Single-cell optoporation and transfection using femtosecond laser and optical tweezers

Muhammad Waleed,¹ Sun-Uk Hwang,² Jung-Dae Kim,¹ Irfan Shabbir,¹ Sang-Mo Shin,³ and Yong-Gu Lee¹,*
¹Department of Mechatronics, Gwangju Institute of Science and Technology, 123 Cheomdan-gwagiro, Buk-gu, Gwangju, 500-712, South Korea
²Production Engineering Research Team, Samsung SDI Co., Ltd., 428-5 Gongse-dong, Yongin-si, Gyeonggi-do, 446-577, South Korea
³Department of Medical System Engineering, Gwangju Institute of Science and Technology, 123 Cheomdan-gwagiro, Buk-gu, Gwangju, 500-712, South Korea
*lygu@gist.ac.kr

Abstract: In this paper, we demonstrate a new single-cell optoporation and transfection technique using a femtosecond Gaussian laser beam and optical tweezers. Tightly focused near-infrared (NIR) femtosecond laser pulse was employed to transiently perforate the cellular membrane at a single point in MCF-7 cancer cells. A distinct technique was developed by trapping the microparticle using optical tweezers to focus the femtosecond laser precisely on the cell membrane to puncture it. Subsequently, an external gene was introduced in the cell by trapping and inserting the same plasmid-coated microparticle into the optoporated cell using optical tweezers. Various experimental parameters such as femtosecond laser exposure power, exposure time, puncture hole size, exact focusing of the femtosecond laser on the cell membrane, and cell healing time were closely analyzed to create the optimal conditions for cell viability. Following the insertion of plasmid-coated microparticles in the cell, the targeted cells exhibited green fluorescent protein (GFP) under the fluorescent microscope, hence confirming successful transfection into the cell. This new optoporation and transfection technique maximizes the level of selectivity and control over the targeted cell, and this may be a breakthrough method through which to induce controllable genetic changes in the cell.

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Transfection is the process of deliberately introducing external genes into living cells. After introducing external genes into the cell, genetic changes occur within the cell. There are many situations when it is desired to deliver a gene into the cell, such as in the case of gene therapy. This process is becoming increasingly accepted as a possible approach for the treatment of various and specific genetic diseases [1]. There are many transfection techniques that can be essentially classified into single-cell transfection and population/multiple-cell transfection. Population cell transfection techniques are employed where a number of cells need to be transfected at the same time. Major methods employed for multiple cell transfection are chemical-based transfection [2–6], electroporation [7], sonoporation [8–12], hydrodynamic [13–15], magnetofection [16] and gene gun techniques [17]; however, these techniques cannot be used where specific individual cells present in clusters of other cells need to be transfected.

Single-cell transfection techniques enable the individual monitoring of genetic changes in a specific cell. The advantage of single-cell transfection over the population/multiple-cell transfection method is that multiple controlled biological processes can be applied in a single unit (Petri dish). In other words, this technique allows for genetic changes to be made in a specific single cell present in a cluster of other cells that are not to be treated. Untreated cells that are present in the vicinity of treated cells act as a control and serve as confirmation that transfection occurred in the treated cells. Microinjection was the first technique to be used for single-cell transfection. Although it is a well-established technique to introduce foreign substances into targeted individual cells, it requires that the cover of the Petri dish be opened, which can result in contamination [18]. A plasmid decorated AFM probe has also been used for puncturing and insertion of plasmids into the cell; however, using AFM is difficult in cells immersed in medium and can result in permanent cell membrane damage [19].

Successful transfection results were achieved by laser-assisted optoporation [20–33] of the cell membrane, which intakes external DNA present in the medium through diffusion. In the case of laser-assisted optoporation of the cell membrane, nanosecond or femtosecond laser pulses have also been used to create pores or to alter the permeability of the cell membrane at a specific time to make it possible to intake external substances such as plasmids. This perforation is due to various localized effects, including the formation of plasmas with high free-electron density that eventually becomes a pore. This phenomenon was first discovered by Tsukakoshi et al. in 1984 [20].

