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Microsurgery of Cell Membrane with Femtosecond Laser Pulses for Cell Fusion and Optical Injection

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Abstract

We report on results of using femtosecond laser scalpel for microsurgery of plasma membrane of living cells. Femtosecond laser pulses were applied to initiate cell fusion as well as to perform reversible permeabilization of cell membrane required for efficient injection of extrinsic substances into the target cells. Laser-based cell fusion of mammalian embryo blastomeres as well as fusion of cell bodies of neurons of mollusk Lymnaea stagnalis were successfully carried out by applying single femtosecond laser pulses (second harmonic of a Cr:Forsterite laser system) 620 nm, 100 fs with the fluences of 0.42-0.71 J/cm^2. It was shown that the fusion of cells was completed within 5-60 minutes depending on the cell type. Successful permeabilization of a cell membrane and optoinjection of a membrane impermeable dye was performed with the help of a compact laser system for cell microsurgery DissCell-F (ytterbium laser, 1050 nm, 75 MHz, ~115 fs). In both cases the laser irradiation parameters were thoroughly optimized to achieve high viability of treated cells and high efficiency of the procedures of cell fusion and optical injection.

Introduction

Cell microsurgery with ultrashort laser pulses has become an important tool in various biological studies and medicine. Femtosecond (fs) lasers are widely used for high resolution imaging[1] of cells and tissues, for corneal surgery[2] in ophthalmology, for efficient caries removal in dentistry[3]. One of the most promising applications of femtosecond lasers is dissection[4] of cellular organelles and selective permeabilization[5] of cell membrane, so-called optical perforation (optoporation), enabling introduction of foreign reagents and compounds into the cells. In this paper we demonstrate the possibilities of reversible and irreversible cell membrane perforation with femtosecond laser pulses that can be effectively used for cell fusion and optoinjection of different extrinsic substances into living cells.

Femtosecond Laser-based Cell Fusion

Application of laser enables performing cell fusion with high selectivity as compared to standard fusion techniques that utilise chemical reagents or electric fields to initiate cell fusion. Laser-based cell fusion requires optimization of various parameters, such as laser pulse energy, exposure time or laser focus adjustment. To simplify the process of parameters optimization we used 2-cell mouse embryos for cell fusion according to the fact that embryos at this stage of development had a well-defined interface between two blastomeres. The interface can be easily visualized, exposed to laser radiation and the process of cell fusion can be monitored in real time. Schematic diagram of the experimental set-up used for these experiments is presented in Fig.1.

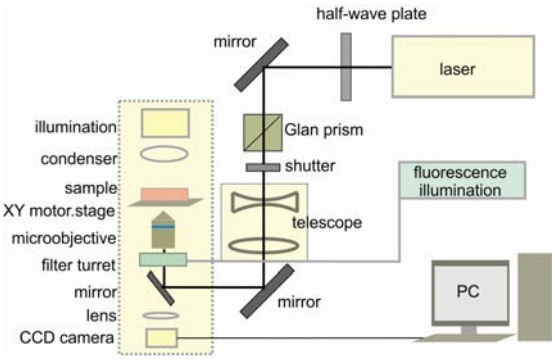

Figure 1: Experimental set-up for femtosecond laser-based cell fusion and optoporation

Femtosecond laser pulses were directed toward specimen through a 40x (NA=0.85) microobjective of an inverted optical microscope Axiovert 40 (Carl Zeiss). Two-cell mouse embryos were placed into the Petri dishes mounted on the X-Y motorized translation stage. Central region of plasma membrane in the interface between two blastomeres (indicated by white dot in Fig.2A) was exposed to femtosecond laser pulses at a wavelength of 620 nm (second harmonic generation, Cr:Forsterite seed oscillator and regenerative amplifier (Avesta Ltd.), 1240 nm, 100 fs, 10 Hz). Application of a single femtosecond laser pulse gently ruptured membrane and initiated the merging of the two blastomeres into one (Fig.2B-C). Cell fusion process was usually completed within 20-60 minutes (Fig.2D).

