



## Gene delivery process in a single animal cell after femtosecond laser microinjection

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### ABSTRACT

Microinjection of extracellular molecules into a single animal cell was performed by an amplified femtosecond laser irradiation. When a single-shot laser pulse was focused on the plasma membrane of a single fibroblast from the mouse cell line NIH3T3 with a high-numerical aperture objective lens, a transient hole with a diameter of 1  $\mu\text{m}$  was formed. The delivery process of extracellular molecules immediately after the hole formation was monitored by a fluorescence staining with fluorescein-isothiocyanate–dextran (FITC–dextran). Then the gene expression was confirmed using a DNA plasmid of an enhanced green fluorescent protein (EGFP). The gene expression was observed when the laser pulse was focused first on the cellular membrane and then on the nuclear membrane, while the gene was not expressed when the laser was focused only on the cellular membrane. On the basis of these results, the efficiency of gene delivery by the femtosecond laser microinjection and the subsequent gene expression were clarified.

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## 1. Introduction

The injection of genes into cells is an indispensable method for investigating and controlling the individual functions of living cells. Various kinds of gene injection techniques, such as lipofection, electroporation, sonoporation, virus vector, and particle gun injection, have been already established and applied, but they cannot realize injection to an individual targeted cell. On the other hand, various microinjection techniques for single cells under a microscope have been developed and applied. While micro-needles and micro-pipettes have been used in the conventional microinjection procedure, laser microinjection has recently received much attention as a promising method for easy and efficient gene injection into a single cell.

An effective laser microinjection for tissues and organs has been established by applying laser-induced stress waves. The stress waves are generated by focusing the laser on a black rubber sheet overlaid on tissue, causing a DNA plasmid placed between the tissue and the rubber sheet to permeate into the tissue. This method has been reported to result in effective gene expression in tissues and organs [1–4]. The limitation of this method is that the stress waves cannot be focused on a specific single cell through the rubber sheet. Therefore, an alternative technique using direct laser irradiation to individual targeted cells has been proposed. An ultraviolet laser was initially used for the targeted gene transfection in the method [5], but it was determined that the laser irradiation disrupted the cellular integrity [6]. To overcome this limitation, a femtosecond laser has been applied as an alternative. When an infrared femtosecond laser is focused on the cell, a deformation of the cell is induced at the laser focal point due to the effective multi-photon absorption, and gene delivery from outside the targeted cell is promoted. König et al. were the first to demonstrate that a gene can be injected by focusing a pulse train from a Ti:Sapphire femtosecond laser oscillator on an individual cell [7,8]. When the femtosecond laser is focused by a

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high-numerical aperture objective lens, the deformation area is three-dimensionally confined within a sphere of 1  $\mu\text{m}$  diameter. Since most animal cells are larger than 10  $\mu\text{m}$ , we can selectively modify the specific position of the plasma membrane without destroying other components of the cell. This spatial selectivity also overcomes the geometrical limitations of microinjection using a micro-needle. Moreover, the femtosecond laser is advantageous not only because it permits effective multi-photon absorption but also because it realizes photomechanical ablation, thereby avoiding photothermal damage at the laser focal spot [9–11]. These noticeable characteristics have been widely exploited not only for laser microtransfection [12,13] but also for laser microsurgery [14–16].

In order to elucidate the dynamical processes of femtosecond laser injection, we must clarify how the gene is delivered into the cell as an event of the single-shot irradiation. In this work, the delivery process after the single-shot laser irradiation was investigated in detail by using an amplified Ti:Sapphire femtosecond laser, whose single-shot pulse makes a hole for cellular and nuclear membranes. As a sample, mouse fibroblast NIH3T3 cells were used. A pulse train from an amplified 800 nm-femtosecond laser was focused on the individual NIH3T3 cell with a high-numerical objective lens and the deformation process of the cell was monitored by a high-speed camera. The permeation of the extracellular materials immediately after the laser irradiation was investigated by a fluorescein isothiocyanate molecule conjugating a sugar chain of dextran (FITC-dextran), whose electrostatic properties and size are similar to those of a DNA plasmid used in our experiment. The gene expression after the injection was confirmed by using a DNA plasmid of an enhanced green fluorescent protein (EGFP). On the basis of these results, the delivery process of the DNA plasmid after the laser irradiation was revealed.