Transfection by optoporation has proven to be a successful and excellent technique due to its high selectivity and controllability over the treated cell. For optoporation, a femtosecond laser has been used by a number of research groups for a number of cell lines, and cell viabilities have ranged from 50% to 100% [27–32]. However, these studies do not adequately explain the process by which the femtosecond laser focuses exactly on the cell membrane.
Due to this problem, the targeted cell has to bear repeated and unnecessary exposure of femtosecond laser energy as it takes many trials to focus the femtosecond laser on the cell membrane. To solve the problem associated with repeatedly focusing the femtosecond laser beam on the cell membrane, optoporation is demonstrated by using the Bessel beam [33] which has multiple focusing points. However, improvements should also be made to the femtosecond laser based optoporation systems in terms of focusing it on the cell membrane.

In the last decade, optical tweezers have been used in many biological applications. Optical tweezers are instruments that use tightly focused laser beams to exert an attractive or repulsive force to physically trap and manipulate microscopic dielectric objects. This phenomenon was first reported by Arthur Ashkin in 1970 [34]. Years later, Ashkin reported a tightly focused beam of light capable of holding microscopic particles stable in three dimensions, which are now known as optical tweezers [35,36]. Following that, optical trapping and the manipulation of viruses, bacteria, and silica-coated particles was also demonstrated [37,38]. Recently, optical tweezers were employed by Arita et al. [21] for transfection of single as well as multiple cells. In this process, optoporation is achieved by laser induced breakdown of an optically trapped nanoparticle with a nanosecond laser pulse. In above mentioned laser based perforation techniques, plasmids are added in the media of cell culture dish which slips into the cell through the cell membrane when it is made permeable. In these techniques, plasmid may or may not diffuse into the punctured cell depending upon the concentration of the plasmid in the media.

In present work, we have developed a new optoporation and transfection technique by trapping and inserting the polystyrene-based plasmid-coated microparticle into the single layer adhered MCF-7 cell [39] using optical tweezers. The cell membrane is first punctured and plasmid-coated microparticle is inserted into the cell using optical tweezers. Contrary to the previously discovered techniques in which plasmids need to slip into the cell, our technique removes the possibility of non-insertion of external gene. Besides optical tweezing the plasmid-coated microparticle into the cell, our technique also opens possibilities to discover new features and take measurements inside the cell using the inserted particle. To achieve this goal, we have employed three laser beams. The first one is an 800 nm femtosecond laser, where 75 mW exposure power and a 100 ms pulse is used to puncture the cell membrane. The second laser used is a 1064 nm continuous wave (CW) laser, whose purpose is to trap and insert the plasmid-coated microparticle into the cell. The third laser is a 685 nm CW laser, which detects the exact position of the cell membrane so that the 1064 nm trapping laser can insert the plasmid-coated microparticle in the cell through the puncture site in the cell membrane. For optoporation, the behavior of irradiated cells was thoroughly analyzed to optimize various sets of parameters such as exposure time, energy, perforation diameter, and cell healing time. The trapped plasmid-coated microparticle not only helps to produce genetic changes in the cell, but it also acts as a probe through which to focus the femtosecond laser precisely on the cell membrane to puncture it upon first exposure. To detect the trapped particle position during the experiment, a 685 nm CW laser beam was used to generate the respective signal on Quadrant Photodiode (QPD).

2. Materials and methods

2.1 Preparation of cells

MCF-7 cells were cultured using cell culturing techniques on a grid Petri dish (ibidi, grid size = 50 µm). A grid Petri dish is used to relocate or revisit the cell after optoporation or transfection experiments to check the behavior of the experimented cell after a specified time period. Cells were incubated at 37°C, 5% CO₂, with 2 ml culture medium, which was prepared by mixing Dulbecco’s Modified Eagle Media (DMEM) with 10% v/v Fetal Bovine Serum (FBS) and 1% antibiotic solution (Penicillin). Cells were cultured on the glass bottom of the Petri dish for 48 hours to achieve good adhesion of cells at the bottom of the dish. Prior
to and following the experiment, cells were washed with Phosphate Buffered Saline (PBS) and culture media was also changed so that non-sticky cells floating in the cell culture media would filter out.

