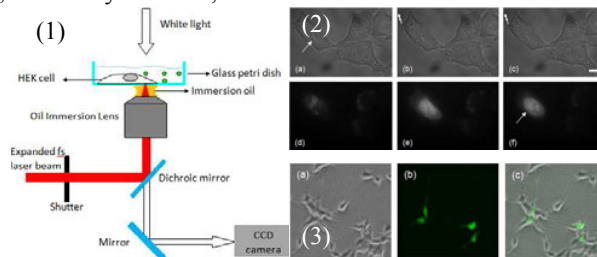


Femtosecond laser-assisted optoporation for drug and gene delivery into single mammalian cells

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Statement of Purpose: Targeted and reversible permeabilization of the plasma membrane is of paramount interest in today's biology and biotechnology. Transfection of cultured mammalian cells is also one of the indispensable techniques in biomedical research laboratories. In conventional methods, cells are treated as a population and the produced data only represents the average of the whole group. Single-cell manipulation and analyses has been drawing attention recently since it is well accepted that there are cell-to-cell variabilities in many biological aspects, and analyses of individual cells could often provide a wealth of information [1]. Optical-poration or optoporation using femtosecond laser has recently been receiving much attention as a promising method for easy and efficient delivery of extrinsic compounds into single live cells. Laser can produce a tiny, submicrometer-sized pore, which lasts for a fraction of a second, at a defined location on the plasma membrane of a target cell to facilitate introduction of membrane impermeable substances such as foreign DNA. Lack of standardization of various experimental parameters seems to be the reason for discrepancies among various labs. In this work, we present a detailed experimental study on standardization of the protocol for the femtosecond laser-assisted optoporation using human embryonic kidney (HEK) cells. We used both a membrane impermeable fluorescent dye and a plasmid DNA carrying the coding sequence for green fluorescent protein (GFP) to analyze the entry of extrinsic molecules into the target cells. We also simulated the near infrared (NIR) femtosecond laser ablation process.

Methods: All optoporation experiments were performed using 35 mm petri dishes with poly-D-lysine coated glass bottom. Human embryonic kidney (HEK) 293H cells were maintained in Dulbecco's Modified Eagle Media with 10% v/v Fetal Bovine Serum and 1X Antibiotic/Antimycotic Solution in a humidified incubator. Cells were seeded on the glass bottom dish and grown for 48 hours to achieve 10-30 % confluency before laser treatment. (Fig.1) Ti:sapphire femtosecond laser (Coherent Inc.) produce 100-femtosecond duration pulses at with a central wavelength of 800 nm at 80 MHz frequency. The glass bottom dish containing cultured HEK cells was mounted on the stage. The laser beam was focused on the cell membrane by a 100X oil-immersion objective lens which was mounted in an inverted microscope. The laser power, target location and laser exposure time was optimized for consistent results. SYTOX® Green nucleic acid stain was added to the culture medium before each optimization experiment. Cell viability was tested using Trypan Blue. For the transfection experiments, plasmid DNA pEGFP-N1 was added to the culture medium and targeted cells were imaged after 24-48 hours of incubation.



Results: We optimized the parameters for cellular optoporation by monitoring the influx of a fluorescent indicator SYTOX into the targeted cells. Upon entering the cells, the dye binds to nucleic acids and produce fluorescence signal. The optimal and most reproducible results were obtained from the laser exposures at 60 mW for 35 ms (Fig. 2). An increase of fluorescence inside the cell was observed as a function of time, indicating successful perforation of the plasma membrane. The SYTOX dye mainly accumulated in the nucleus as expected. As control, no sign of dye uptake was observed by the adjacent non-treated cells, indicating that the laser exposure on the plasma membrane is required to change the membrane permeability. Also, no change was observed when the laser focus was in the vicinity of the cell but not on the cell. The cells were negative to the Trypan blue stain which verifies the integrity of the cell-membrane and overall cell viability after laser treatment. Cells when exposed to higher values of laser parameters were permanently damaged (death, blebbing at multiple locations) (Data not shown). With lower than optimum conditions, no change was observed.

We used the optimized laser parameter (laser power = 60 mW; exposure time = 35 ms; location = protruding edge of cells) to perform DNA transfection experiments with HEK cells. Cells were irradiated in the presence of plasmid DNA pEGFP-N1 in the culture medium and then returned to the incubator. Positive expression of GFP in the irradiated cells and their daughter cells after cell division indicates that the plasmid DNA was successfully introduced into the cell interior by optoporation treatment (Fig. 3). No fluorescence was observed in the surrounding cells not exposed to the laser. Using simulations, we also demonstrate that the transient perforation created by the laser can even be smaller than the size of the laser focal volume.

Conclusions: In this work, a detailed experimental procedure was used to standardize the various parameters for targeted optoporation of human embryonic kidney (HEK) using femtosecond laser. A membrane impermeable fluorescent dye and a plasmid DNA carrying the coding sequence for green fluorescent protein (GFP) were used to verify successful and repeatable optoporation of HEK cells.

References: Sims CE, Allbritton NL. 2007. Analysis of single mammalian cells on-chip. Lab Chip