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Noncontact microsurgery and delivery of substances into stem cells by means of femtosecond laser pulses

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Abstract. **We have studied the efficiency of microsurgery of a cell membrane in mesenchymal stem cells and the posterior cell viability under the localised short-time action of femtosecond IR laser pulses aimed at noncontact delivery of specified substances into the cells.**

Keywords: *femtosecond laser pulses, laser microsurgery, stem cells, optoinjection, cell membrane.*

1. Introduction

Promising expectations in the field of regenerative medicine are associated with stem cells. Due to such properties of stem cells as the ability of self-maintenance and differentiation into mature specialised cells, new possibilities pave the way in the field of genetic and cell therapy of cardiologic [1], oncologic [2], neurodegenerative [3–4] and other socially significant diseases [5–6]. The implementation of these possibilities depends on how efficient the protocols of genetic manipulations with stem cells will be [7]; this particularly relates to the translation of genetic material (therapeutic gene) into the cells, on which the genetic therapy is based. The therapeutic gene can be injected either into cells preliminarily extracted from the organism and cultivated outside it (*ex vivo* therapy), or directly into the specified area of the organism (*in vivo* therapy). The success of the treatment depends not only on the used therapeutic gene and gene carrier, but also on the efficiency and safety of gene delivery into the cells.

The problem of efficient gene delivery into the cells can be solved by using modern laser technologies, particularly femtosecond laser pulses (FLPs), in cell membrane microsurgery to provide a short-time change in the membrane permeability. The technique of laser delivery of specified extracellular substances into cells possesses several advantages. First, it is noncontact, does not require mechanical instruments and keeps the sterility of the procedure. Second, the possibility to focus the laser radiation into a spot of submicron size provides high selectivity of the impact, i.e., it becomes possible to treat individually not only the selected cells, but also the selected part of a cell. Finally, the use of ultrashort laser pulses allows minimisation of the cell damage risk due to nonlinear processes and relatively small pulse energy, necessary to perform the 'surgical' procedure.

The possibilities of using laser radiation for efficient noncontact optoinjection of substances and gene transfection into cells are widely studied at the present time [8–20]. Initially it was important to demonstrate the possibility of *in vitro* cell transfection in principle, without quantitative estimates of the method efficiency, as, e.g., in Ref. [8], where the use of cw radiation of an argon laser and addition of the phenol red dye made it possible to deliver a plasmid construction into fibroblasts. In further studies the authors tried to obtain quantitative data on the efficiency of optoinjection and cell transfection. While, as a rule, the use of cw [8, 9] and nanosecond [10, 11] lasers did not allow high efficiency (typically the efficiency did not exceed 5%), the application of femtosecond lasers made it possible to improve this indicator by an order of magnitude (up to 50%–90%) in cell transfection *in vitro* [12–15] and stimulated the interest of researchers to further studies of the capabilities of laser-assisted delivery of substances into cells *in vivo* [16, 17].

To perform the femtosecond optoinjection and transfection the Ti:sapphire laser systems are mainly used [12–15] that produce FLPs at the centre wavelength of 800 nm. Probably, this is explained by the fact that these laser systems were among the first to become commercially available. However, because of the construction features and the necessity to use a cw pump laser operating at the wavelength of 532 nm, such systems are still rather expensive. In this connection it is urgent to apply different sources of femtosecond laser radiation, which are cheaper and more reliable in exploitation. Earlier [18] we demonstrated the possibility of a successful use of a laser scalpel based on an ytterbium femtosecond laser (1048 ± 2 nm, 75 MHz, ~115 fs) with a built-in diode pump unit for optoinjection and cell transfection, which significantly increases the stability of the entire system and facilitates the laser manipulation.

