

Insertion of Microscopic Objects through Plant Cell Walls Using Laser Microsurgery

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Abstract: A detailed protocol is presented for precisely inserting microscopic objects into the periplasmic region of plant callus cells using laser microsurgery. *Ginkgo biloba* and *Agrobacterium rhizogenes* were used as the model system for developing the optical tweezers and scalpel techniques using a single laser. We achieved better than 95% survival after plasmolyzing *G. biloba* cells, ablating a 2–4- μm hole through the cell wall using a pulsed UV laser beam, trapping and translating bacteria into the periplasmic region using a pulsed infrared laser beam, and then deplasmolyzing the cells. Insertion of bacteria is also described. A thermal model for temperature changes of trapped bacteria is included. Comparisons with other methods, such as a reverse-pressure gradient technique, are discussed and additional experiments on plants using laser microsurgery are suggested.

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INTRODUCTION

Since the first reports of cutting (Leppard and Raju, 1965) and trapping (Ashkin et al., 1986) of biological materials using laser beams, many optical micromanipulation techniques have been demonstrated (for recent reviews, see: Berns et al., 1991; Greulich and Weber, 1992; Hoffman, 1996; Leitz et al., 1994; Svoboda and Block, 1994). For example, optical scissors have been used to ablate portions of plant cell walls to access the plasma membrane for patch-clamp experiments (Taylor and Brownlee, 1992; De Boer et al., 1994; Hendriksen et al., 1996; Kurkdjian et al., 1993). Optical tweezers techniques were used to manipulate bacteria and viruses (Ashkin and Dziedzic, 1987) and test bacterial flagella compliance (Block et al., 1989). Recently, combined cutting and trapping aided cell fusion of myeloma cells (Steubing et al., 1991). Liang and co-workers (1993)

used lasers to cut and rotate large chromosomes, while Schütze and colleagues (1994) drilled a hole in the zona pellucida of bovine oocytes and manually inserted sperm into the opening.

Laser microsurgery techniques provide one of few methods for precise, sterile placement of microscopic objects inside the plant cell's rigid wall. Alternative microinjection techniques (Nims et al., 1967), although allowing specific cell targeting, have several disadvantages. The glass capillaries are prone to breakage while penetrating rigid cell walls (Leitz et al., 1994). Microinjection must be accomplished in a sterile environment if aseptic conditions are desired, the method is limited to objects no larger than the inside diameter of the capillary (typically $\sim 1 \mu\text{m}$), and microinjection is difficult with suspended target cells. A single cell must be immobilized with a vacuum pipette while the microinjection pipette is simultaneously manipulated into position. In addition, cells can stick onto the microinjection pipette, making manipulation more difficult. Light beams, on the other hand, have the advantage of being sterile, require no mechanical contact, and can be used within cells in their natural state (Ponielis et al., 1994). Furthermore, large irregular-shaped particles, including yeast cells to $10 \mu\text{m}$ (Ashkin et al., 1987), may be manipulated by optical traps.

Laser ablation techniques were used to breach the cell wall barrier aiding the transformation of plant cells (Guo et al., 1995; Weber et al., 1988). The cells were placed in a hypertonic solution with naked plasmid DNA, and then the cell walls and plasma membranes were ablated with a laser beam. The DNA was pulled into the plant cells as the cells returned to their normal size, due to a reverse-pressure gradient rather than using optical trapping techniques. While this method provided an efficient and somewhat targeted means of transformation, it required separating the plasmids from the bacteria and may not be suitable for plasmids larger than 15 kilobase pairs due to isolation and manipulation problems.

We report here a protocol, combining optical tweezers and scissors techniques, to insert microscopic objects across living plant cell walls using *Ginkgo biloba* callus cells and intact *Agrobacterium rhizogenes* as the model system. The

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determination of optimum osmotic conditions, a method of readily changing osmotic solutions, and optical setup are described for this system. This procedure may be generalized for other plant species after determining the species specific osmotic conditions and for inserting other objects such as polystyrene microspheres. For example, we have also demonstrated this procedure with *Nicotiana tabacum*, *Cucumis melo*, and *Arabidopsis thaliana*. This is the first time, to our knowledge, that objects of any type have been manually moved through a plant cell wall using only laser microsurgery techniques.

MATERIALS AND METHODS

Lasers and Optics (Fig. 1)

All lasers and optics were mounted on an air-suspension table (Newport Research Corp., Newport, CA) to eliminate vibrations. A 30-Hz pulsed Nd:YAG laser (Model GCR 170, Spectra-Physics, Mountain View, CA) with second and third harmonic generation capabilities (KD₂OP₄ crystal, HG-4, Spectra-Physics) produced the beams. The fundamental beam (1064 nm, long pulse mode, 200- μ s duration) was used for trapping, and the frequency-tripled beam (355 nm, Q-switched, 6-ns duration pulse) was used for cutting. The laser beams were collimated with uncoated lenses (BK-7 glass), directed through a series of prisms (BK-7 glass), and focused with a 100 \times Neofluar (numerical aperture 1.3) oil immersion microscope objective (Carl Zeiss, Thornwood, NY) to a nearly diffraction-limited spot (about one wavelength in diameter). The unused beam was blocked to prevent unwanted interactions.