Fused blastomeres were placed in an incubator and cultured until the blastocyst stage. Finally, the hybrid embryos were stained with Hoechst 33258 (Sigma) and Propidium Iodide and viewed under a fluorescent microscope to estimate the survival rates of treated embryos. It was determined that cell fusion occurred when energy of laser pulses was in the range of 30-50 nJ that corresponded to laser fluences of 0.42-0.71 J/cm^2. Peak intensity in these cases was close to 10^{13} W/cm^2. Laser-induced optical breakdown is supposed to be the main mechanism caused irreversible plasma membrane perforation. Instead of using fs laser pulses with repetition rates of tens of MHz, we obtained cell fusion when only one fs laser pulse was applied. The highest cell fusion rate (88.9%) was achieved when a single laser pulse with energy of 35 nJ (0.5 J/cm^2) was applied to initiate the fusion of the blastomeres. About 50% of the fused embryos in this case have demonstrated normal development for at least 3 days post fusion.

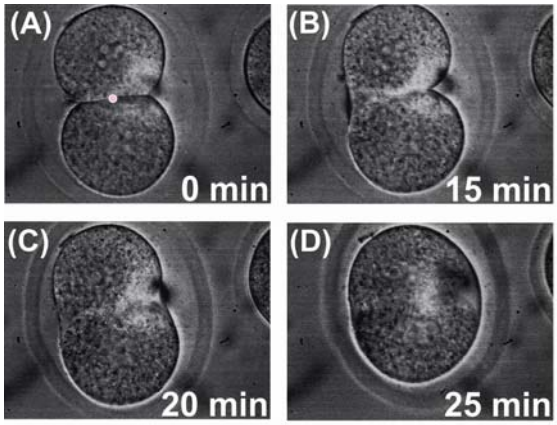

Figure 2: Embryo blastomere fusion by applying femtosecond laser pulses: (A) - two-cell mouse embryo (fs laser scalpel was directed at the position indicated by the white circle marker), (B-C) the process of cell fusion 15 min and 20 min after laser irradiation respectively, (D) - two blastomeres merged into one cell within 25 minutes.

Cell staining with a cell permeable nucleic acid dye Hoechst 33258 was done right after the blastomere fusion to demonstrate the formation of a "hybrid" cell. As can be seen in Fig.3A-B in the case of successful cell fusion two nuclei (appear in blue) inside single a "hybrid" cell were observed.

The developed technique of laser-based cell fusion was also applied to fuse cell bodies of two neurons of mollusk *Lymnaea stagnalis* (data not shown). Laser-mediated merging of the contents of two neurons was usually completed within ~5-7 minutes. It is believed that the laser-based technique of neuronal fusion has a great potential because it can significantly help in solving the problem of nerve damage repair.

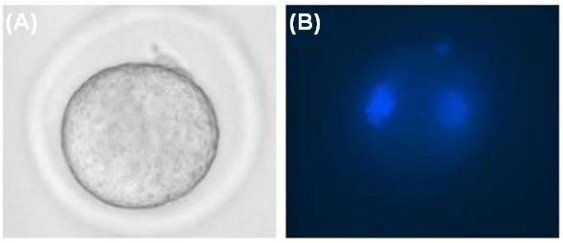

Figure 3: Brightfield image of the embryo after laser-induced blastomere fusion (A) and fluorescence image of the embryo stained with Hoechst 33258 (B) that demonstrates the appearance of two nuclei inside the "hybrid" cell.