To provide clear comparison, our experiments were carried out with the cells of the CHO line (Chinese hamster ovary cells), often used by other authors [14, 15] as model ones. A series of successful experiments with the cells of this line allowed further studies of femtosecond laser-assisted delivery of substances directly into the stem cells, because in the work with stem cells that belong to the category of hard-to-transfect ones the application of femtosecond lasers may appear to be most reasonable [13, 19]. In spite of wide interest in the technology of laser-assisted delivery of substances into cells, only a few authors [13, 20] could demonstrate the possibility of successful femtosecond laser transfection in stem cells. The positive results (transfection efficiency of ~25% [20] and 70% using the FLPs with the duration ~12 fs [13]), obtained by the

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authors in certain categories of stem cells, confirm the urgency of further studies in the field of laser-assisted transfection and optoinjection of stem cells of various origin.

This paper presents the results of using femtosecond laser pulses of the IR range for affecting the membrane of mesenchymal stem cells and introducing extracellular substances into them. The cells were subjected to the injection of the propidium iodide fluorescent dye that simultaneously served as an indicator of successful membrane 'perforation' (i.e., the creation of a micropore in the cell membrane for transporting extracellular substances) by means of the FLP, and an indicator of the cell viability after the laser impact. The experimental studies were performed by using the special laser system including two femtosecond lasers, the ytterbium TeMa laser (Avesta) and the TiF-20F Ti:sapphire laser (Avesta).

The possibility of a successful use of radiation from the femtosecond ytterbium laser (1048 nm) for delivering not only the fluorescent dye, but also the pEGFP-N1 plasmid, encoding the green fluorescent protein, into the CHO line cells was demonstrated by us earlier [18]. In the present paper the radiation of this laser was used for microsurgery of cell membranes in stem cells; however, the efficiency of the extracellular substance delivery into the stem cells was found to be not high. The main reason was the reduction of viability of the cells, subjected to the laser impact. In order to increase the safety of the laser pulse effect on the stem cells, we decided to use the radiation from the femtosecond Ti:sapphire laser (800 nm) that produces pulses of shorter duration (50 fs instead of 115 fs). The experimental data presented below confirm the growth of efficiency of the dye optoinjection into the stem cells and the improvement of the indicators of cell viability in the case of using such a laser system.

2. Experimental setup

The schematic of the experimental setup is presented in Fig. 1. Initially, the laser system used in cell microsurgery was based on the femtosecond ytterbium TeMa laser (1048 nm, ~115 fs, 75 MHz) with the maximum mean power of 3 W, which corresponds to the maximum pulse energy of 40 nJ. Later, the laser system was equipped with an additional unit, including the TiF-20F Ti:sapphire laser (~50 fs in the sample plane, 800 nm, 80 MHz, mean power ~1 W). The radiation from both ytterbium and Ti:sapphire lasers was injected into the inverted Axiovert40 microscope (Carl Zeiss) via the side port, and by means of dielectric mirrors installed in the three-position turret of the microscope the radiation was directed into the microscope objective and focused on the sample.

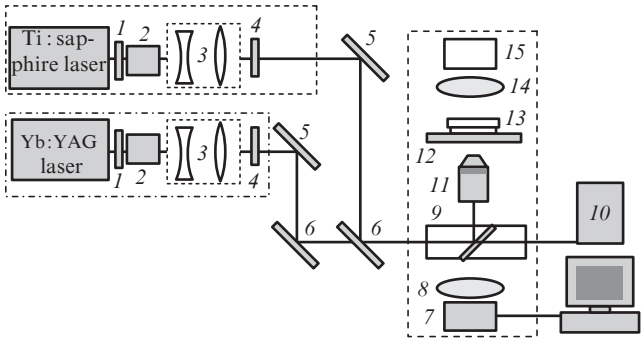

Figure 1. Schematic of the experimental setup on the basis of the femtosecond laser for injecting extracellular material into cells: (*1*) half-wave plate; (*2*) Glan prism; (*3*) telescope; (*4*) mechanical sutter; (*5*, *6*) mirrors; (*7*) CCD camera; (*8*) lens; (*9*) three-position turret with mirrors for directing the radiation and sets of bandpass filters and beam splitters for fluorescence analysis; (*10*) metal-halogen lamp; (*11*) microscope objective;(*12*) motor-driven stage; (*13*) Petri dish; (*14*) condenser; (*15*) illuminator lamp.