## 2. Experiments

Mouse NIH3T3 fibroblast cells were cultured on a glass-bottom dish (Matsunami Glass, Osaka, Japan) coated with an extracellular matrix of fibronectin (5 mg/ml). The dish was filled with phosphate buffered saline (PBS) solution. Fundamental pulses from a regeneratively amplified femtosecond Ti:Sapphire laser (Hurricane: 800 nm, 120 fs; Spectra physics, Mountain View, CA) were introduced into an inverted microscope (IX71; Olympus, Tokyo, Japan), and focused on the sample through an oil immersion objective lens (100 $\times$ , N.A. 1.25) as shown in Fig. 1. The laser pulse energy was tuned to 5 nJ/pulse, which is slightly above the threshold energy to induce bubble generation in water (4 nJ/pulse). Since the spot size of the laser focal point was about 1  $\mu\text{m}$  in diameter, the laser fluence at the center of the laser focal point would reach a few hundred  $\text{mJ}/\text{cm}^2$  even when the laser energy was 4 nJ. The time evolution of the deformation of the cellular

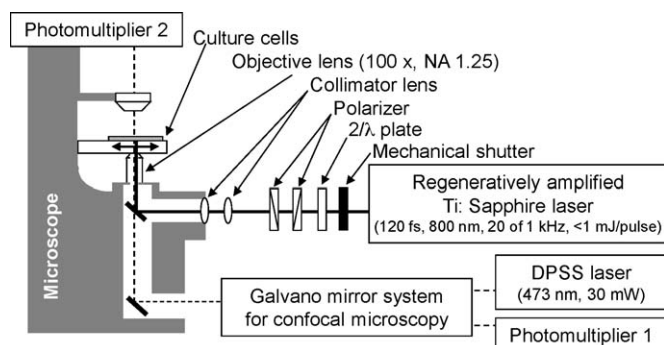
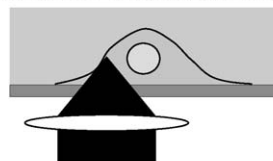
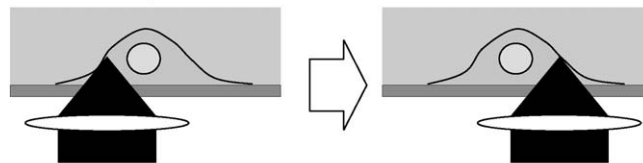


Fig. 1. Experimental setup for femtosecond laser microinjection.

Procedure A: One-point irradiation on cellular membrane



Procedure B: Two-points irradiation on cellular membrane



Procedure C: Sequential irradiation on cellular and nuclear membranes

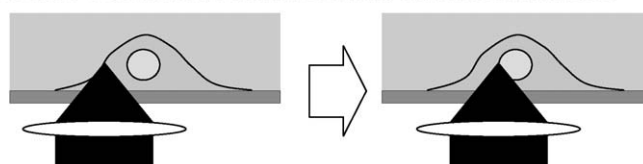


Fig. 2. Experimental procedure of femtosecond laser irradiation to a single cell.