### 2.2 Preparation of plasmid-coated microparticles

The main purpose of this transfection technique is to deliver the external gene into the selected cell. For this purpose, we used 1 µm amino-based polystyrene microparticles (Spherotech, Cat. No. AP-10-10) and a plasmid named pAcGFP1-C1 (Clontech). Plasmids are coated on the microparticle which acts like a carrier of gene. This plasmid vector encodes a green fluorescent protein (GFP, excitation maximum = 475 nm; emission maximum = 505 nm) from a jelly fish named Aequorea coerulescens. It forms a true monomer and has been human codon-optimized for increased translational efficiency, resulting in a higher level of expression in mammalian expression systems. Once inserted into the cell, this vector will produce GFP, a protein that will fluoresce in a green color under ultraviolet excitation. In this experiment, a microparticle carrying the pAcGFP-C1 vector is inserted into the prokaryote cell through a small passage temporarily created by a femtosecond laser. The plasmid-coated microparticle is optically tweezered through the punctured cell membrane and is inserted into the cytoplasm. After insertion, the DNA produces GFP in the cytoplasm. This type of gene insertion is also established by the gene gun method [17], in which plasmid-coated gold particles are inserted into the population of cells for transfection.

In our procedure, plasmid and microparticles solutions are mixed well using vortex shaker. The large amount of plasmids is used so that all of the particles are coated by plasmids. However, a calculated amount of plasmids can also be coated on the microparticle, as microparticle carries specific amount of amino groups which bind to the external gene. Therefore, calculated amount of plasmids can also be delivered into the targeted cell. The plasmid and particle solution is mixed well and kept in a CO₂ incubator for 4 hours for adequate coating. Then this solution is centrifuged at 15,000 rpm for 15 minutes to separate the plasmid-coated microparticles and the unbound plasmids present in the same solution. These plasmid-coated microparticles are resuspended in deionized (DI) water by vortex mixing for use in the optoporation and transfection experiment. The plasmid-coated particle solution can be sonificated for 1–2 seconds if the aggregation remains after vortex shaking. Prior to the transfection experiment, 10 µl of plasmid coated microparticles having concentration 3.39 × 10³ particles/µl were added into the Petri dish containing 2 mL of media. With this concentration of particles, we found an average of 3 particles present in 100 × 100 µm area of Petri dish near the cell surface. The average 2D size for single MCF-7 cell is comparable to grid of 50 × 50 µm. Therefore for a cluster of 4 cells, 3 particles were present near the cell surface during the experiment.

### 2.3 Experimental laser setup

The complete experimental setup for both the optoporation of MCF-7 cells and the manipulation of the plasmid-coated microparticles is shown in Fig. 1. For the optoporation of MCF-7 cells, a femtosecond laser beam (Pump Laser-Laser Quantum Ltd, Finesse 532, and Oscillator-Trestles-100) is employed, whose power is 400 mW; the duration of the pulses is 100 femtoseconds. The central wavelength is 800 nm, and the repetition rate is 80 MHz. A 800 nm femtosecond laser beam can also be used as a CW laser beam for bead trapping. The femtosecond laser beam is delivered to the periscope, which is used to make its height equal to other laser beams. Neutral density (ND) filters are used to attenuate the femtosecond laser power, and a beam shutter is used to expose the femtosecond laser on MCF-7 cells, only for the desired time. Optical trapping and manipulation of the plasmid-coated microparticles are accomplished by a CW, near infrared (NIR) fiber laser (B&W Tech, 1064 nm, 5W, TEM00). Femtosecond puncturing and trapping laser beams are then directed to a beam expander
(Thorlabs, BE02-05-B) through a dichroic mirror (DM1). The beam expander is installed in the setup to broaden the incident laser beams (3X).

These broadened laser beams arrive at the dichroic mirror (DM2) though the mirror (M1) and lens (L1). This expansion is necessary to overfill the objective lens to maximize the trapping force at the point of focus. For the detection of the relative position of the trapped plasmid-coated microparticle, a diode laser beam (Power Tech, 685 nm, and 50mW) is extended by the lens (L3) through a combination of puncturing and trapping laser beams at the dichroic mirror (DM2). The puncturing (800 nm), trapping (1064 nm), and detection (685 nm) laser beams pass through the lens (L2) and are then directed to the oil immersion objective lens (Olympus, 100 x , 1.4 NA), which is positioned on a piezoelectric objective positioner (OP) through another dichroic mirror (DM3). The purpose of this dichroic mirror is to reflect the laser beams and transmit the image plane of the microscope. All laser beams are made to be concentric at the lateral axis (x and y axes) using optical alignment techniques.