In order to obtain a cell monolayer with the required concentration, the samples under study (mesenchymal stem cells) were transferred from the cultural vials into Petri dishes (the diameter of 35 mm, the glass bottom thickness of 170 μm) a day before the experiment and were kept in the CO_2 incubator (37 °C, 5% CO_2). For the laser treatment the Petri dish was fixed in the holder of the $X-Y$ motorised stage (Merzhauser), moving which along the specified trajectory one could successively irradiate the chosen target cells (the stage motion velocity 10 mm s^{-1}, the minimal step 1 μm). The top of the dish was covered with a portable thermostat that kept the temperature at the level of 36–37 °C. Using the monochrome AxioCam Icm1 camera (Carl Zeiss) replacing one of the binoculars, we recorded the cell exposure process. To perform the fluorescence analysis of the irradiated cells in real time, the microscope was equipped with a fluorescence unit. The radiation from the halogen lamp (120 W, C-Cite, EXPO) was passed to the back port of the microscope via an optical fibre. The set of bandpass filters and a beam splitter, necessary for the fluorescence analysis, were installed in the microscope turret.

The energy of laser pulses was controlled by a polarisation attenuator consisting of a half-wave plate and a Glan prism (the output energy of laser pulses was varied by rotating the phase plate). To provide the maximum filling of the entrance aperture of the microscope objective with laser radiation and, therefore, to reduce the light spot size on the object, the telescope was used consisting of a diverging and a converging lenses. The telescope also allowed correction of the laser beam divergence, providing the coincidence of the beam waist with the object plane after focusing by the microscope objective. When proceeding from one cell to the other in the process of laser treatment, a minor focus adjustment was necessary because of the different cell thickness and the curvature of the glass substrate surface, which is slightly bent from the centre to periphery. The time of the sample exposure to the train of laser pulses was controlled by using the electromechanical shutter (Uniblitz, USA, minimum exposure time 6 ms) placed behind the telescope.

3. Femtosecond laser microsurgery of stem cell membranes for optoinjection of a fluorescent dye

In the studies we used multipotent mesenchymal stromal cells obtained from the human umbilical cord tissue (more than 800 cells). A detailed description of the culture characteristics, specific features of the extraction and cultivation can be found in Refs [21, 22]. The propidium iodide fluorescent dye (the absorption maximum at the wavelength of 536 nm, the fluorescence maximum at the wavelength of 617 nm) was used as a diagnostic tool to determine the optimal parameters of the laser radiation and the exposure regimes that allow high-efficiency membrane microsurgery and conserve the cell viability. Before the experiment the cells were washed and the cultural medium was changed for the OptiMEM medium, containing propidium iodide (Sigma) in the required concentration (5 g mL^{-1}). High-intensity fluorescence of the propidium iodide in the cell nucleus is observed in the case of death or severe damage of the cell [Fig. 2, cell (*1*)], because in living

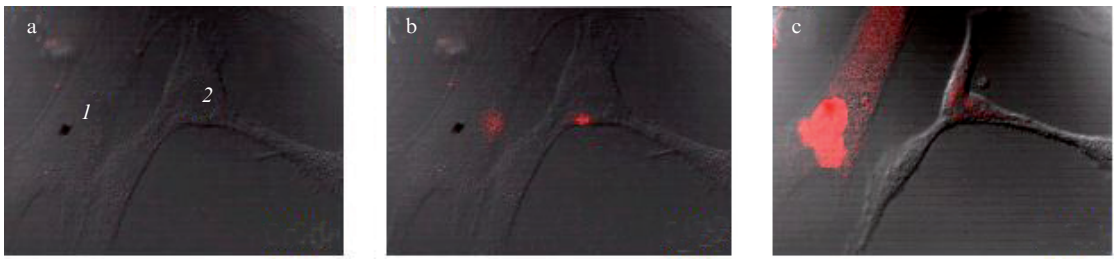

Figure 2. Dynamics of filling the cells with the propidium iodide fluorescent dye as a result of laser irradiation at (a) 0, (b) 5 s and (c) 5 min after the impact [high-intensity fluorescence of dye inside cell (*1*) indicates the irreversible damage of cell membrane, weak fluorescence intensity inside the cell (*2*) confirms its viability].