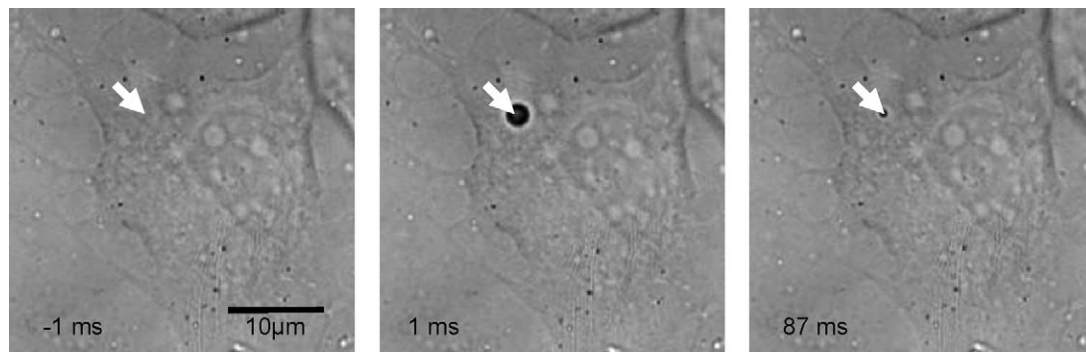
membrane was observed with a high-speed CMOS camera (FASTCAM-APX RS 250K; Photron, San Diego, CA) whose frame rate was 1000 frames/s. A halogen lamp was used as a light source to obtain transparent images.

The femtosecond laser irradiation for the microinjection was performed using the three procedures described in Fig. 2. In procedure A, the laser was introduced at a point on the cellular membrane. In procedure B, the laser was sequentially focused at two different points on the cellular membrane. In procedure C, the laser was first focused at a point of the cellular membrane and then at a point on the nuclear membrane. Two experiments were conducted for each of the three procedures—an experiment with single-shot irradiation and an experiment with 125 shots of irradiation to each irradiation point. The single pulse was picked up from the pulse train with a repetition rate of 20 Hz by a mechanical shutter whose gate time was 50 ms. The 125 shots of irradiation were performed using a pulse train with a repetition rate of 1 kHz, which was gated by a mechanical shutter with a time of 125 ms.

The distribution of molecules injected by the laser irradiation was monitored by adding FITC-dextran to the buffer solution at a concentration of  $5.0 \times 10^{-7}$  M. Immediately after the laser irradiation, the buffer solution containing FITC-dextran was replaced with intact buffer solution, and the fluorescence of the cells was measured by a confocal laser microscope system (FLUOVIEW FV300; Olympus, Tokyo, Japan) whose excitation light source was a 473 nm diode pumped solid-state c.w. laser (BL473T; Shanghai Laser & Optics Century, Shanghai, China). The setup is described in Fig. 1.

The gene expression after the laser irradiation was investigated by using pEGFP-actin plasmid (Clontech, Mountain View, CA), which was added to the buffer solution at a concentration of  $4.8 \times 10^{-8}$  M. The cells processed by the laser irradiation procedures, A, B, and C were cultured in a  $\text{CO}_2$  incubator for 24 h, respectively. Then the expression of the plasmid was investigated by the confocal laser microscope.

For each experimental condition, cells with number larger than 100 were tested and a histogram was produced. Each experiment was performed in triplicate, and the same tendency about the statistical distribution was obtained.



**Fig. 3.** Morphological change of the cellular membrane monitored by a high-speed imaging. The white arrow indicates the laser focal point.

