. No attempts to introduce larger molecules (*e.g.* plasmid DNA) were made in this study. Thus, at present we are unable to predict whether or not this technique will be useful for introducing compounds such as polypeptides, plasmid DNA and other large molecules into cells.

Thus, at present we are unable to assess the usefulness of this technique for introducing compounds such as polypeptides, plasmid DNA and other large molecules into cells. However, the results of the present study suggest the potential of our technique as to the introduction

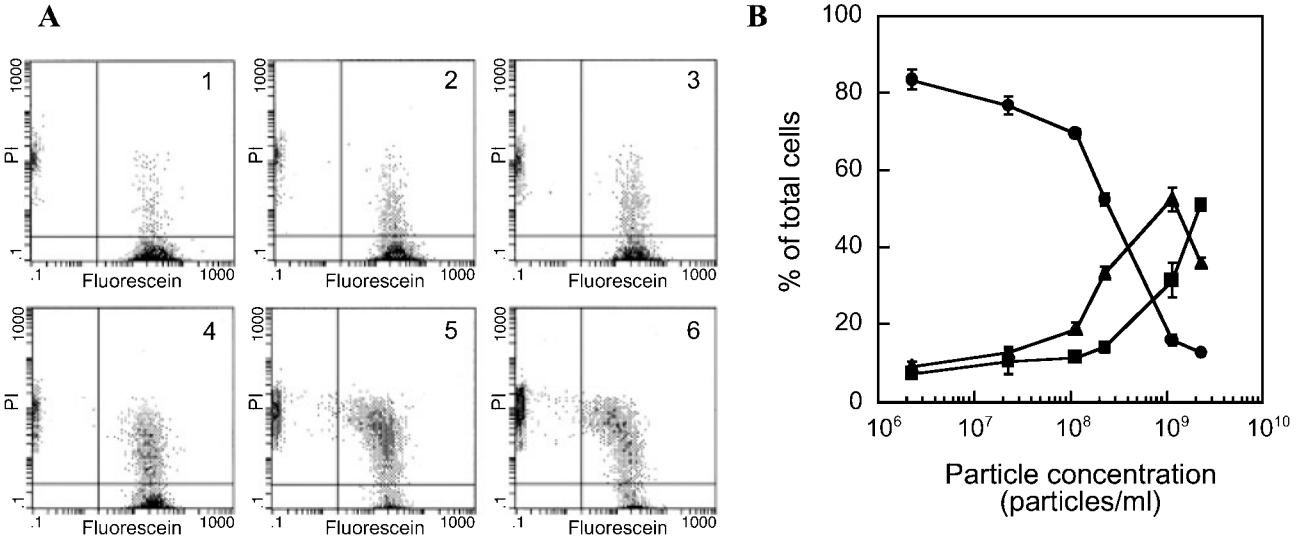


Fig. 5. **The effects of various concentrations of latex particles on permeabilization efficiency under fixed irradiation conditions (780 mW/cm², 30 s).** (A) Typical two-dimensional flow cytometry histograms of PI against fluorescein (see Fig. 3). (1) 2.3 × 10⁶ particles/ml, (2) 2.3 × 10⁷ particles/ml, (3) 1.1 × 10⁸ particles/ml, (4) 2.3 × 10⁸ particles/ml, (5) 1.1 × 10⁹ particles/ml, (6) 2.3 × 10⁹ particles/ml. (B) The relative percentages of unpermeabilized cells (circles), permeabilized-resealed cells (triangles), and dead cells (squares) obtained with the histograms.

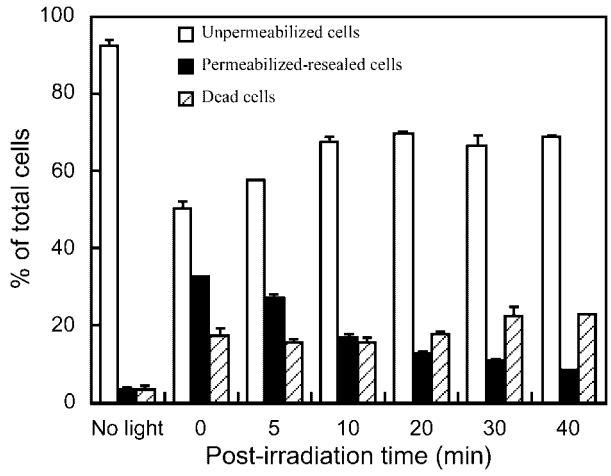


Fig. 6. **Time course of resealing of permeabilized membranes.** Cells were exposed to laser light (780 mW/cm$^2$ for 30 s) in latex-containing PBS ($2.3 \times 10^8$ particles/ml). PI was added after the light irradiation at the time shown. Subsequently, the cells were incubated with FDA, and three populations were distinguished by using a flow cytometer (see "MATERIALS AND METHODS" for details).

