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Noncontact microsurgery of cell membranes using femtosecond laser pulses for optoinjection of specified substances into cells

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Abstract. **IR femtosecond laser pulses were used for microsurgery of a cell membrane aimed at local and short-duration change in its permeability and injection of specified extracellular substances into the cells. The possibility of noncontact laser delivery of the propidium iodide fluorescent dye and the pEGFP plasmid, encoding the green fluorescent protein, into the cells with preservation of the cell viability was demonstrated.**

Keywords: *femtosecond laser pulses, laser microsurgery, optical transfection, cell membrane, optoporation, optoinjection.*

1. Introduction

At present lasers are widely used for performing surgical operations not only at the tissue level [1], but also at the cellular and sub-cellular levels [2]. Laser radiation sources are successfully used to modify the smallest structure elements of the cell [3–5], which offers the possibility of studying intracellular processes and revealing the interconnection between the structure and the functions of the cell components. One of the important problems of modern biology and medicine, for the solution of which the laser technologies are applied, is the intracellular delivery of various extracellular compounds [6], for which the natural transport into the cell is initially hindered. In this case the use of femtosecond lasers seems particularly promising, because they allow performing surgical procedures with submicron resolution and minimise the risk of damaging both the processed cells and the adjacent tissues as a result of nonlinear processes, since the energy of a femtosecond pulse is considerably smaller than the energy of a nanosecond one. The laser IR radiation is often used [7–9], which itself is poorly absorbed by the biological objects; however, if the intensity exceeds the threshold value, then nonlinear interactions of the laser radiation with the substance occur, giving rise to a considerable growth of absorption. Due to the nonlinear mechanism of interaction, the laser radiation affects only a small region near the focal spot, leaving the adjacent areas unaffected.

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As a rule, in foreign literature the laser technique of delivering various compounds, such as fluorochromes, proteins, ions, and even nanoparticles, is referred to using the general term 'optoinjection' [10]. The term 'optical transfection' [11] is used for a class of laser techniques used to introduce nucleic acids (RNA, DNA) into cells. The transfection procedure is a powerful tool for investigating the functions of genes and proteins. Using this technique it becomes possible to enhance or suppress the expression of certain genes, which may be used in genetic therapy when assigned genes are purposefully introduced into cells to treat certain diseases.

Among the available biological, chemical, and physical methods of transfection [12] the laser method is distinguished for several considerable advantages. First, it is noncontact and does not require the use of additional mechanical instruments or chemical agents. Second, this method can be applied for efficient transfection of cells of different types, including the stem ones [9]. Third, it provides high selectivity of cell transfection, which in some cases is one of the key requirements that determine the choice of the method. For example, in gene expression studies or operations with neurons [13], where each cell domain executes a definite function, just the selective transfection, rather than the mass one, is preferable. That is why the laser transfection method that allows not only individual processing of target cells, but also local action on the chosen cell zone, is the most promising technique.

The possibilities of successful optoinjection of various substances into the cells were demonstrated in [7–11]. In the majority of optoinjection cases the Ti : sapphire laser systems were used that generate femtosecond laser pulses at a centre wavelength of 800 nm. Probably this is because these systems were among the first to become commercially available. Nevertheless, they still remain expensive because of the necessity to use cw pump lasers operating at a wavelength of 532 nm. In this connection, it is important to study the possibilities to implement the cell optoinjection using alternative laser radiation sources with femtosecond pulse duration, less expensive and more reliable in operation. In the present work the injection of specified extracellular compounds into cells was implemented using the laser scalpel on the basis of an ytterbium femtosecond laser (1048 ± 2 nm, 75 MHz, ~115 fs) with a built-in diode pump unit, which significantly enhances the stability of the entire system and simplifies the laser exploitation. As far as we know, this is the first report of using laser radiation at this wavelength for optoinjection of extracellular compounds into cells. That is why in the paper we experimentally determine the optimal parameters of laser radiation and irradiation regime, necessary for membrane microsurgery and local short-time enhancement of the membrane permeability. The effect of laser pulses gives rise to for-

mation of self-contracting pores in the membrane. The efficiency of their formation was estimated by introducing into the cells the propidium iodide fluorescent dye, followed by fluorescence analysis. The viability of cells after the impact of laser radiation was assessed using additional staining with the vital dye calcein AM. The possibility of femtosecond laser cell transfection is demonstrated by the example of introducing the plasmid DNA pEGFP-N1, encoding the green fluorescent protein (GFP), into the cells of the CHO line (Chinese hamster ovary cells).