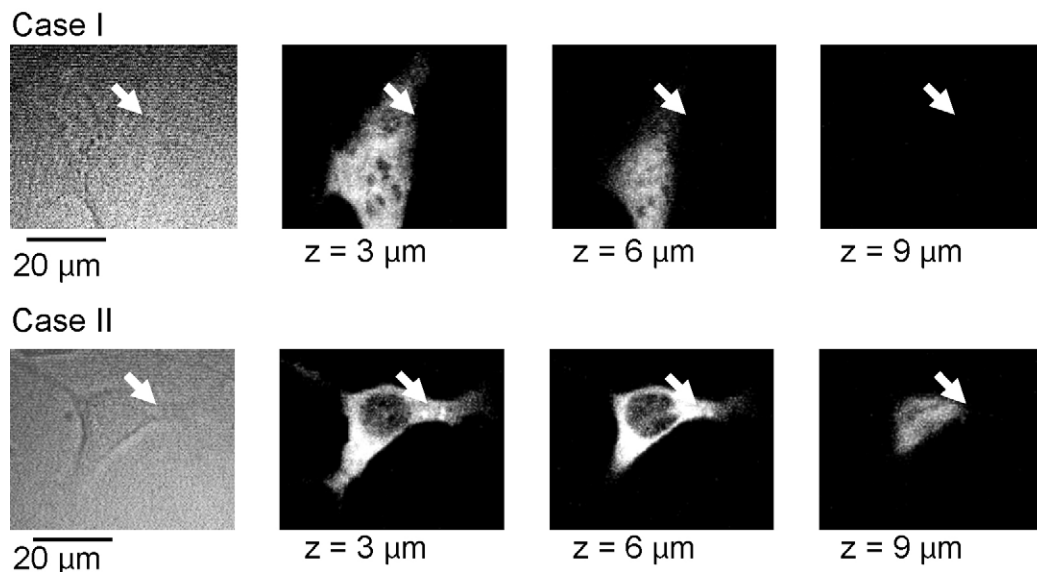
### 3. Results and discussion

**Fig. 3** shows high-speed images of the cellular membrane during the deformation process using the procedure A. When a one-shot laser pulse with energy of 5 nJ/pulse was focused on the membrane, a black spot was observed at the laser focal spot. The spot shrunk and disappeared within a few tens of ms. When a single-shot laser pulse with an energy above 4 nJ/pulse was focused on water, a similar bubbling was observed. This bubbling process by the single-shot laser irradiation has been explained as a lasting bubble formation process after shockwave and cavitation bubble generations [17,18]. Since the threshold energy required to cause the morphological change of the cellular membrane shown in **Fig. 3** was approximately the same as that required to cause bubbling in water, the cellular membrane would be deformed due to the generation of bubbles in water, which is the main component of the cell. In fact, not only the cellular membrane but also all the other parts of the cell, including the nuclear membrane, have been shown to have the same threshold.

When the femtosecond laser was focused in water, cavitation bubble generation was observed less than 10  $\mu$ s after shockwave propagation, and then the cavitation bubble collapsed within 100  $\mu$ s. On the other hand, in the case of high viscosity solution, e.g. poly(ethylene glycol) aqueous solution, the cavitation bubble does not collapse but rather remains for several seconds. Therefore the black spot on the cellular membrane would be attributed to

this lasting bubble. It is worth noting that the damaged membrane is repaired within a time scale comparable to that of the shrinking of the bubble.

The molecular injection was first examined by the procedure A with the single-shot laser irradiation. When the femtosecond laser was focused on cells immersed in buffer solution containing FITC-dextran, the FITC-dextran was introduced into the cells. This behavior was classified into two cases as shown in **Fig. 4**. In case I, the fluorescence of FITC-dextran was observed throughout all of the intracellular space. In case II, on the other hand, the fluorescence was observed throughout all of the intracellular space except for the nucleus. Such fluorescence inside the cell was only observed after the laser irradiation. After the observation by the confocal microscopy, Trypan blue was added to the culture medium. Since the cells in cases I and II were not stained by the Trypan blue, the cell would not lose its activity. In addition to cases I and II, there was another case in which the fluorescence was not observed inside the cell after the laser irradiation. This case was further classified into two types: one in which the cell was stained by Trypan blue and one in which it was not. In the former case the cell had died as a result of laser irradiation and in the latter the cell was alive. These differences in the results of the injection would be due to individual differences of the particular cell. The experiment using procedure A was also performed in several hundred cells by using EGFP plasmid instead of the FITC-dextran but no expression was observed.