Optical Injection with Femtosecond Laser Pulses

The delivery of exogenous DNA and various membrane-impermeable substances into living cells is essential for a variety of applications in genetics, cell biology and molecular biology. For this purpose, several techniques have peen proposed, including electroporation, microinjection, viral vectors, and chemical agents. However, all of these methods have some drawbacks. Some of them don't provide high selectivity (unsuitable for a single cell treatment) and high optoinjection/transfection efficiencies. Other methods are invasive and may cause cytotoxicity or mechanical damage to cells. An alternative optoinjection/transfection technique that is based on using femtosecond laser pulses for cell membrane perforation offers several key benefits over the methods listed above. It is minimally invasive technique and allows for very precise and gentle processing of preselected target cells. It has been shown to be useful even for introduction of extrinsic molecules into the so-called "hard-to-transfect" cells[6].

We have also obtained some promising results on laser-based delivery of exogenous substances into living cells of different cell lines, including Chinese hamster ovary cells, mouse fibroblasts, mesenchymal stem cells etc. In these experiments, we used a compact system for cell microsurgery DissCell-F (BML Technologies, Russia) that is available in two configurations. It can be based on a titanium-sapphire femtosecond laser (wavelength 800 nm, pulse frequency 77 MHz, pulse duration 40 fs, output power up to 650 mW) or on an ytterbium femtosecond laser (wavelength 1050 nm, pulse frequency 75 MHz, pulse duration ~115 fs, output power up to 4W). In the literature, typically Ti:Sapphire femtosecond lasers with a wavelength of 800 nm have been used to date in order to perform cell membrane perforation[7-8]. In this paper we present the results on laser-based optoinjection with fs laser pulses with the wavelength of 1050 nm. To our knowledge this is the first time that the cell membrane perforation has been performed using a NIR fs laser pulses with a wavelength of 1050 nm.

The photograph of the DissCell-F system used is shown in Fig. 4 (schematic diagram of the system is

similar to the scheme depicted in Fig.1). The infrared laser beam is firstly directed into the beam control unit, containing the beam expanding telescope, the beam attenuator and the mechanical shutter. After that the laser beam is guided to the objective of the inverted microscope using a dichroic mirror placed in the turret of the microscope. A brightfield or fluorescent image is acquired by means of a CCD camera attached to the right side port of the microscope.

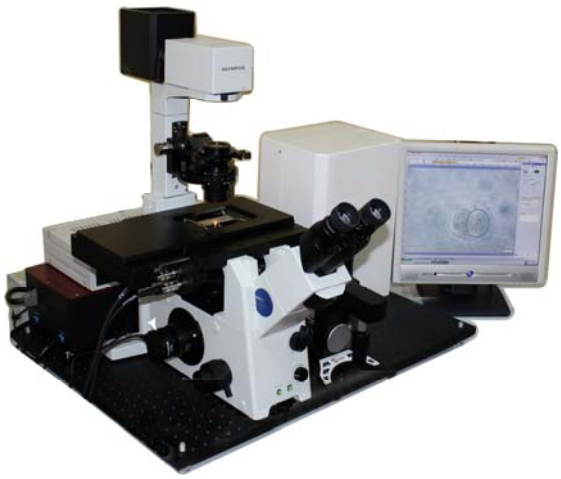

Figure 4: Photograph of the DissCell-F system for cell microsurgery (BML Technologies Ltd., Russia) based on ytterbium femtosecond laser

Before the experiments, the cells were incubated (37° C, 5% CO_2) for 1-2 days to allow cell attachment to the bottom of the glass Petri dishes with the thickness of 170μm (Ø35 mm). During the experiments the cells were placed on an X-Y-translation stage covered by a portable mini-incubator. Femtosecond laser pulses were precisely focused on the top of the cell membrane in order to create a transient pore in the membrane and initiate the diffusion of extrinsic molecules into the cell. To confirm the fact of successful membrane optoperforation we used a propidium iodide (PI) dye which is membrane impermeable in viable cells. The detailed protocol of cell preparation and staining can be found elsewhere[8]. Chinese hamster ovary cells were exposed to fs laser irradiation in order to inject propidium iodide dye (Fig.5A). Small amount of PI dye diffuses through the transient hole created in the cell membrane and fluoresces in red when exposed to green light. Low level of fluorescence indicates successful optoinjection (cells #1,2, Fig.5B) while intensive fluorescence of the nuclei of the cell indicates cell death (cell #3). As can be seen in Fig.5A, damaged cell#3 has demonstrated significant morphological changes within 30 minutes after laser irradiation.