2. Experimental methods and objects of study

2.1. Experimental setup

The schematic diagram of the experimental setup is presented in Fig. 1. The femtosecond laser system (*1*) on the basis of the active medium with ytterbium ions generates laser pulses at the wavelength 1048 nm with the duration ~115 fs and repetition rate 75 MHz ('Avesta', Russia). By means of dielectric mirrors (*2*) and (*7*), efficiently reflecting the laser radiation at 1048 nm, the laser beam was introduced into the optical scheme of the AxioObserver Z1 microscope (Carl Zeiss, Germany). The radiation was directed into the microscope objective (*12*) and focused at the sample (*14*) by means of the mirror, mounted in the hexagon turret head. In the course of experiments the studied samples (CHO cell line) were placed in Petri dishes 35 mm in diameter with glass bottom 170 μm thick. The Petri dish was mounted in the holder of X–Y motor-driven stage (*13*), the movement of which along the specified trajectory allowed sequential irradiation of the chosen target cells (the velocity of the stage motion 10 mm s^{-1}, the minimal step width 1 μm).

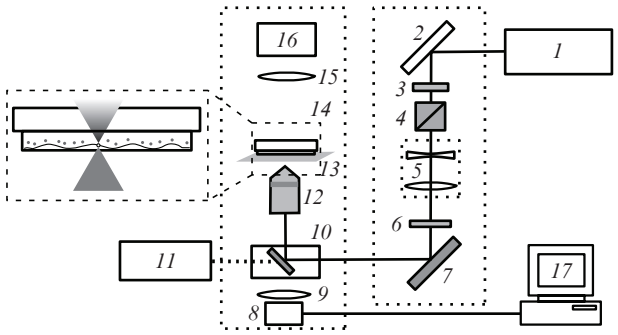

Figure 1. Schematic diagram of the experimental setup on the basis of a femtosecond laser for injecting extracellular material into cells: (*1*) femtosecond ytterbium laser; (*2*, *7*) mirrors; (*3*) half-wave plate; (*4*) Glan prism; (*5*) telescope; (*6*) mechanical gate; (*8*) CCD camera; (*9*) lens; (*10*) hexagon turret head carrying the mirror for directing the radiation beam and the sets of bandpass filters and beam splitters; (*11*) metal-halide lamp; (*12*) microscope objective; (*13*) motor-driven stage; (*14*) Petri dish; (*15*) condenser; (*16*) illuminator lamp; (*17*) personal computer.

To focus the laser radiation onto the object, we used a 40× LD Plan-NeoFluar microscope objective (Carl Zeiss) with the numerical aperture NA = 0.6 and a 100× AC Plan-NeoFluar microscope objective with the high numerical aperture NA = 1.3. The maximal output power of the laser system approached 3 W, which corresponded to the laser pulse energy of 40 nJ and repetition rate of 75 MHz. To control the laser pulse energy, the polarisation attenuator was used, consisting of the half-wave plate (*3*) and the Glan prism (*4*). By rotating the phase plate it was possible to change the output energy of the laser pulse. The telescope (*5*), consisting of the defocusing (f_1 = –100 mm) and focusing (f_2 = 400 mm) lenses, was used to enlarge the diameter of the laser beam in order to optimally fill the entrance aperture of the microscope objective and, therefore, reduce the diameter of the laser spot at the object. Besides, the telescope allowed correction of the laser radiation divergence aimed at superposing the plane of the laser beam waist with the object plane. Proceeding from cell to cell required minor tuning of the focus, since the surface of the glass substrate on which the cells are located was not ideally plane and had a certain inflection from centre to periphery. The necessity of focus tuning could be also caused by different thickness of the cells.