The optical setup included two CCD cameras (FlexCam,

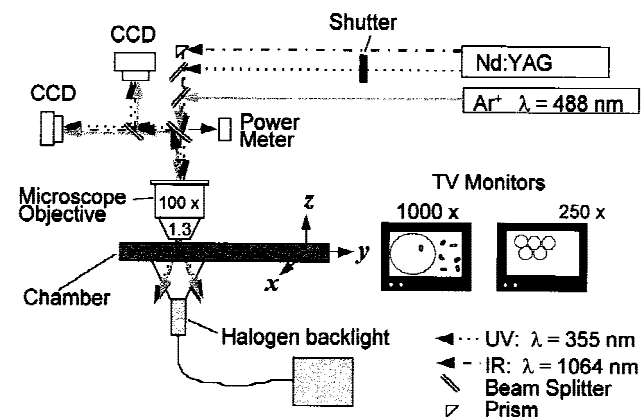


Figure 1. Schematic of laser setup. Ablation was achieved with a Q-switched 355-nm beam and trapping was accomplished with a long pulse mode 1064-nm beam. The 488-nm Ar⁺ beam was used to excite hydrolyzed fluorescein diacetate to determine plant cell viability. The x,y,z positioning is provided by a micromanipulated stage allowing precise movements and is calibrated for recording and relocating cell positions in the chamber. The microscope objective was a 100 \times oil immersion Zeiss Neofluar with a 1.3 numerical aperture. Cells were backlit with a halogen lamp and visualized by CCD cameras on TV monitors.

Video Labs, Minneapolis, MN) to visualize the cells at low (250 \times) and high (1000 \times) magnification. The high magnification camera was mounted on a translation stage such that the image plane could be shifted to coincide with the 355- or 1064-nm wavelengths' focal planes. Time course events were recorded with a VCR. An electromechanical shutter controlled the cutting beam duration. An Ophir power meter (Model PE-50, Peabody, MA) was used to determine the laser power entering the microscope objective. The amount of light reaching the focal plane was estimated using the objective's transmission characteristics. Transmission of $63 \pm 5\%$ at 1064 nm was measured using the technique of Misawa et al. (1991). This value is consistent with other evaluators (Liang et al., 1996; Liu et al., 1995; Wright et al., 1994). The transmission at 355 nm was estimated at $20 \pm 10\%$ as provided by the manufacturer (I. Seidler, Zeiss, USA, representative. Personal communication with K.T.G., 1996).

The wavelengths and power levels used require some special safety considerations. The CCD cameras allowed viewing without directly observing the laser beams. Color glass filters were used to prevent light damage to the CCD cameras. Standard protective eye wear was used to avoid eye damage by stray radiation.

Flow Chamber

The flow chamber (Fig. 2) was a modified version of Walcerz and Diller's (1991) perfusion chamber. The major changes were the elimination of the bubble trap by a T-fitting, removal of the thermocouple, and the use of Lexan (General Electric Plastics, Pittsfield, MA) versus stainless steel for the cover. The chamber was washed with 70% ethanol and placed in a sterile hood with a bactericidal UV light source (8 h minimum) to achieve sterilization. Callus

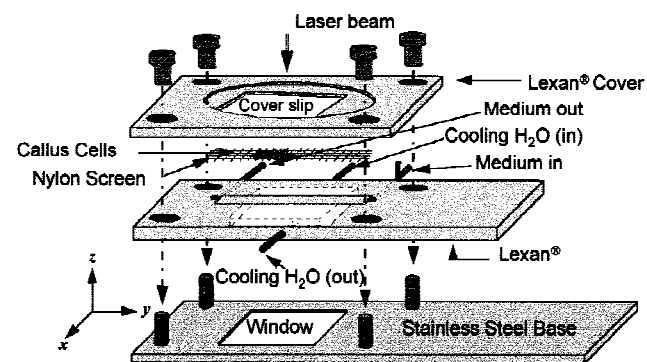


Figure 2. Exploded view of the flow chamber for immobilizing *G. biloba* callus cells and introducing different osmotic solutions. Design is a modified Walcerz and Diller (1991) perfusion chamber. Dimensions: cover, 6.3 \times 5 cm; the No. 1.5 cover slip (22-mm square) is glued (UV cured, NOA 68, Norland Products Inc., New Brunswick, NJ) into a recess; an o-ring provides a seal to the center assembly; center assembly, 8.3 \times 5 cm; a cover slip is glued into a recess in the top and bottom to seal the cooling chamber; entrance and exit tubing are 3 mm OD; bottom assembly, 12 \times 5 cm with a 22-mm square opening.

cells were immobilized in the chamber on a nylon mesh, and the unit was assembled aseptically. The osmotic solutions and bacteria were introduced and removed through the media inlet and outlet. A peristaltic pump forced water through the cooling compartment to maintain a constant temperature (25°C).