A DSP controller (nPoint, C.300 series) is employed for high-speed control of the piezoelectric device to move the lasers’ foci in an axial direction. Finally, puncturing, trapping, and detection laser beams are tightly focused onto the MCF-7 cell Petri dish. This Petri dish is placed at the motorized stage, with a 50 nm resolution and capable of moving in the x, y, and z axes. The scattered and transmitted light of the laser beams at the sample specimen are captured by the condenser lens (CL), reflected by the dichroic mirror (DM4), and focused by the lens (L4) on the quadrant photodiode (QPD, Thorlabs, PDQ80S1) mounted on a manual xyz stage. A band-pass filter (F) is also installed to block all the laser beams, except the detection laser beam, to reach the QPD. The QPD is located above the condenser lens and on the right-hand side of the band-pass filter. It is used to detect the relative position of the trapped microparticle during the experiment. Use of this QPD is critical because its signal will help monitor the relative position of the trapped particle. Furthermore, the cell demonstrates variable shape at every point on its surface; therefore, detection of the cell membrane location is also mandatory to successfully insert the plasmid-coated microparticle into the cell. Moreover, the QPD signal will help determine the exact location of the cell membrane. To get real-time bright-field images of the sample plane, a CCD camera (Cooled Retiga EXI, RBRC 03-10) is also installed, which is imaged through the tube lens (L5).
2.4 Measurement of relative distance between puncturing and trapping laser foci

2.4.1 Theoretical calculations

In the experimental laser setup, 800 nm femtosecond puncturing and trapping lasers are focused on the specimen by the objective lens. However, there exists both lateral (x and y-axes) and axial (z-axis) differences between the foci of puncturing and trapping lasers. This difference is majorly introduced by thick lenses L1 and L2. However, beam expander, objective lens and the properties of the focused Gaussian beam also contribute to this foci position difference. Lateral position difference between the laser foci can be corrected by good alignment of the optical setup, but the axial difference cannot be addressed because of the chromatic aberration behaviors of lasers of different wavelengths, which result in the axial differences between their foci. The relative foci difference between the puncturing and trapping laser needs to be measured for exact focusing of the 800 nm femtosecond puncturing laser on the cell membrane. Using the following two equations, the theoretical value of the laser foci wavelength differences across varying beams that are focused on by the same lens can be calculated:

Sellmeier Equation [40]:

\[ n^2(\lambda) = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4} - \frac{D}{\lambda^6} \]  

(1)

Thick lens equation [41]:

\[ \frac{1}{f} = (n-1) \left( \frac{1}{R_1} - \frac{1}{R_2} + \frac{(n-1)r}{nR_1R_2} \right) \]  

(2)

where \( \lambda \) is the wavelength of the incident laser beam, \( n \) is the effective refractive index of the lens, depending on the wavelength (\( \lambda \)) of incident laser beam. A, B, C, D, and E represent the Sellmeier constants of a certain material. In our case, the values for these constants are used for BK7 [42]. According to the supplier information for lens L1 (\( R_1 = 128.2 \) mm, \( R_2 = -128.2 \) mm, \( t = 3.3 \) mm), lens L2 (\( R_1 = 76.6 \) mm, \( R_2 = -76.6 \) mm, \( t = 4.1 \) mm) and in our optical setup, the distance between L1 and L2 is 200 mm and the distance between L2 and the objective back aperture is 85 mm. Using the above mentioned values, effective refractive index \( n(\lambda) \) and focal length ‘\( f \)’ for lens L1 and L2 is calculated for both laser beams. Finally, the foci difference is calculated by employing the matrix method for ray tracing [43]. The theoretical difference between the puncturing laser and the trapping laser foci was found to be approximately 892 nm. Furthermore, it was also observed that an 800 nm femtosecond puncturing laser spot is closer than the 1064 nm trapping laser to the sample specimen placed on the motorized stage.

2.4.2 Experimental measurement

In order to measure the axial offset between the two lasers’ foci, a new technique has been developed using optical manipulation of the trapped microparticles. Basically, a femtosecond laser (800 nm) is used to make a hole on the cell membrane, and the CW laser (1064 nm) is used to tweeze and insert the plasmid-coated particle into the cell. A third laser (685 nm) with much weaker power is also used to sense the bead touching the cell membrane. When measuring the relative distance between the foci of the 800 nm femtosecond laser and the 1064 nm CW laser, the femtosecond laser is switched to CW mode. Here, the femtosecond laser was used in CW mode to trap a 1 \( \mu \)m particle. It is only during this measurement of the foci distances when the femtosecond laser is switched to CW mode. Before the experiment, the objective positioner was set at its home position, and this position was used as the
reference point to measure the relative distance between the lasers’ foci. At first, a 1 µm polystyrene particle was trapped by an 800 nm femtosecond laser used in CW mode, as shown in Fig. 2(a).