We also used another fluorescent dye, Calcein AM, in order to assess the viability of the cells exposed to laser radiation. Viable cells usually fluoresce in green when exposed to UV light, whereas low level or no fluorescence is registered in dead or damaged cells (cells #1 and 2 are viable, and cell #3 is dead, Fig. 5C).

It should be noted that the effect of PI dye uptake was only detectable when the cavitation bubble was formed as a result of laser exposure. Several parameters, such as laser power, exposure time and focal spot size should be optimized in order to gently optoporate the cell membrane with minimal detrimental effects on the cell viability. Individual properties of each treated cell should also be taken into account. Nevertheless, it was possible to determine typical conditions required to create the creation of cavitation bubble and to inject the dye.

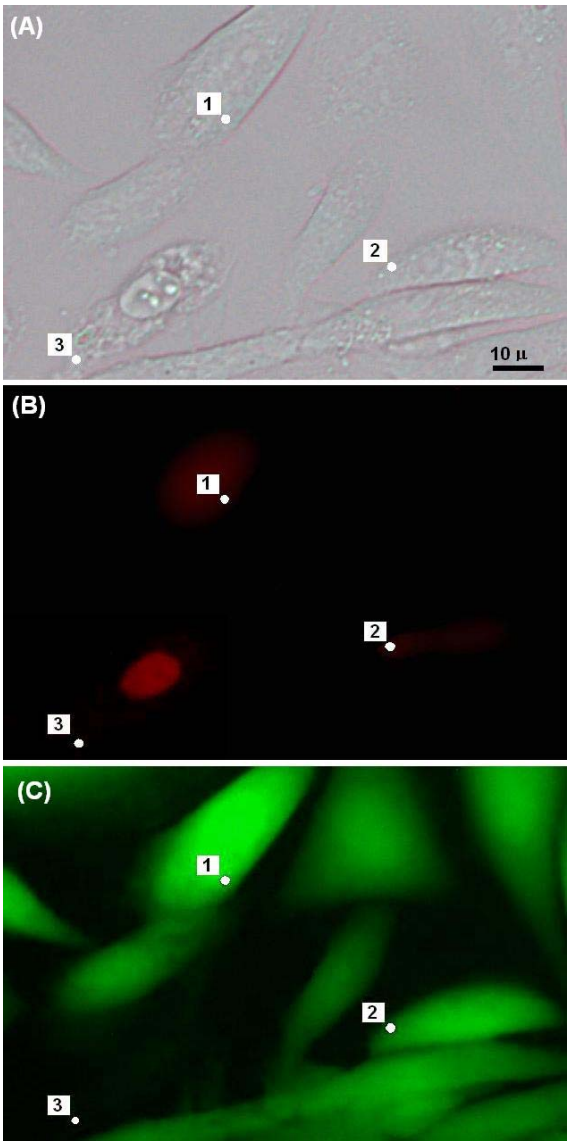

Figure 5: Femtosecond laser-based optoinjection: (a) Chinese hamster ovary cells within 30 minutes after irradiation with fs laser pulses, (b) optoinjection of membrane impermeable dye propidium iodide through the laser-induced pore in the plasma membrane, (c) analysis of cell viability by cell staining with Calcein AM fluorescent dye (cells #1,2 remain viable, cell #3 is dead)