The chamber was mounted on a three-axis micromanipulator equipped with micrometer scales. Recording cell coordinates facilitated relocation for further experimentation and evaluation. Movement in the *z* direction (focus) was achieved with a computer-controlled actuator (Stepper-Mike, Model 18510, Oriel, Inc., Stratford, CT) with a step resolution of $\pm 1 \mu\text{m}$. Coarser motion was allowed in the *x, y* plane; thus, we used manually controlled micrometers.

Plant Cell Cultures

G. biloba stem callus, initiated from seedlings, was grown according to Carrier et al. (1990) on semisolid (0.3% w/v Gelrite, Adam Scientific, West Warwick, RI) or liquid Murashige and Skoog Minimum Organics (MSMO) basal salts (Linsmaier and Skoog, 1965) supplemented with 3% sucrose (w/v) under cool white fluorescent lights (continuous $20 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C. NAA (1-naphthaleneacetic acid, 2 mg L^{-1}) and kinetin (1 mg L^{-1}) were added as plant growth regulators. Suspension cultures were agitated at 100 rpm on a rotary shaker. Subculturing was accomplished every 2–3 weeks for both types of cells.

Bacterial Culture

A. rhizogenes strains ATCC (American Type Culture Collection) 11325, ATCC 15834, and A4, each harboring wild-type plasmids, were used in the insertion procedures. The cultures were inoculated from frozen glycerol stocks and incubated on rotary shakers at 150–200 rpm at 25°C. The bacteria were grown in liquid YMB medium (in g L^{-1} : K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 0.2; NaCl, 0.1; mannitol, 10.0; yeast extract, 0.4; pH 7.0, according to Hooykaas et al., 1977) at 25°C in 50 mL ($2 \times 15 \text{ cm}$) test tubes with 10 mL of medium. The bacteria were centrifuged (3000g, 10 min) and placed in a 0.45 M sorbitol solution for suspension cultures or 0.75 M sorbitol solution for semisolid cultures (Table I).

Polystyrene Microspheres

Polystyrene microspheres ($3 \pm 0.3 \mu\text{m}$ diameter, Duke Scientific, Palo Alto, CA) were also used in the insertion protocols. A drop of H_2O suspended microspheres was added to the 10 mL of osmotic solution instead of bacteria.

Osmotic Solutions

Various media (Table I) were tested to determine the experimental conditions for maximum *G. biloba* cell survival. CaCl_2 (10 mM) was added to stabilize the plant's plasma

Table I. Osmotic solutions and pH levels tested for *G. biloba* callus cell survival. Cells were grown in/on MSMO liquid or semisolid media. Conditions underlined are the osmotic solutions chosen for continued experimentation.

Experiment	Osmotic solution	pH
Suspension cultured callus		
A	10 mM Tris; ^a 0.3–0.45 M sorbitol; 10 mM CaCl_2	7.2
B	10 mM Tris; 0.3 M sorbitol; 10 mM CaCl_2	7.2
C	Distilled H_2O	6.0
Gelrite cultured callus		
D	10 mM Tris; 0.3–0.75 M sorbitol; 10 mM CaCl_2	7.2
E	10 mM Tris; 0.3 M sorbitol; 10 mM EDTA ^b	7.2
F	10 mM Tris; 0.3 M sorbitol; 10 mM CaCl_2	7.2
G	10 mM Tris; 0.6 M sorbitol; 10 mM CaCl_2	7.2
H	10 mM Tris; 0.3–0.45 M sorbitol; 20 mM CaCl_2	7.2
I	Distilled H_2O	6.0
J	10 mM Tris	7.2
K	2.6 mM MES; ^c 0.3 M sorbitol	6.0
L	10 mM Tris; 0.3 M sorbitol; 10 mM CaCl_2 ; ice–water cooling bath	7.2
M	MSMO media; 6% sucrose	6.0

^aTris (Trizma–base buffer).

^bEDTA (ethylenediaminetetraacetic acid).

^cMES (2-[*N*-morpholino]ethanesulfonic acid buffer).

membrane (Kurkdjian et al., 1993). The various osmotic solutions were transferred (10 mL) into stoppered test tubes ($2 \times 15 \text{ cm}$) with a glass tube and flexible tubing, permitting removal of the solution. A 22-gauge needle, with glass wool stuffed in the end and covered with aluminum foil, pierced the stopper (for pressure equalization), allowing sterile transfers to the flow chamber. The osmotic solutions were drawn into the flow chamber using a Pipet Pump (Bel-Art Prod., Pequannock, NJ). Test tubes containing the various osmotic conditions were autoclaved at 121°C for 21 min at 15 psi and stored at 4°C until used.

Cell Viability Assay

G. biloba callus cell viability was assayed with fluorescein diacetate (FDA, Fluka Biochemika, Buchs, Switzerland) following the protocol of Widholm (1972). The osmotic solutions had FDA aseptically added to them (final concentration 0.01% w/v) immediately before use. The solution remains active approximately 3 h (Widholm, 1972). A continuous wave argon ion laser (Fig. 1), tuned to 488 nm, was used to excite hydrolyzed FDA fluorescence, indicating a viable cell.