Then, the trapped particle was manipulated in 50 nm steps of objective positioner towards the cover slip, which was placed at the specimen holder mounted on the motorized stage. Meanwhile, the detection laser focus is present on the particle and the scattered light from the trapped particle reaches the QPD which generates voltage in arbitrary units. If the particle is trapped and manipulated in the medium, detection laser focus position on the trapped particle will remain same and QPD will produce same amount of voltage. But when the trapped particle touches the coverslip and is further tried to push by displacing objective positioner, then the particle is hindered by the coverslip. However detection laser focus position is displaced from its previous position. Due to this, the scattered light is also changed and hence QPD shows a sudden change in its output voltage.

At this point, the laser focus position was measured by recording the position value of the objective positioner. Then, the trapped particle was brought back to its initial position, which was the home position of the objective positioner. In the second step, the 800 nm femtosecond laser is turned off and the 1064 nm laser is turned on immediately, as shown in Fig. 2(b). Then, the same particle was trapped by the 1064 nm laser, and the same procedure that was conducted for the 800 nm laser was repeated. The difference between the two recorded values of the position of the objective positioner will determine the relative difference between focus of the 800 nm and 1064 nm lasers, as shown in Fig. 2(c), 2(d). This experiment was performed multiple times, and average value was 1.2 µm. We have calculated the theoretical difference 892 nm caused by thick lenses L1 and L2 using Sellmeier and thick lens equations.
whereas the measured difference is 1200 nm. The extra 308 nm difference in laser foci could be produced by beam expander and objective lens.

2.5 Optimization of laser exposure parameters for the cell’s viability

As the MCF-7 cell membrane is made permeable by exposure of the 800 nm femtosecond laser, it is mandatory to optimize the different parameters such that the punctured hole is large enough to insert the plasmid-coated microparticle into the cell, keeping the cells 100% viable. These parameters could change between the different cell lines. Different parameters related to the cell’s viability, such as optimum laser exposure power, exposure time, puncture hole diameter, and healing time should be optimized before transfection [20–28]. This punctured hole is clearly visible through the CCD. The video graphic observation of cell during optoporation helps to determine the cell condition [44]. During the experiment, the cells are being observed from the top through CCD. When the cell is exposed to 800 nm femtosecond laser, a hole is produced on the cell membrane due to localized thermal effects. The field of view of CCD is calibrated. One pixel of the captured image corresponds to 127.5 [nm]. The images of punctured cell are captured during optoporation. The punctured hole size is determined by counting the number of pixels occupied by the punctured hole and multiplying it with unit pixel length. These are approximate measurements and have been taken to make experimentally easy the insertion of 1 µm particle into the cell. If laser exposure is appropriate, the punctured hole on the cell membrane is closed within few moments. We defined this as healing time. Moreover, the cell is “healed” means that after the laser exposure, the targeted cell remained healthy and grew like un-exposed cells.

Since we are introducing the 1 µm particle into the cell, the diameter of the punctured hole should be more than 1 µm. To increase the chances of particle insertion, we found it better to use 75 mW laser exposure for 100 ms to produce a 3 µm hole on the cell membrane so that a 1 µm particle could be tweezed easily inside the cell. The cell membrane healed completely in 3.5 seconds without any damage. The optoporated cells at above mentioned conditions are post analyzed for 96 hours. The cells grew like the normal un-optoporated normal cells depicting no damage to the cells. However, above the 3.5 µm punctured hole diameter, damage to the cell membrane was observed. Table 1 describes the cell’s viability on the different laser exposure conditions.