of such macromolecules. As shown in Fig. 4A (*3*, *4*, and *5*), the fluorescence intensity of cytoplasmic fluorescein of a partial cell population decreased as the light irradiation time increased. One interpretation of this finding is that the leakage of endogenous esterase led to a decrease in the quantity of intracellular fluorescein. Therefore, it is quite likely that extracellular molecules, which are about the same size as esterase, can enter the cytoplasm through sites of damage to the cell membrane. Moreover, as the cytogram indicates (Fig. 4A; *6*), excessive irradiation both enlarges the area of membrane damage and increases the degree of damage. Thus, the cells should die if the damaged area is not repaired and/or a large amount of cytosol is lost. We can conclude that optimization of the light irradiation time for our technique enables the introduction of macromolecules into cells and the consequent maintenance of cell viability.

From this discussion, it is clear that cells permeabilized due to overstimulation with our technique should die because of a failure or merely a delay in resealing of the membrane. The problem that we have to consider next is what should be done to decrease the number of dead cells. First, the light dose to which individual cells are exposed must be made homogeneous. In the present study, we could not satisfy this need because we exposed a cell suspension containing latex particles to the laser light; that is, the light decreased gradually as it progressed through the suspension. Second, to apply stress to each cell uniformly, it is necessary for the number of latex particles adhering to the cell surface to be equal. Finally, we must carefully determine the composition of the exposure medium, which includes cell-latex complexes and is exposed to the laser light, because a change in the composition would change the time during which a permeabilized membrane could reseal (*25*, *26*).

The fundamental mechanisms underlying the membrane permeabilization process in our experimental model are not known. Doukas *et al.* reported a transient increase in plasma membrane permeabilization caused by laser-generated stress waves (LSW) (*27*, *28*). In their experimental model, LSW were generated by laser ablation of a polyimide film, and membrane permeabilization recovered within 80 s after LSW (*29*, *30*). They concluded that the mechanisms involved in the permeabilization and recovery of the plasma membrane caused by LSW were different from those involved in electrical pulses, a method called electropulsation, since the resealing of membranes permeabilized by electropulsation takes minutes to hours (*26*, *31*). Our experimental design also employs laser light; hence, LSW may be one of the factors causing the membrane permeability. However, membrane permeabilization persisted up to 20 minutes after light irradiation (Fig. 6). In this way, the type of membrane injury in our method may more closely resemble the effect of electropulsation.

It is also probably that a temperature increase would have contributed to the increase in membrane permeabilization. The absorption of high intensity laser light by dyed latex particles at the interface with the cell membrane should induce localized changes in the local properties of the membrane bilayer. In synthetic membranes, the 'melting' temperature is dependent upon the chain lengths of the fatty acids, the amount of cholesterol, and the level of hydration. In these systems, the transition temperature is, in fact, about 60°C (*32*). It seems quite probable that exogenous materials can enter cells through these areas of increased permeability. Measurement of the superficial temperature of latex particles could be a subject of further study.

The advantages of the present technique over the others are various. First, it eliminates the need for extraneous, potentially harmful substances such as high concentrations of salts (especially calcium salts), polyions, detergents, lipids, and/or bacterial toxins to be added to the cell culture. Second, since the radiation used is in the visible region, in which neither endogenous nor exogenous DNA and proteins are able to interact, no adverse effects on cells are reasonably expected. Third, because the stresses that increase membrane permeability are produced on the cell surface, they are effectively applied to the plasma membrane. In addition, the effects are localized to particle contact sites, thereby limiting the size or extent of plasma membrane damage sites. For all of these reasons, this method has the potential to be used for *in vivo* applications.

In summary, this report is the first description of the combined use of dyed latex particles and laser light for increasing plasma membrane permeability. As expected, extracellular membrane-impermeable molecules could be introduced into living cells. If this strategy proves useful, additional applications based on this technique could be developed, such as selective introduction to target cells, by coating particles with monoclonal antibodies, or perhaps delivery of drugs via particles to the cell surface.

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