We studied the efficiency of pore generation with laser beams focused to a different diameter on the cell membrane. Firstly we used a microobjective with a low NA = 0.6 and observed a formation of a cavitation bubble. However, in these cases creation of cavitation bubble can usually cause an irreversible damage to cell membrane and lead to a cell death within 30-40 minutes post irradiation. We have determined the mean power threshold for bubble formation. There was no cavitation bubble observed when target cells were exposed to laser radiation with mean power less than 300 mW even for 100ms (laser beam diameter in these experiments was close to 2 μm). Formation of cavitation bubbles started with 50% probability when we applied femtosecond laser pulses with a mean power of 320 mW and exposure time >75 ms. Using of exposure times less than 75 ms could also lead to creation of cavitation bubbles. However, in these cases very precise adjustment of laser beam focus on the target cell membrane should be performed. Applying fs laser pulses with a mean power >340 mW with exposure times of 50 ms initiated formation of a huge bubbles (d~10 μm) and inevitably led to 100% cell mortality. Thus, using fs laser pulses with average powers in the range of 320-340 mW and exposure times of 75-100 ms (30-50ms with tight alignment of laser beam focus on the cell membrane) can initiate the poration of cell membrane and promote the diffusion of extrinsic substances into the target cells. Nevertheless, care should be taken even when fs laser pulses with average power of 320mW (t=75-100ms) are used because of a risk of unexpected bubble formation with relatively large diameters of more than 3-5μ. The creation of such a bubble could potentially lead to a cell death. This risk can be avoided by using microobjective with a high numerical aperture. We used a 100x oil-immersion microobjective with NA=1.3. The beam diameter in the focal plane was slightly larger than 0.5 μm. The cavitation bubble was created when fs laser pulses with mean powers significantly lower than that in the first case were applied. As a rule, 50% probability of bubble formation was obtained when the cells were exposed to laser pulses with average power of 27-30 mW for 50-75 ms. Tight focusing of laser beam allowed one to reduce the exposure time required for the creation of cavitation bubble and poration of the cell membrane. As a rule, bubble formation was achieved in 100% cases when fs laser pulses with average power higher than 35 mW and exposure times >50 ms were applied. It should also be mentioned that the deviation between the sizes of the bubbles created was significantly smaller than in the first case and the standard size of the bubble was found to be ~1 μm. Thus, tight focusing of fs laser pulses as well as optimization of laser irradiation parameters (the laser power and the exposure time) allows one to control the process of cell membrane poration and obtain highly predictable results of optoinjection.

Conclusions

In this study, we have demonstrated the possibilities of applying femtosecond laser pulses for reversible as well as for irreversible cell membrane perforation. Laser mediated fusion of two-cell embryo blastomeres and laser-assisted delivery of cell-membrane impermeable dye into the living cells are shown. For these purposes we employed two different laser systems. The self-built Cr:Forsterite laser system was used for the cell fusion and the microsurgery system DissCell-F (BML Technologies Ltd.) equipped with an Ytterbium femtosecond laser was used for the targeted optoinjection. The laser radiation parameters also differed in these two tasks, but they were thoroughly optimized in order to achieve not only efficient cell fusion and optoinjection respectively but also to preserve the viability of the treated cells. We have demonstrated that the cell fusion can be achieved by applying a single laser pulse (100 fs, 620nm) with the fluence in the range of 0.42-0.71 J/cm^2. For the purpose of dye optoinjection we successfully exposed the cells to fs laser pulses with a wavelength of 1050 nm, and a repetition rate of 75 MHz. Depending on the numerical aperture of the microobjective used, the average laser power required for the cell membrane poration varied in the range of 320-340 mW (40x objective, NA = 0.6, the focal spot diameter ~2 μm, the exposure time 75-100 ms) and 25-35 mW for the microobjective with high NA (100x, NA = 1.3, the focal spot diameter ~0.5μm, the exposure time 50-100 ms). We have demonstrated that application of fs laser pulses allows precise, delicate, efficient and noninvasive performing of cell-membrane microsurgery. We believe that the presented techniques have great potential for gene therapy, bioengineering, biotechnology and basic researches in the field of cell and molecular biology.

Acknowledgements

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