Table 1. Viability of the MCF-7 cell on different laser exposure parameters.

| Exposure Power (mW) | Exposure Time (ms) | Exposure Energy (mJ) | Punctured Hole Diameter (µm) | Healing Time (s) | Cell Membrane (Damaged/Not Damaged) |
|---------------------|-------------------|----------------------|-----------------------------|-----------------|-------------------------------------|
| 75                  | 20                | 1.5                  | 0                           | 0               | ND                                  |
|                     | 40                | 3.0                  | 0.45                        | ~1              | ND                                  |
|                     | 60                | 4.5                  | 1.3                         | 1               | ND                                  |
|                     | 80                | 6                    | 2.7                         | 2.5             | ND                                  |
|                     | 100               | 7.5                  | 3                           | 3.5             | ND                                  |
|                     | 120               | 9                    | 4.8                         | 20              | D                                   |
|                     | 140               | 10.5                 | 8.1                         | 300             | D                                   |
|                     | 160               | 11.25                | 9.3                         | 550             | D                                   |

2.6 Single-cell optoporation and transfection (TDFPI sequence)

For this purpose, an 800 nm femtosecond laser was mode-locked and set into a Gaussian beam profile. Propagation of this 800 nm laser beam was stopped by the beam shutter, which was opened for specific time (75 ~100 ms) to puncture the MCF-7 cells’ membranes. The 1064 nm and 685 nm lasers were also turned on, and the foci of all lasers were made in a concentric fashion using laser alignment techniques. The QPD signal upon exposure of the 685 nm detection laser was also checked and verified by trapping the microparticle. The MCF-7 cells were grown on the bottom of the Petri dish, and the plasmid-coated microparticles were added in the media of the Petri dish before the transfection experiment began. The Petri dish was
placed on the motorized stage, and the MCF-7 cells present on the grids were located by viewing them through a CCD camera using a custom-made user interface program.

2.6.1 Trapping

Before the experiment, plasmid coated microparticles are added into the MCF-7 cells’ Petri dish which are floating in the media. The first step is to trap single 1 µm plasmid-coated microparticle present in the Petri dish medium using the 1064 nm laser.

2.6.2 Detection

The shape of the cell varies from point to point and cannot be measured or estimated by viewing the cells through the CCD camera. Conventional femtosecond optoporation methods do not adequately explain the exact focusing of the puncturing laser on the cell membrane at different points on the cell. These conventional methods do not consider the variance in the shape of the cell, and expose the laser on different points on the cell membrane without taking this fact into consideration. As a result, the laser-focused puncture will sometimes occur inside or outside the cell, which is not accurate. Therefore, it is necessary to find the exact location of the cell membrane so that the focal point of the femtosecond laser can be positioned exactly on the cell membrane.

For this purpose, the trapped plasmid-coated microparticle is manipulated towards the cell membrane using the 1064 nm laser, and when the trapped plasmid-coated microparticle touches the cell membrane, the signal created by the 685 nm detection laser on the QPD shows a sudden change. At this point, the position of the objective positioner shows that the cell membrane is present at this position and the trapped plasmid-coated microparticle touches the cell membrane. We know that there is a relative distance between the foci of the 1064 nm trapping laser and the 800 nm femtosecond puncturing laser, meaning that at this position when the particle is touching the cell membrane and if the puncturing laser is turned on the laser will focus inside the cell at a 700 nm distance; the objective lens focuses the 800 nm femtosecond puncturing laser behind the 1064 nm trapping laser. Figure 3 presents a schematic representation, illustrating the position of the lasers’ foci inside the cell.

2.6.3 Focusing

Now, the 800 nm femtosecond puncturing laser needs to be focused on the cell membrane. For this purpose, the objective positioner is moved away from the cell membrane by 700 nm. At this position of the objective positioner, the focus of the 800 nm laser will be 700 nm inside the cell and it is not focused exactly on the cell membrane. Hence, by optical manipulation of the trapped particle, a new technique has been developed to focus the femtosecond laser exactly on the cell membrane. Figure 3(b)
shows that the focal position of the 800 nm femtosecond puncturing laser will be exactly on the cell membrane at this position of the objective positioner.

2.6.4 Puncturing

From this position, the beam shutter is actuated for 100 ms, at which point the 800 nm femtosecond puncturing laser is exposed to the cell membrane. The optoporation process is closely observed by CCD camera, which shows that a hole is created on the cell membrane when the focused femtosecond laser is exposed.