Cutting and Trapping Procedure

Two types of *G. biloba* cells were immobilized on nylon mesh (suspension culture, thread diameter 94 μm , mesh opening 149 μm , P/N E-CMN 149; Gelrite grown callus, thread diameter 160 μm , mesh opening 250 μm , P/N E-CMN 250, Small Parts, Inc., Miami Lakes, FL). Callus

grown on semisolid media was removed from plates, placed in a drop of sterile distilled H₂O, and broken into small pieces before aseptic mounting on the nylon mesh. Suspension callus cells were placed directly on the mesh with a sterile Pasteur pipette by wicking extra media away on a sterile paper towel. All tissues were then placed in the flow chamber (Fig. 2).

Laser procedures were performed with 0.75 M sorbitol for semisolid cultured cells and 0.45 M sorbitol for suspension cultures, corresponding to the point at which at least 50% of the cells were visibly plasmolyzed to $\leq 85\%$ of their original volume. A sorbitol gradient was introduced stepwise into the flow chamber. The gradient increments were 0.3–0.5–0.75 M for semisolid cultured cells and 0.3–0.45 M for the suspension cultures with 5-min intervals between steps. Cell wall ablation was aided by the addition of calcofluor (Sigma Chemical Co., final concentration, 0.01% w/v) which selectively binds to cellulose and increases the cell wall's absorption of UV light (De Boer et al., 1994). Calcofluor was sterile filtered and aseptically added along with bacteria to the highest necessary osmotic condition, depending on which plant cells were used.

The *G. biloba* callus cells were plasmolyzed, 20 viable

cells were selected by fluorescence, and their positions recorded before the bacterial solution was introduced. Of the 20 viable plasmolyzed cells, half were selected for bacterial insertion and half became uncut controls for the osmotic conditions. The cutting beam (355 nm) was then used to ablate a small hole (2–4 μm across) tangential to the wall of the selected cells (Fig. 3). The location of the hole was typically chosen near a point of contact between the cell wall and the plasma membrane. This was done to ensure the plasma membrane would quickly cover the hole upon deplasmolysis.

Following the ablation procedure the optical tweezers were used to manipulate bacteria through the hole into the periplasmic space. Positive insertion of bacteria was confirmed by attempting to move the bacteria back across the cell wall. Normally only one bacterium was trapped in the focal region, but it was possible to trap two or more cells concurrently. On one occasion we inserted two bacteria into a plant cell simultaneously. The insertion procedure was repeated until 5–10 bacteria were inside the cell wall. The bacteria were manipulated to a position as far from the hole as feasible and wedged between the plasma membrane and the cell wall, minimizing escape possibilities. The entire