The duration of the laser pulse train action on the samples was regulated using the electromechanical gate (*6*) (Uniblitz, USA, minimal exposure time of 6 ms) located behind the telescope. Together with the mirror, the hexagon turret head carried sets of bandpass filters and beam splitters that were used for fluorescent analysis of the samples, subjected to the laser impact. The change of filters was implemented by rotating the turret head. To excite the fluorescence, we used the metal-halide lamp (*11*) (120 W, X-Cite, EXFO). The process of cell irradiation was recorded in real time using the CCD camera (*8*) (AxioCam, Carl Zeiss), attached to the exit port of the microscope and transmitting the signals to the personal computer (*17*).

2.2. Cell culture

In the experiments the cell line CHO-K1 was used. The data, presented in the paper, were obtained for the sample of 1000 cells. The CHO-K1 cells were cultured in the DMEM/F12 medium with 10% fetal bovine serum (Invitrogen, USA) in culture bottles with the growth zone 25 cm^2 (Greiner, Germany) in the CO_2 incubator (37 °C, 5%CO_2). A day before the experiment the cells were transferred to Petri dishes having the dimensions 35×10 mm with grass bottom and placed into the incubator for 24 hours. The obtained monolayer of cells was washed and incubated in the medium OptiMEM (Invitrogen, USA), to which, depending on the procedure type, we added fluorescent dyes (propidium iodide or calcein AM) and plasmid vector pEGFP-N1 that encodes the green fluorescent protein (Clontech, USA). After finishing the experiment the cells were washed with the OptiMEM medium, placed into the culture medium DMEM/F12 with 10% fetal bovine serum and transferred into the incubator. To confirm the optoinjection of the propidium iodide into the cells, during 40–50 minutes after the laser exposure the emission of light by fluorochromes accumulated within the cells was recorded. A similar method using fluorescent analysis was used to diagnose the viability of irradiated cells, but in this case instead of propidium iodide the vital dye calcein AM was added to the OptiMEM medium. The efficacy of the vector DNA transfection was assessed using the fluorescence of the GFP in cells in 24–48 hours after the laser impact.

3. Optoinjection of fluorescent dye and transfection of cells using femtosecond laser pulses

Fluorescent dyes introduced into cells can be used for efficient visualisation of various cell structures and investigation

Table 1. Basic parameters of laser radiation, causing the formation of cavitation bubbles.

Beam diameter/μm	Pulse duration/fs	Mean power/mW	Pulse energy/nJ	Intensity/10^{12} W cm^{-2}
2.0	100	215	2.8	0.9 ± 0.05
2.0	115	320	4.2	1.1 ± 0.1
0.75	115	37	0.5	1.0 ± 0.1

of their function [14]. In the present work the fluorescent dyes that cannot penetrate through the membrane of living cells were used as a diagnostic tool for increasing the efficacy of optoinjection by optimising the laser radiation parameters and irradiation regimes. To determine the optimal conditions for efficient poration of the membrane and increasing its permeability, the propidium iodide dye (Sigma) was used. The maximal excitation of the dye occurs at the wavelength 536 nm, while the maximal emission takes place at the wavelength 617 nm. This dye cannot penetrate through the membrane of living cells. If the membrane of the cell is irreversibly damaged, the dye penetrates into the cell and is bound by nucleic acids. If the integrity of the cell membrane was violated locally and during a short time interval, then the bright fluorescence of the nucleus is not observed. Instead in this case one can see a weak glow of the entire cell. This confirms the fact of successful permeabilisation (temporal change of permeability) of the cell membrane and preservation of the cell viability after the impact of laser pulses.

In the fluorescent dye optoinjection experiments the cells were incubated in the medium OptiMEM with propidium iodide (5 μg mL^{-1}). In 10 minutes the cells were analysed using the fluorescent Axio Observer microscope (Carl Zeiss). The absence of cell fluorescence confirmed the cell viability. Then a part of cells was subjected to the impact of femtosecond laser pulses. After a single opening of the electromechanical gate during a specified time interval and irradiation of the target cell the Petri dish was moved to a new position and a new cell was processed. The temperature during the experiments was kept 36–37 °C using a portable thermostat.