cells this dye does not penetrate through the membrane. That is why before the beginning of the experiment the fluorescence analysis of the cells was carried out. The absence of high-intensity fluorescence confirmed the viability of cells, after which a part of the cells was subjected to the FLP impact. After opening the electromechanical shutter for the specified time interval and irradiation of the target cell, the Petri dish was moved to a new position to treat the next cell. To provide reliable identification of treated cells, the Petri dishes were first specially prepared. Using a nanosecond UV laser (355 nm, 2 ns, 100 Hz) an array of horizontal and vertical lines was drawn on the inner surface of the Petri dish. The array mesh dimensions were 50–100 μm. On average, each mesh contained 5–15 cells, which was enough to make their further identification possible. An example of the Petri dish prepared for the experiment is shown in Fig. 3.

After the experiment the cells were washed with the

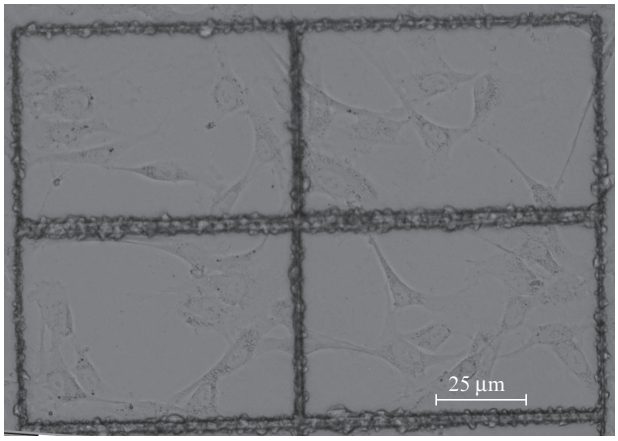

Figure 3. Surface of the Petri dish glass bottom with a monolayer of mesenchymal stem cells and the grid drawn to identify the cells after laser impact.

OptiMEM medium, placed into the cultural medium, and transferred into the incubator for some time. After 5, 20, and 40 minutes the emission of the fluorochrome inside the cells was recorded. An indicator of successful microsurgery of the cell membrane and injection of the dye into the cell was the presence of weak glow of the cell [Fig. 2, cell (*2*)]. The low-intensity fluorescence of the cell also confirmed its viability after the impact of laser pulses.

3.1. Use of the ytterbium laser for optoinjection of a dye into stem cells

The experiments carried out by us earlier with the cells of the CHO line, more viable that the stem cells, confirmed the possibility of successful cell transfection and optoinjection of extracellular substances by means of FLPs with the duration of ~115 fs, generated by the ytterbium TeMa laser. The microscope Olympus objective (UPlanFL, NA = 0.9) with the magnification 60× was used to focus the radiation onto the cells, which allowed focusing the laser radiation into a spot with the diameter of 1.8 μm. The efficiency of optoinjection approached 50% for single irradiation of CHO line cells during 50 ms by laser pulses with the energy of ~3 nJ (the mean power of ~220 mW), which corresponded to the intensity of $\sim 10^{12}$ W cm^{-2} on the sample.

For similar parameters of laser radiation, considered as optimal ones, a series of experiments with mesenchymal stem cells were performed. In the majority of cases the increase in the membrane permeability was observed together with the appearance of cavitation bubbles in the area of FLP action. The formation of cavitation bubbles having small size (~1–2 μm) lead to the local change in the membrane permeability and facilitated the transport of extracellular substances into the cell. The increase in the exposure time (as well as the increase of laser pulse energy) caused the formation of larger cavitation bubbles with the diameter of ~3–5 μm that irreversibly damaged the cell membrane and lead to the cell death.