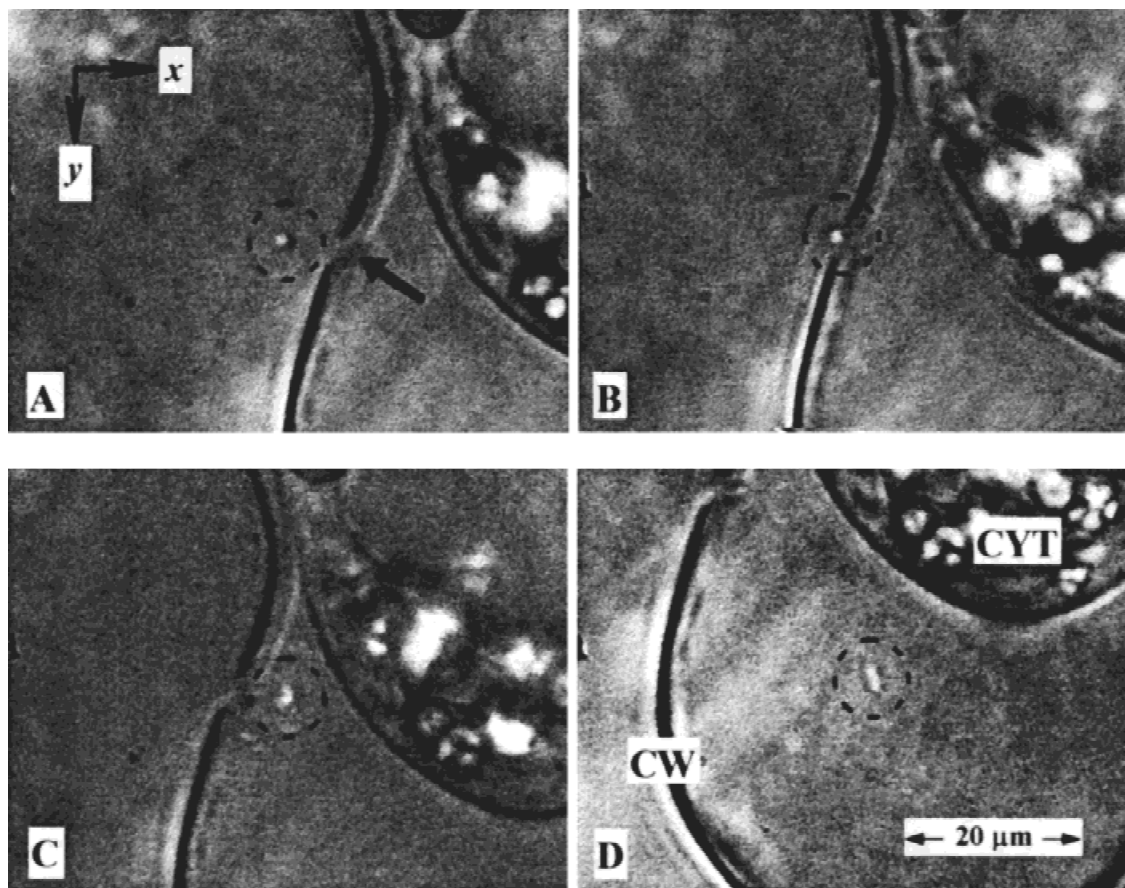


Figure 3. Sequence of images from video tape of a plasmolyzed *G. biloba* cell showing ablated portion of cell wall (arrow) and bacterial insertion (A–C). The bacterium is highlighted by a broken circle. The cutting light beam was directed tangential to the cell wall directed into the page. The decreased resolution of the bacterium is attributed to refraction of the cell wall. (D) Bacterium after the laser beam was shut off; CW, cell wall; CYT, cytoplasm.

cutting and insertion procedure took about 5–10 min and was repeated for the 10 selected plant cells.

RESULTS AND DISCUSSION

Substantial differences were observed between *G. biloba* callus cells grown on semisolid media and in suspension cultures. Sorbitol at 0.75 M was necessary to plasmolyze ~50% of the semisolid grown cells. In contrast, 0.45 M sorbitol plasmolyzed more than 70% of suspension grown callus cells, and the cells were much more efficacious. Callus cells grown on semisolid media had cell walls that appeared thick and encrusted, while those from suspension cultures were thin and smooth.

The ability to sufficiently plasmolyze cells from suspension cultures with 0.45 M sorbitol improved the survival of the control cells to 100%, significantly better than 70% for the 0.3–0.75 M sorbitol step gradient for semisolid grown callus (Fig. 4). Incipiently plasmolyzed suspension culture cells in 0.3 M sorbitol survived significantly better after laser ablation than cells cultured on semisolid media. Comparisons between both cell types were not significantly different after laser ablation in water, probably due to nonplasmolysis of the cells and subsequent cytoplasm leakage.

Plasmolysis of suspension cultured cells resulted in significantly increased survival after ablation compared to un-plasmolyzed cells. This difference was also apparent when using cells from semisolid media but was not statistically significant. Cytoplasm was forcefully ejected from un-plasmolyzed cells and leaked from the cells for more than 20 min after cutting. Cytoplasmic expulsion seemed more forceful after ablation at hypotonic conditions (water) than after cutting in 0.3 M sorbitol. It was nearly impossible to selectively cut the cell wall (and not the plasmalemma) with the cytoplasm appressed.

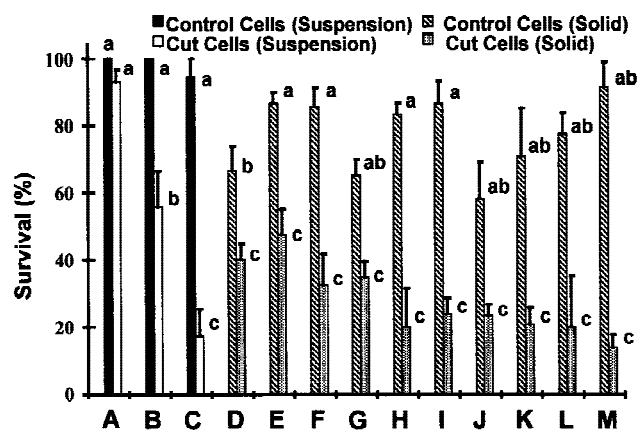


Figure 4. *G. biloba* callus cell survival under different osmotic conditions from semisolid and suspension cultures after 3–4 h. Key to the different solutions is in Table I. Error bars are the standard deviation of the mean of at least three replicates (≥ 10 cells per trial) for each condition. Significance was determined by heteroscedastic Student's *t* test at the 95% confidence level. Different letters above the bars indicate a significant difference existed between conditions.