It was noticed that when the intensity of laser radiation exceeds the threshold value at the moments of laser impact, the cavitation bubbles appear at the surface of cell membranes. Subsequent fluorescent analysis confirmed the penetration of dye into the cell. Thus, the presence of bubbles can be a distinctive indicator of successful optoinjection of the propidium iodide into the cell. The effect of formation of cavitation bubbles depending on the intensity of laser radiation was investigated at the diameters of laser beam (at the level 1/e of the maximal intensity) 2.0 and 0.75 μm at the surface of the cell membrane. In the first case the focusing of radiation onto the cell membrane was implemented using the 40× microscope objective with the numerical aperture NA = 0.6, in the second case we used the 100× microscope objective with the high numerical aperture NA = 1.3. For example in Fig. 2 the formation of a cavitation bubble under the irradiation of the cell membrane with femtosecond laser pulses is shown. Table 1 summarises the experimentally measured threshold values of the laser radiation intensity (for different diameters of the focused laser beam and pulse durations), for which the formation of cavitation bubbles occurs with the probability of 50%. The error in determination of the laser pulse intensity was comprised of the errors of measuring the beam diameter and the radiation power. These results were obtained under the irradiation of the studied samples during 50 ms. At shorter exposure times, the formation of a cavitation bubble was observed extremely rare (probably, because of too small size of the bubble), and the subsequent fluorescent analysis did not confirm the fact of penetration of the fluorescent dye into the cell.

On the contrary, the increase in the exposure time leads to formation of bubbles of relatively large size (no smaller that 3–5 μm), which cause considerable damage of cells. Thus, the exposure time 50 ms was chosen as optimal, because the fluorescent analysis, performed in 40–50 minutes after the laser impact, confirmed the viability of the cells.

It is also worth noting, that despite the possibility of successful injection of the fluorescent dye into cells in both cases, the use of the objective with a large numerical aperture for focusing the laser beam is preferable. This is associated not

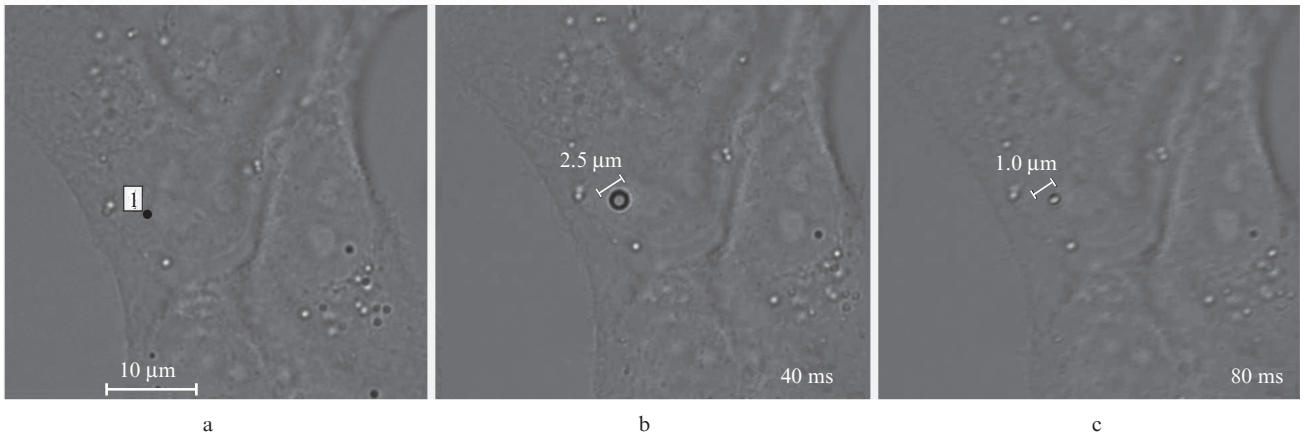

Figure 2. Formation of a cavitation bubble under the action of femtosecond laser pulses on the cell membrane: (a) the cell before the action of a train of laser pulses (the target area is marked with the black dot with number 1), (b) the formation of a cavitation bubble at the moment of laser action, and (c) the disappearance of the cavitation bubble.