**Fig. 4.** Transmittance and confocal microscopic images of the NIH3T3 cell after FITC-dextran was injected by femtosecond laser irradiation. The white arrow indicates the laser focal point. In case I, the whole intracellular space was stained, while the nucleus was not stained in case II.

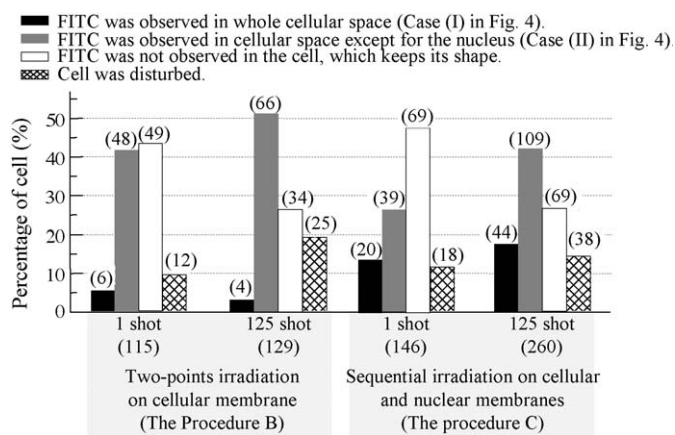


Fig. 5. Histogram indicating the cell behavior after femtosecond laser irradiation.

We therefore performed additional trials using the procedures B and C. Although the behavior of the tested cells could be categorized by the cases mentioned above, the rate of each case was dependent on the procedure and the laser-shot number. These results are summarized in Fig. 5. The rate at which FITC was injected into the cells (the sum of cases I and II in Fig. 4) was increased with the laser shot number, although the rate of increase was not linearly proportional to the shot number. It is worth noting that the ratio at which FITC was observed in nucleus (case I) was dramatically increased when the laser was sequentially focused on the cellular and nuclear membranes (procedure C). This indicates that the FITC–dextran was injected into the cell when the nuclear membrane was directly damaged by the laser.

The ratio of gene expression of pEGFP–actin after the femtosecond laser irradiation is summarized in Fig. 6. Clear gene expression, like that indicated by the inset microphotograph in the statistical chart, was only observed when the 125 pulses were shot using procedure C. This result suggests that the gene is only expressed when sequentially delivered across the cellular and nuclear membranes.

The efficiencies of the molecular injection by procedure C using 125 shots of laser irradiation are summarized in Fig. 7. The rates

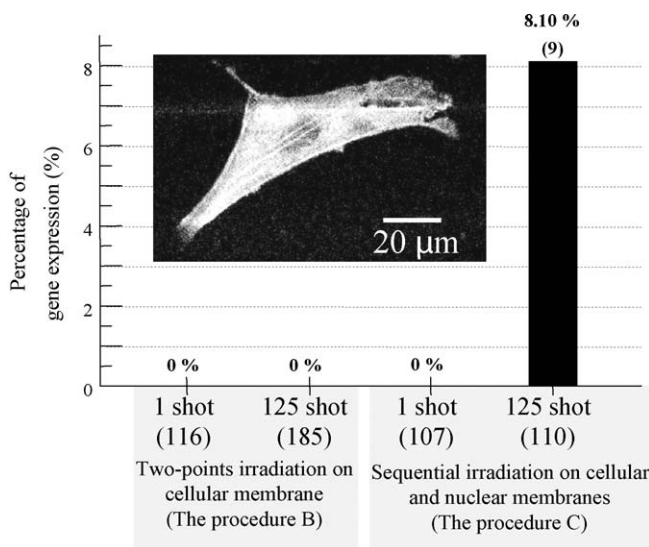


Fig. 6. Ratio of the gene expression of pEGFP–actin after the femtosecond laser irradiation. The inset microphotograph is a representative example of a pEGFP–actin expressing cell.