2.6.5 Insertion

Before the cell membrane heals itself, a trapped plasmid-coated microparticle is manipulated towards the pore and inserted. The plasmid-coated microparticle is optically tweezed immediately by the 1064 nm tapping laser and inserted into the cell before the cell membrane heals itself. Physically, after this insertion process, the presence or absence of the plasmid-coated microparticle is carefully observed by a CCD camera, and its absence shows that the plasmid-coated microparticle is inserted into the cell by this TDFPI process. If the particle is
not successfully inserted, then it will remain above the cell membrane and this can be viewed easily from the CCD camera. Complete TDFPI sequence for single cell transfection is shown in Fig. 4.

3. Results and discussion

3.1 Confirmation of transfection

A plasmid-coated microparticle is inserted into the cytoplasm through the punctured hole on the cell membrane, and the gene is transfected into the cytoplasm of the cell. The targeted cell exhibited green fluorescent protein (GFP) expression under fluorescence microscope, hence establishing this technique as an optimal way to achieve single-cell transfection. For the desired manipulation of optical devices, a customized user interface is also developed. From this user interface, we can easily perform experimental tasks: remotely controlling the trapping laser for the trapping and manipulation of plasmid-coated particles; establishing focus and exposure of the femtosecond laser on the cell membrane for optoporation; and visualizing/monitoring of the viability of the cells.

After the plasmid-coated microparticle is inserted into the cell, it is difficult to see the particle by CCD. We know that some researchers were able to move the trapped particles inside the cell, but they were not able to do this in a repeated manner. We think the green fluorescence from the transfected cell is proof of delivery of the microscopic particle inside the cell. To confirm the cells’ viability and the insertion of a plasmid-coated particle into the cell, images of the treated cells have been captured by phase contrast and confocal laser scanning microscopes, which show that the plasmid-coated microparticle is inside the cell and that it has been transfected into the cell. Figure 5 and Fig. 6 show the laser scanning microphotographs of the transfected cells.

Fig. 5. Confocal laser scanning microphotographs of MCF-7 cell 96 hours after optically tweezing the plasmid coated 1µm particle into the cell. (a) Transmitted light image with the focal plane above the top surface of the cell. (b) Transmitted light image of the cell with the focal plane inside the cell showing that particle is inside the cell. The inserted particle is marked by the circle. Horizontal and vertical lines are grid lines marked on the Petri dish. (c) Confocal fluorescence image. (d) Merged image.

Fig. 6. Confocal laser scanning microphotographs of MCF-7 cell after 72 hours of optically tweezing the plasmid coated 1µm particle into the cell. Two attempts were made on the same cell to insert the plasmid coated microparticle (a) Square boxes show the particles floating in the media. Encircled are the optically tweezed plasmid coated microparticles into the cell. (b) Confocal fluorescence image. (c) Merged image.
The efficiency of some transfection methods using optoporation techniques is tabulated in Table 2. Efficiency is defined as the ratio of the number of transfected cells versus the number of affected cells. The transfection efficiency for MCF-7 cells using our method is 12.7%, which was obtained from a population of 47 cells with six successful transfections. Although 12.7% efficiency is low compared to other methods, it may not be fair to compare these techniques in terms of efficiency alone. The transfection efficiency of mammalian cells varies from one cell line to another and MCF-7 cells are one of those aggressive cells that are very hard to transfect [17]. Moreover, there are other factors such as the type of gene, number of plastic-coated particles; amount of adhered gene onto them also influence the transfection efficiency. Our method is different from other methods in that we are treating a single cell, and the amount of the vector that is introduced in the cell is very small. The amount used is such that it only coats a single 1 µm diameter particle, and this is a significantly lower amount than is used by all of the other methods shown in Table 2. Furthermore, if in future, DNA capsule is invented encapsulating the concentrated and specific amount of gene, then our method will be highly suitable to insert calculated amount of gene into the cell that would be really helpful to study the cell genetics.

| Transfection by optoporation | % efficiency |
|------------------------------|--------------|
| Tirlapur et al. [29]         | 90%          |
| He et al. [22]               | 77%          |
| Sagi et al. [23]             | 41%          |
| Our technique                | 12.7%        |
| Mohanty et al. [24]          | <2%          |
| Badr et al. [25]             | 0.50%        |
| Tsukakoshi et al. [20]       | 0.3-0.6%     |