only with a lower energy of the pulses, required for successful permeabilisation of the membrane, but also with the size of the produced cavitation bubbles. When the microscope objective with the numerical aperture 0.6 was used, it was found that the size of the produced bubbles can significantly vary and in some cases reach 5–10 μm in diameter. The formation of cavitation bubbles of such diameter inevitably causes irreversible damage of the membrane followed by the cell death. The use of a microscope objective with a large numerical aperture allowed control of the size of the produced cavitation bubbles (typical size of a bubble in this case was ~1 μm) and safely perform the procedure of optoinjection of the fluorescent dye.

Figure 3 presents the result of successful optoinjection of the propidium iodide fluorescent dye into the CHO cells 1 and 2. This result was obtained by irradiating the cells by laser pulses having the duration 115 fs and the energy ~0.5 nJ during 50 ms. Cell 3 is also shown, for which the irradiation was performed during 100 ms. One can see from Fig. 3a that cell 3 differs in morphology from cells 1 and 2 and from the surrounding cells, not affected by the laser pulses. The low fluorescence intensity of cells 1 and 2 (Fig. 3b) confirms the fact of successful optoinjection of dye and the cell viability, while the nucleus of the damaged cell 3 demonstrates high-intensity fluorescence.

To obtain reliable information about the cell viability after the laser impact, we stained the cells with the calcein AM dye (the maximal excitation occurs at the wavelength 496 nm and the maximal emission at the wavelength 517 nm). After the experiment on optoinjection of propidium iodide, the cells were washed with the OptiMEM medium, placed in the culture medium and kept in incubator for 30–40 min. Then, the culture medium was replaced with the OptiMEM medium, containing the vital dye calcein AM. The fluorescent analysis of the stained cells was performed in 20 min. Calcein AM easily penetrates through the living cell membrane. In this case the intense fluorescence of living cells is observed, while the dead and damaged cells do not fluoresce. One can see from Fig. 3c that the fluorescence intensity from cells 1 and 2, affected by the laser radiation, is comparable with the intensity from intact cells. Cell 3, damaged as a result of laser action, does not fluoresce. Hence, using double staining of the cells with the fluorescent dye calcein AM, we confirmed the viability of irradiated cells. This make it possible to conclude, that at the chosen parameters of laser radiation and the regime of cell irradiation it is possible to deliver into the cell the molecular compounds, not transmitted by the membrane, the viability of the target cells being preserved.