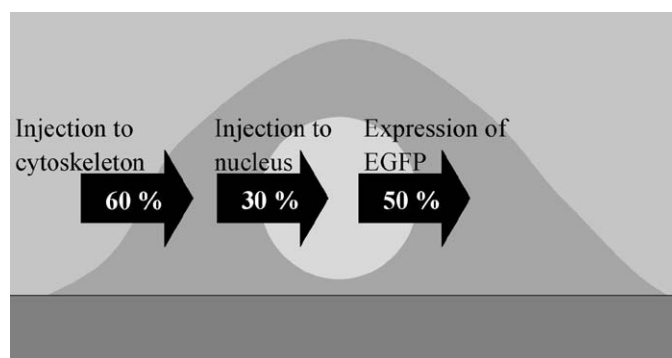


Fig. 7. Schematic for the efficiencies of gene delivery by the femtosecond laser injection.

were estimated from the right-side histograms in Figs. 5 and 6. The percentage of cells exhibiting effective injection from outside to inside the cytoskeleton was calculated as

$$\left[ \frac{X + Y}{Z} \right] \times 100 \sim 60[\%],$$

where X, Y, and Z are cell numbers of case I (44), case II (109), and the total (260) in Fig. 5, respectively. The percentage of cells exhibiting effective injection from cytoskeleton to inside the nucleus was calculated as

$$\left[ \frac{X}{X + Y} \right] \times 100 \sim 30[\%]$$

The percentage of cells exhibiting effective gene expression was calculated as  $\left[ \frac{\zeta / \Sigma}{(X/Z)} \right] \times 100 \sim 50[\%]$ , where  $\zeta$  and  $\Sigma$  are numbers of cells with gene expression (9) and tested total cells (110) in Fig. 6, respectively. This result suggests that the gene expression does not always occur in the nucleus even when the gene is injected into the nucleus. Since proteins of EGFP will be multiplied during the process of the gene expression, the fluorescence of EGFP (Fig. 6) should be more sensitive than that of FITC–dextran (Fig. 5). However, such enhancement was not observed in Fig. 6.

There is a probability that both cellular and nuclear membranes are deformed when the laser is focused on the nuclear membrane. Especially when the laser is tightly focused by the high-numerical objective lens through several different kinds of materials, as under our experimental conditions, it is difficult to avoid aberrations [19,20], which would stretch the laser focal point in the direction of the optical axis. However, the delivery of FITC–dextran and pEGFP plasmid was hardly observed when the laser was only focused on the nuclear membrane. Therefore, it was considered that nuclear membrane was only deformed when the laser was focused on the nuclear membrane in the above estimation of the efficiencies of the molecular injection.

On the basis of these results, we have come to the conclusion that, in order to realize effective gene injection, it is indispensable to make a hole not only on the cellular membrane but also on the nuclear membrane. If the hole is too large to repair itself quickly, the cell will be fatally damaged. So it is suggested that an effective gene injection can be realized by making small holes on both the cellular and nuclear membranes over a relatively long period of time. In fact, very effective gene expression has already reported by Köning et al., who irradiated a pulse train with a high repetition rate from a Ti:Sapphire femtosecond laser oscillator to individual cells over several



seconds. Conclusively, our experiments clearly indicate dynamic processes of gene delivery in the laser microinjection.

#### 4. Conclusion

The molecular delivery process in a single animal cell after the femtosecond laser microinjection was confirmed by investigating the distribution of fluorescence molecules in the cell. The gene expression was initiated when the gene was delivered into the nucleus, though the gene was not expressed when the gene was only delivered into the cytoskeleton. Even when the gene was delivered into the nucleus, many genes should be injected in order to lead an exact gene expression. Such problem would be common not only for the direct laser microinjection but also for other gene injection enforced by physical perturbation, such as that by laser-induced stress wave, electroporation, or, particle gun injection. Our approach will contribute to a mechanistic understanding of how DNA can be input and expressed in living cells.

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