We estimate a pulse energy of 1 μJ at the cell wall was necessary to achieve thermal ablation (wavelength 355 nm), a value consistent with Greulich and Weber (1992) and Leitz et al. (1994). The fluence threshold, ψ_{th} (J cm^{-2}), necessary to evaporate water from biological tissue may be estimated from the rule given by Welch et al. (1991), $\psi_{\text{th}} = 2500\delta$, where δ is the penetration depth (in cm) into the tissue at which the beam intensity drops to 1/e of the incident intensity. For a strongly focused beam the fluence decreases rapidly as one moves away from the beam focus. This permits ablation of a small volume of tissue surrounding the focal spot while leaving nearby tissue undamaged. For instance, the optical energy fluence of a 1 μJ pulse through a nearly diffraction limited focal spot of radius $w_0 \cong 0.5\lambda$ is $\psi \cong 2000 \text{ J cm}^{-2}$ in the focal region. Just 1 μm along the optical axis, the fluence drops to $\psi \cong 140 \text{ J cm}^{-2}$. The δ for cellulose and our system (calcofluor bound to cellulose) is unknown. On the basis of our visual observation of an ablation volume of $\sim 1.0 \mu\text{m}^3$ for each pulse, we estimate $\delta \approx 0.06 \text{ cm}$. To ablate a 2–4- μm diameter hole in the 1–3- μm thick cell wall and clear the hole of debris, we exposed the region to 10–100 pulses.

Differences in cutting energy corresponding to depth below the cover slip were determined with and without the use of calcofluor (Fig. 5). Without calcofluor we required pulse energies of 2–6 μJ at the focal plane for interactions with the cell wall. Selective binding of calcofluor to cellulose of the cell wall allowed us to operate in the range of 1 μJ . Fewer spontaneous deplasmolysis events occurred while using calcofluor.

Liquid flowed outward through the hole of many plasmolyzed cells following ablation, lasting from a few seconds to several minutes. Other authors also observed this

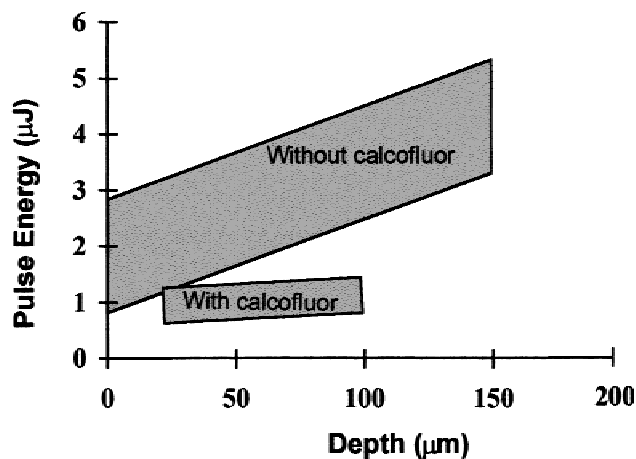


Figure 5. Depth dependence of the minimum cell wall ablation energy at 355 nm with and without calcofluor. Shaded areas represent the standard deviation around the average pulse energy for each case. The energy at the input of the objective was measured with a power meter and the manufacturer's specifications of transmission through the objective ($\sim 20\%$) was used to determine the ablation energy. The considerable difference in the absorption of light energy between different cell walls, cell position, and cell wall composition accounts for the high degree of power variability at similar depths below the cover slip.

flow (De Boer et al., 1994; Kurkdjian et al., 1993), although Weber et al. (1988) and Guo et al. (1995) did not mention it in their work. The flow could result from an increase in osmotic pressure inside the plasma membrane, movement of the protoplast, microperforations of the plasma membrane from shock waves, or thermally induced active transport. The initial flow was usually strong enough to prevent immediate insertion of the bacteria with the optical tweezers. After 1–2 min, the flow decreased to a manageable rate. Measurements immediately following ablation resulted in a maximum volumetric flow rate of $7 \times 10^{-10} \text{ cm}^3 \text{ s}^{-1}$. The measurements were made by timing small particles as they flowed through the cell wall opening.

The use of a pulsed infrared beam was more for convenience than to create ideal trapping conditions. While absorption of light by biological substances is low at 1064 nm, the high instantaneous power of a pulsed beam may harm the trapped bacterium by generating transitory temperature increases compared to a continuous beam at the same average power. Temperature effects on bacteria over long time scales (minutes) have been studied (Ashkin et al., 1987). Consequences of short-lived temperature increases associated with energy deposition into a bacterium at faster rates than dissipation are not well understood. However, virulence is known to be adversely affected in *Agrobacterium* at temperatures above 32°C (Fullner et al., 1996), and at higher temperatures ($\approx 80^\circ\text{C}$) DNA dissociation begins (Tamarin, 1993). It was useful to estimate the magnitude of the transient temperature increases to assess the possibility of damage.