Based on the number of cells transfected per hour, it is important to introduce the term, “transfection frequency”; this definition is different from that used in the literature. Tao et al. [45] and Guo et al. [46] reported that the transfection frequency is $8 \times 10^{-4}$ and $4.8 \times 10^{-3}$, respectively. In these methods, the transfection frequency is calculated as the inverse of number of cells irradiated per hour in the presence of an ambient high concentration of plasmids in the medium. However, we would like to define transfection frequency in a different manner. Here, we express it as the number of cells that are treated in 1 hour. Using this definition, Tao et al. [45] and Guo et al. [46] methods can be stated as 1250 and 208 cells per hour. In our method, we were able to puncture and insert 20 cells an hour with plasmid-coated particles, which means that the transfection frequency is 20 cells per hour. Although, fewer cells are treated per hour, the magnitude of control of our approach is much more sophisticated. We have exact control of the amount of plasmid vectors that are introduced into the cell, while other methods blindly inject plasmid vectors with no control in terms of the quantity. We believe that in future applications the ability to precisely control the injected amount would be crucial. To our knowledge, our method is the only one that can control the amount of injected plasmid vectors. Second, our method is characterized by the absence of a high concentration of plasmids in the medium. High concentration of plasmids in the medium can have unknown effects to the cell. Furthermore, we can also devise experiments with the use of multiple and different plasmids acting on different cells located in the same Petri dish. In summary, the exact focusing of a femtosecond laser on the cell membrane, the insertion of a microparticle into the cell, as well as the level of selectivity and control over the treated cell are the major advantages of this technique when compared to other types of previously
discovered techniques for optoporation and transfection. We believe that these major advantages can make significant contributions in genetic engineering.

3.2 Transfection control experiment

As the last point of caution, we need to verify the fact that plasmids are introduced into the cell only through the insertion of the plasmid-coated microparticle, while excluding the possibility of that the plasmid-coated microparticles were inserted by other means. For this purpose, we conducted a transfection control experiment. For the control experiment, MCF-7 cells are prepared and plasmid-coated microparticles are added into the solution having concentration equal to that used for transfection experiment. This time, the trapping laser (1064 nm) and detection laser (685 nm) were turned off. The cells were only optoporated by femtosecond laser without using the probe just next to the plasmid-coated microparticle present near the cell membrane; however, the microparticle was purposely not optically tweezed into the cell. Although it took a few trials to puncture the cell membrane (which is unlike puncturing while using the probe, which requires only one-time laser exposure), the cell membrane was punctured with a hole size of 3 µm, which healed itself within 3.5 seconds. Cells were washed with PBS and the cell culture media were also changed after this experiment to remove plasmid-coated microparticles from the Petri dish. As a result of this optoporation, and after observing the cells under fluorescence microscope after 48-96 hours, the cells did not exhibit any green signals. This confirms that while plasmids were previously inserted into the cell only through the use of optical tweezers (resulting in successful transfection), transfection cannot be achieved in this experiment through optoporation alone without inserting the plasmid-coated microparticle into the cell.

Another control experiment was performed to justify the fact that particles that optoporation is necessary to insert the plasmid coated microparticle into the cell and without optoporation; particle cannot diffuse into the cell. In this control, we brought plasmid coated microparticle above the surface of the cell using optical tweezers and did not punctured the cell membrane. After observing the cell under confocal laser scanning 96 hours of post experiment, the cells did not exhibited any fluorescence which confirms that optoporation is also necessary to insert the plasmid coated microparticle into the cell. Figure 7 shows the microphotographs of the particles and cells in which plasmid coated microparticle was not inserted purposely. The cells did not exhibit any transfection result.

Fig. 7. Confocal laser scanning microphotographs of MCF-7 cell 96 hours after the control experiment. (a) The plasmid coated microparticles were brought above the cell membrane. Cells were not punctured and particles remained above the surface of the cell. (b) Fluorescence image of the control cells that did not exhibit any fluorescence.

4. Conclusions

In this paper, we have demonstrated a new technique that can be used to focus the femtosecond Gaussian beam exactly on the cell membrane. Furthermore, optoporation and transfection of a single mammalian cell is achieved by inserting the plasmid-coated microparticle into the specific cell using a femtosecond laser and optical tweezers. This technique is highly suitable for treating specific, individual cells in a cluster of other cell types.
that are not to be treated. Furthermore, this method facilitates the best level of control over the cell, as we can insert a plasmid-coated microparticle into a cell through any single point on the cell membrane.

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