Table 1 presents the data on the optoinjection efficiency and cell survival depending on the exposure time. It is seen that the increase in the cell irradiation time leads to the growth of the membrane permeability and increases the optoinjection efficiency, but at the same time the cell survival rate is essentially reduced. Therefore, it is reasonable to introduce

Table 1. Efficiency of optoinjection of the fluorescent dye into mesenchymal stem cells at different times of cell exposure to the pulses of ytterbium laser.

Exposure time/ms	Number of cells subject to successful injection (%)	Number of viable cells (%)	Final opto-injection efficiency (%)
6	0	100	0
10	0	100	0
20	18	84	15.1
40	50	34	17
50	65	16	10.4
60	85	12	10.2
80	90	5	4.5
100	100	0	0

the final efficiency criterion of optoinjection as $E_{inj} = (N_{inj}N_{via})/100\%$, i.e., the number of cells that experienced successful injection N_{inj} multiplied by the number of cells that remain viable after the irradiation N_{via}. The maximal final efficiency of the optoinjection E_{inj} achieved using the laser system described above amounted to 17% and corresponded to the exposure time 40 ms. In order to increase E_{inj} we decided to use laser pulses of smaller duration for the cell treatment. This was expected to reduce the risk of undesirable cell damage and thus to improve the survival rate indicators after the irradiation by FLPs.

3.2. Use of a Ti:sapphire laser for optoinjection of a dye into stem cells

Irradiation of mesenchymal stem cells with laser pulses having the wavelength 800 nm and the duration 50 fs was performed sequentially ('cell by cell') during a single opening of the electromechanical shutter for 6–100 ms. The same microscope Olympus objective with the magnification 60× was used to focus the radiation onto the objects. The laser beam diameter in the object plane (at the 1/e level of the intensity decrease) was equal to 1.6 μm. As a result of the experiments it was found that the probability of successful injection of the dye into the cell attains 50%, when the laser pulses acting on the cells have the energy not smaller than 1.4 nJ (i.e., for the peak intensity not less than 1.5×10^{12} W cm^{-2} and the mean power not less than 115 mW) and the cell exposure time ~20 ms. When the exposure time is increased beyond 40 ms or the pulse energy exceeds 1.9 ± 0.1 nJ, the cell viability sharply degrades due to the formation of cavitation bubbles of considerable size (greater than 3 μm), although the efficiency of optoinjection is high. Therefore, in order to save the viability of cells the exposure time and the pulse energy were restricted to 20 ms and 1.4–1.5 nJ.

For clarity Fig. 4 presents the data on the efficiency of dye delivery into the cells and the resulting survival rate after the irradiation with the pulses having the energy 1.5 nJ as a function of the exposure time. As it is seen, the successful optoinjection was observed in 65% of the cases for the exposure time 20 ms, and 50% of the cells remained viable (for smaller exposure times the final optoinjection efficiency remains low because of small probability of successful optoinjection, while for the times, greater than 20 ms, the viability of the cells drastically decreases). Thus, it was possible to attain $E_{inj} = 32.5\%$, which is by the factor of 1.9 greater than in the case of using the FLPs from the ytterbium laser (115 fs, 1048 nm). The experimental data demonstrate that the increase in the final efficiency of optoinjection when using the Ti:sapphire laser is related to the decrease in the pulse energy and, therefore, the mean power affecting the cells, as a result of reducing the pulse duration, which is a positive factor for the viability of cells. The reduction of the mean power of impact occurs also due to the decrease in the focused beam spot by ~1.25 times because of shorter wavelength of the radiation. Hence, to increase the final efficiency of optoinjection it is reasonable to use sources of femtosecond laser radiation with shorter wavelengths and pulse durations.