We estimated the temperature increase in the focal region by modeling the radiant energy deposited as an infinite cylindrical heat source with a Gaussian profile of radius $w_0 = 0.5\lambda$. We further assume the time dependence of the source as a square wave pulse of 200 μs . With this assumption, the transient temperature rise during the pulse may be modeled as a continuous wave source. Gordon et al. (1965) derived the transient temperature distribution as a function of the distance, r , from the optical axis and exposure time, t , for a cylindrical continuous wave source:

$$\Delta T(r,t) = \frac{0.48\alpha P}{8\pi k_T} \left[\text{Ei} \left(-\frac{2r^2}{w_0^2} \right) - \text{Ei} \left(-\frac{2r^2}{8Dt + w_0^2} \right) \right],$$

where P is the instantaneous beam power, α the absorption coefficient, k_T the thermal conductivity, D the thermal diffusion coefficient of the medium, and Ei the error function. The decrease in temperature following the exposure of time $t = t_p$ may be calculated using a two-dimensional Green's function (Carslaw and Jaeger, 1963) with heat source $Q(r) = \rho c_p \Delta T(r, t_p)$:

$$G(r,r',t) = \frac{1}{4\pi k_T t} e^{-(r^2+r'^2)/4Dt} I_0 \left(\frac{rr'}{2Dt} \right),$$

which gives a temperature distribution of $t > t_p$ of

$$\Delta T = 2\pi \int_0^\infty Q(r,t_p) G(r,r',t) r' dr'.$$

The temperature rise and decay at the focal point $w_0 = 0.5\lambda$ of an infrared pulse ($\lambda = 1064 \text{ nm}$) of duration $t_p = 200 \mu\text{s}$ and $E_p = \sim 170 \mu\text{J}$ ($P_{\text{avg}} = \sim 6 \text{ mW}$), focused in water ($\alpha = 0.1 \text{ cm}^{-1}$, $k_p = 5.87 \times 10^{-3} \text{ J cm}^{-1} \text{ s}^{-1} \text{ K}^{-1}$, $D = 1.4 \text{ cm}^2 \text{ s}^{-1}$) is shown in Fig. 6. A maximum temperature rise of $\sim 7^\circ\text{C}$ occurs over the period of the pulse after which the temperature decays to a fraction of a degree ($\approx 0.01^\circ\text{C}$) above the original temperature before the arrival of the next pulse 33 ms later. Experimentally, the beam diverges and the temporal profile of the pulse is Gaussian. The model, therefore, overestimates the temperature changes. Under typical average operating powers of $P_{\text{avg}} = 1\text{--}10 \text{ mW}$, we may expect a temperature rise for the trapped bacterium of no more than a few degrees.

The bacteria are probably not adversely affected by trapping with the pulsed beam. In a few cases the bacteria exhibited an increased level of motility following prolonged trapping (suggesting some level of discomfort). We did not, however, attempt to assay bacterial viability following trapping. Ashkin et al. (1987) observed bacteria undergo binary fission while trapped. In a run of almost 5 h about 2.5 life cycles were observed with all four of the bacteria remaining in the trap. No damage to bacteria was observed up to their maximum power of $\sim 80 \text{ mW}$ at $1.06 \mu\text{m}$ with a continuous wave laser. Liu et al. (1995) predicts a temperature rise of $1.2\text{--}1.5^\circ\text{C}$ per 100 mW, or $10\text{--}13^\circ\text{C}$ under our conditions. However, the experiments of Liu et al. (1995) were with a continuous wave laser.

Upon entering the trap, the rod-shaped bacterium immediately aligned lengthwise along the beam axis. This effect, first observed by Ashkin et al. (1987) may be understood by modeling the bacterium as a distribution of electric dipoles. The dominant force acting on a single dipole of polarizability, α , in the presence of a strongly focused beam is the gradient force, $F = \frac{1}{2} \alpha \nabla E^2$, where E^2 is proportional to

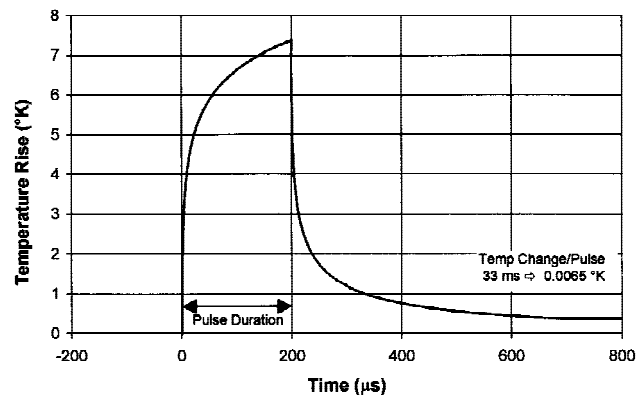


Figure 6. Modeled change in temperature of a bacterium trapped in a 1064-nm laser beam set at the long pulse mode with an energy of $\sim 170 \mu\text{J}$. Upon arrival of the next pulse 33 ms later, the temperature has fallen to $\sim 0.01^\circ\text{C}$ above the original temperature.

the time-averaged intensity of the beam (Ashkin et al., 1986; Gordon, 1973). For positive α (most biological materials), the gradient force tends to pull dipoles into regions of higher intensity. A bacterium oriented at an angle with respect to the optical axis (Fig. 7B) feels a torque that rotates it until parallel with the axis (Fig. 7A). This torque arises from gradient forces acting on the dipoles located near the ends of the bacterium, pulling the ends toward the optical axis.