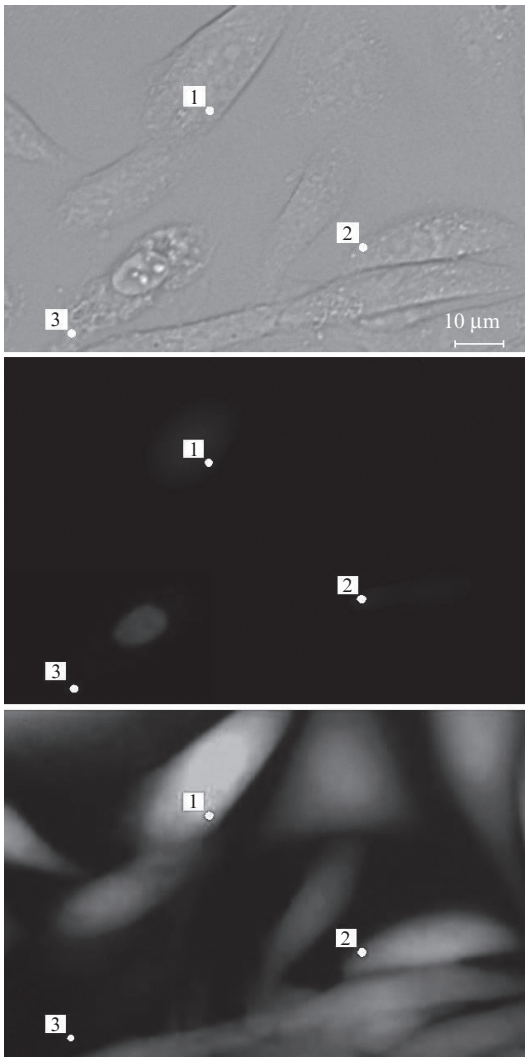

Figure 3. Optoinjection of the propidium iodide fluorescent dye into CHO cells using femtosecond laser pulses: (a) cells 1, 2, 3 after the action of femtosecond laser pulses; (b) fluorescence of propidium iodide in the cells (low intensity of the fluorescence of cells 1 and 2 confirms the fact of successful optoinjection of the dye, while the intense fluorescence of cell 3 nucleus is the evidence of the irreversible cell damage); and (c) staining of the cells with the dye calcein AM for assessing their viability (cells 1 and 2 are living, cell 3 is strongly damaged and does not fluoresce).

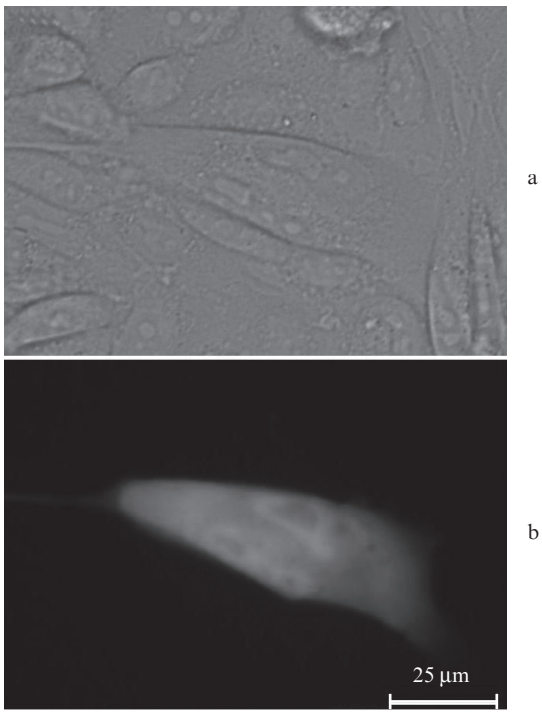

Figure 4. Femtosecond laser transfection into the cell of the plasmid pEGFP-N1 that encodes the green fluorescent protein: (a) the CHO line cells at the next day after the laser transfection experiment and (b) the intense fluorescence of the cell, confirming the introduction of the plasmid and the consequent expression of the GFP in the cell.

For the laser transfection of the cells, we used the irradiation regime and the parameters of laser radiation similar to the optimal regime and parameters, found in the dye optoinjection experiment. Before the beginning of the experiment the culture medium was replaced with the solution, containing the medium OptiMEM and the plasmid, encoding the green fluorescent dye (20–50 μg mL^{-1}). The target cells were subjected to the action of femtosecond laser pulses, after which the OptiMEM medium was replaced with the culture medium and the cells were placed into the incubator. The fluorescent analysis was performed 24 or 48 hours later. As an example, Fig. 4 shows the result of successful transfection of CHO line cells. The intense fluorescence of the laser-affected cell is seen, which confirms the injection of the plasmid and the following expression of the GFP in the cell.

4. Conclusions

In the present paper we report the results of using IR femtosecond laser pulses to provide efficient introduction of the DNA vector into cells. We determined the optimal parameters of laser radiation and the regime of irradiating the CHO line cells, chosen as model ones, for which the optoinjection of the GFP-encoding plasmid into the cells was implemented. It was shown that the viability of the cells is not reduced, if the optoinjection is carried out using laser pulses with the intensity, slightly exceeding the threshold values. In future the developed technique will be tested in other cell lines to obtain the statistical data on the transfection efficacy and the viability indicators of these cell types.

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