In the experiments we studied the possibility of further increasing the final efficiency of optoinjection by means of double and triple irradiation of cells with FLPs. For this goal the electromechanical sutter was opened for 20 ms several times to irradiate the same shutter. Before repeating the cell irradiation we performed the adjustment of the radiation focusing plane to fit the beam waist with the membrane surface. The obtained results are shown in the histogram (Fig. 5). It is seen that the optoinjection efficiency N_{inj} increases from 65% to 70% for double and to 74% for triple irradiation. This can be explained by supposing that at the very first time it was not always possible to focus the laser radiation exactly onto the cell membrane, and the additional adjustment of the beam focal plane to the membrane surface increased the probability of successful outcome of the laser manipulations performed. However, no significant increase in the final optoinjection efficiency could be achieved, which means that the major part of successful injections was already achieved during the single exposure. The subsequent double and triple exposure of the cells allowed an increase in the optoinjection efficiency by 5%–9%, but caused a sharp fall of the cell survival rate indicators (N_{via} decreased to 18% and 5%, respectively), which affected the final efficiency of optoinjection (E_{inj} decreased to 12.6% and 3.7%, respectively). Thus, we can conclude that for the specified parameters of the laser radiation (800 nm, 80 MHz, 50 fs, 1.5 nJ, and 120 mW) and the duration of exposure 20 ms the singe-exposure mode is optimal and allows one to achieve $E_{inj} = 32.5\%$.

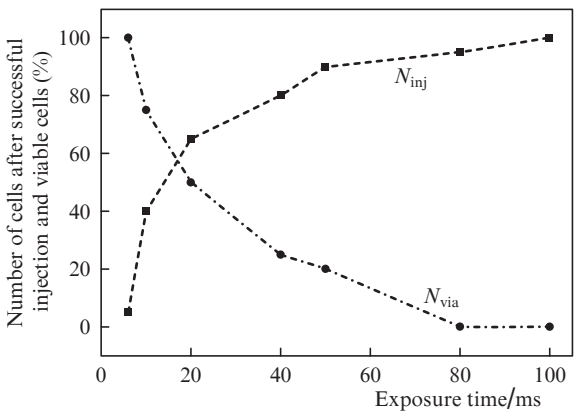

Figure 4. Number of mesenchymal stem cells that undergo successful injection and the number of viable cells vs. the exposure time when using the Ti:sapphire laser pulses.

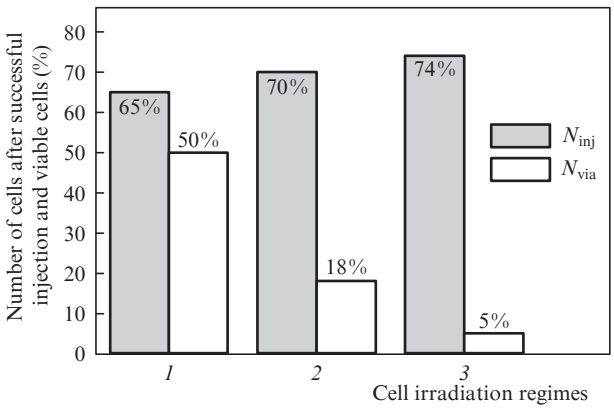

Figure 5. Efficiency of optoinjection of propidium iodide into the mesenchymal stem cells and the posterior survival rate of the cells under their (*1*) single, (*2*) double and (*3*) triple exposure during 20 ms to trains of femtosecond pulses with the energy 1.5 nJ (800 nm, 50 fs).

4. Conclusions

The possibility of successful application of femtosecond laser pulses in the IR range for noncontact injection of extracellular substances into the stem cells is demonstrated. The localised action on the cell membrane was implemented using the radiation from ytterbium and Ti:sapphire lasers. The parameters of laser radiation and the cell exposure regime were optimised such that not only the optoinjection efficiency could be increased, but also the cell viability could be saved. As a result, when using the pulses of the ytterbium laser (1048 nm, 115 fs, ~3 nJ) and single irradiation of the cells during 40 ms the final efficiency of the propidium iodide fluorescent dye optoinjection into mesenchymal stem cells amounted to 17%. It appeared possible to increase E_{inj} up to 32.5% by using the pulses of the Ti:sapphire laser (800 nm, 50 fs, 1.5 nJ). Similar to the first case, the optimal regime appeared to be single-exposure (during 20 ms) rather than multiple-exposure one. The experiments carried out confirmed the efficiency of applying femtosecond laser pulses for performing high-precision microsurgical procedures in stem cells and provided a base for further studies on the delivery of nucleic acids into the stem cells (laser-assisted transfection) with the aim of controlled modification of their properties and functions.