It was often difficult to insert the first bacterium into the plant cell. While ablating the hole the cell wall seemed composed of two layers: an easily visualized outer layer and an inner invisible layer. Ablating the visible layer was generally insufficient to allow insertion; it was also necessary to ablate the inner layer that seemed to form a net-like covering across the hole. It is postulated that ablation of the wall leaves strands of cellulose microfibrils (Wolters-Arts et al., 1993) protruding across what appears to be an open hole. The ablated hole was also probably oval-shaped due to the tangential cut along a round surface. It is possible the peritrichous flagella of the bacterium were being caught on the invisible projections. Further cutting allowed the bacterium to be inserted. The insertion procedure was successful about 80% of the time. Common causes for failure were spontaneous deplasmolysis of the cell, difficulty in cutting because of light scattering by neighboring cells, and transparent barriers in the media blocking hole accessibility.

Orientation in the z direction was sometimes difficult. Callus cell walls are transparent enough to complicate finding the ablated hole. Occasionally insertion was proven false by attempting to manipulate the bacterium back across the cell wall. After determining exactly where the hole was with the first bacterium, the remaining bacteria were easily inserted.

We also investigated inserting bacteria into plant cells using a reverse-pressure gradient similar to Weber et al. (1988) and Guo et al. (1995). They achieved transformation in *Brassica napus* and rice cells, respectively, by using hypertonic conditions and perforating both the cell wall and the plasma membrane. The naked plasmid DNA entered the cell through the opening in the cell wall as the cytoplasm

returned to its original position. In our procedure, the cell walls of slightly plasmolyzed cells were ablated without damaging the plasma membrane. Bacteria were then introduced at a higher osmotic condition. As the cells plasmolyzed further, they drew bacteria spontaneously through the ablated hole. With this method, bacteria entered ~40% of the treated cells. There was no control over the number of bacteria taken up by the plant cell. The number of bacteria in the area around the hole, the volume and rate of media uptake of the plasmolyzing cell, and transparent barriers blocking the movement of the bacteria affected the insertion efficiency. Although the use of this hypertonic method has the advantage of inserting objects simultaneously into several plant cells, targeting of cells is less specific.

We attempted many times to perforate the plasma membrane under controlled conditions. Optical damage was possible but the membrane seemed to close immediately following ablation. The cell would not undergo deplasmolysis in any of these cases. The spontaneous deplasmolysis of some cells while ablating the cell wall is unexplained. There was no apparent damage to the plasma membrane in those cases.

Long-term plant cell survival was not rigorously tested. Test cells survived at least 14 h on a few occasions when cells were left in the flow chamber. Removal of cells from the flow chamber caused orientation changes making further specific cell survival determination impossible. An aeration system would need to be incorporated into the system if one were to leave the cells in the flow chamber for extended periods.

The fate of the inserted bacteria is unknown. Upon deplasmolysis the bacteria are apparently flattened between the cell wall and the plasma membrane and become invisible. Some type of tagging would be necessary to elucidate postplasmolysis events.

The test conditions for *Cucumis melo*, *Nicotiana tabacum*, and *Arabidopsis thaliana* were not optimized. Arabidopsis and melon callus cell walls are more transparent than tobacco and ginkgo making cell wall ablation slightly more difficult to visualize. Plasmolysis was consistently possible in the 0.5–0.6 M sorbitol range.

Laser insertion of foreign particles into plant cells presents a precise means to study plant responses and physiology. The manipulation of particles past the cell wall could be used to insert fungal spores or other bacteria into plant cells for studying plant–microbe interactions. Especially interesting would be the insertion of *Agrobacterium*, *Rhizobium*, and *Bradyrhizobium* bacteria, mycorrhizal fungal spores, cyanelles, or cyanobacteria to observe interactions over time. It is also possible to insert polystyrene spheres into the cell and perhaps test biological forces in situ following attachment to various structures.

We have demonstrated a manual and a reverse-pressure gradient method for inserting microscopic objects across plant cell walls. While using a pulsed laser for trapping particles is not the optimum method, we confirmed that it is possible, providing a more economical venue when working

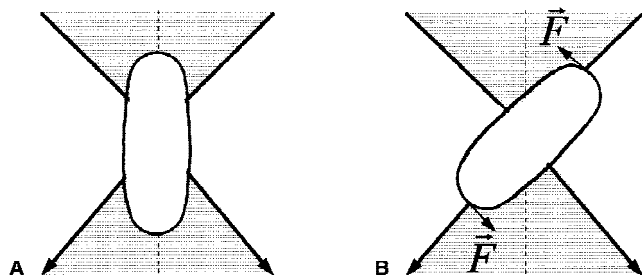


Figure 7. Theoretical location of the bacterium in the trapping beam. (A) Bacterium aligned along the beam axis while stably trapped. (B) Unstable trapping position of a bacterium. A torque is applied to the bacterium, aligning it along the optical axis as in (A). This torque is attributed to an intensity gradient force acting on individual electric dipoles within the bacterium.

under budgetary constraints. High plant cell survival rates were achieved under osmotic conditions necessary for plasmolysis. A method of introducing the various osmotic solutions and microscopic objects has been resolved. While our osmotic conditions may not be optimum for other plant species, our protocol should apply to other species and microscopic objects.

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