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References

1. Bradshaw A.C., Baker A.H. *Vascular Pharmacology*, **58**, 174 (2013).
2. Sagar J., Chaib B., Sales K., Winslet M., Seifalian A. *Cancer Cell International*, **7**, 9 (2007).
3. Muller F.-J., Snyder E.Y., Loring J.F. *Nat. Rev. Neurosci.*, **7**, 75 (2006).
4. Rizvanov A.L., Islamov R.R., Guseva D.S., Kiyasov A.P. *Kletochnaya Transplantologiya i Tkanevaya Inzheneriya*, **11**, 29 (2007).
5. Colella P., Cotugno G., Auricchio A. *Trends Mol. Med.*, **15**, 23 (2009).
6. Kaneda Y., Tamai K. *Arch. Dermatol. Res.*, **295**, S63 (2003).
7. Lakshmipathy U., Pelacho B., Sudo K., Linehan J.L., Coucouvanis E., Kaufman D.S., Verfaillie C.M. *Stem Cells*, **22**, 531 (2004).
8. Palumbo G., Caruso M., Crescenzi E., Tecce M.F., Roberti G., Colasanti A. *J. Photochem. Photobiol. B*, **36**, 41 (1996).
9. Nikolskaya A.V., Nikolski V.P., Efimov I.R. *Cell Communication and Adhesion*, **13**, 217 (2006).
10. Mohanty S.K., Sharma M., Gupta P.K. *Biotechnol. Lett.*, **25**, 895 (2003).
11. Terakawa M., Sato S., Ashida H., Aizawa K., Uenoyama M., Masaki Y., Obara M. *J. Biomed. Opt.*, **11**, 014026 (2006).
12. Baumgart J., Bintig W., Ngezahayo A., Willenbrock S., Escobar H.M., Ertmer W., Lubatschowski H., Heisterkamp A. *Opt. Express*, **16**, 3021 (2008).
13. Uchugonova A., König K., Bueckle R., Isemann A., Tempea G. *Opt. Express*, **16**, 3957 (2008).
14. Ma N., Gunn-Moore F., Dholakia K. *J. Biomed. Opt.*, **16**, 028002 (2011).
15. Stevenson D., Agate B., Tsampoula X., Fischer P., Brown C.T., Sibbett W., Riches A., Gunn-Moore F., Dholakia K. *Opt. Express*, **14**, 7125 (2006).
16. Zeira E., Manevitch A., Khatchatouriants A., Pappo O., Hyam E., Darash-Yahana M., Tavor E., Honigman A., Lewis A., Galun E. *Molecular Therapy*, **8**, 342 (2003).
17. Tsen S.-W.D., Wu C.-Y., Meneshian A., Pai S.I., Hung C.-F., Wu T.-C. *J. Biomed. Sci.*, **16**, 36 (2009).
18. Il'ina I.V., Ovchinnikov A.V., Chefonov O.V., Sitnikov D.S., Agranat M.B., Mikaelyan A.S. *Kvantovaya Elektron.*, **43** (4), 365 (2013) [*Quantum Electron.*, **43** (4), 365 (2013)].
19. König K., Raphael A.P., Line L., Grice J.E., Soyer H.P., Breunig H.G., Roberts M.S., Prow T.W. *Adv. Drug Delivery Rev.*, **63**, 388 (2011).
20. Mthunzi P., Dholakia K., Gunn-Moore F. *J. Biomed. Opt.*, **15**, 041507 (2010).
21. Saburina I.N., Gorkun A.A., Kosheleva N.V., Semenova M.L., Pulin A.A., Repin V.S. *Vestnik Novykh Meditsinskikh Tekhnologii*, **XVI** (4), 9 (2009).
22. Prihod'ko A.V., Isaev A.A., Kiseljov S.L., Lagar'kova M.A., Kosheleva N.V., Saburina I.N., Melikhova V.S. European patent EP 2277994 (A1). Date of publication 26.01.2011.