Noncontact laser microsurgery of three-dimensional living objects for use in reproductive and regenerative medicine

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Abstract. Laser microsurgery has enabled us to make highly precise and delicate processing of living biological specimens. We present the results of using femtosecond (fs) laser pulses in assisted reproductive technologies. Femtosecond laser dissection of outer shells of embryos (so-called laser-assisted hatching) as well as laser-mediated detachment of the desired amount of trophectoderm cells (so-called embryo biopsy) required for preimplantation genetic diagnosis were successfully performed. The parameters of laser radiation were optimized so as to efficiently perform embryo biopsy and preserve the viability of the treated embryos. Effects of application of fs-laser radiation in the infrared (1028 nm) and visible (514 nm) wavelength ranges were studied. We also applied laser microsurgery to develop a new simple reproducible model for studying repair and regeneration in vitro. Nanosecond laser pulses were applied to perform localized microdissection of cell spheroids. After microdissection, the edges of the wound surface opened, the destruction of the initial spheroid structure was observed in the wound area, with surviving cells changing their shape into a round one. It was shown that the spheroid form partially restored in the first six hours with subsequent complete restoration within seven days due to remodeling of surviving cells.

1. Introduction
It is hard to imagine that modern medicine and biology can operate without high-tech equipment and lasers, in particular. Advances in the field of laser technologies make it possible to perform rather challenging and time-consuming manipulations at tissue, cellular and even subcellular levels with unprecedented accuracy and selectivity. In current clinical practice and life sciences, laser microsurgery based on application of pulsed lasers has recently gained popularity [1]. Femtosecond laser sources are considered to be the most promising. They provide high spatial and temporal resolution, and have greater penetration depth, which is extremely important, particularly for affecting interstitial structures. Femtosecond laser systems have been shown...
to be useful and successful in different fields: for subcellular microdissection [2–4], inactivation of cell organelles [5, 6], separating single living cells from the group [7] and even for targeted optoinjection and laser transfection of living cells [8–11].

In recent decades, lasers have also been used for research purposes in the fields of embryology, developmental biology (for stem cell derivation, oocyte enucleation or cellular microsurgery), and in clinical practice, in the field of assisted reproductive technology (ART). This implies performing various micromanipulations on human oocytes and (or) embryos within a laboratory environment that result in successful fertilization, normal developmental physiology of the preimplantation embryo, high implantation rates and, finally, birth of a healthy baby. Various laser systems have been employed to perform different microsurgical procedures on embryos in nearly noncontact mode. Lasers have been used not only for assisted hatching, but also for sperm immobilization and selection, and for embryo biopsy for preimplantation genetic diagnosis as well. In earlier papers it has been shown that nanosecond lasers may be successfully applied for embryo microsurgery [12, 13]. At the present time picosecond [14] and femtosecond (fs) lasers [15–17] are mainly used. It should be pointed out that femtosecond lasers have several advantages as compared to continuous wave (CW) lasers or long-pulsed lasers. The main mechanism of microsurgery with femtosecond laser pulses is based on nonlinear absorption [18]. According to the fact that absorption of laser radiation and heating outside the area of laser focal spot are negligible, extremely high accuracy of laser microsurgery can be achieved with a minimal risk of damaging living cells and tissues. It is crucial when dealing with living cells or organisms. In this paper the results of successful application of femtosecond laser pulses for assisted hatching and embryo biopsy will be presented.

Lasers can be effectively used not only in the field of developmental biology, but also in the regenerative medicine. One such example will be discussed in this paper. Nanosecond laser microsurgery of cell spheroids was carried out in order to develop a new simple reproducible model for studying repair and regeneration in vitro. Repair and restoration of tissues play an important role in the normal functioning of the body. Repair mechanisms and problems of their failure, such as scarring of the parenchymal organs and skin, are not fully understood. A long-surviving, simple, reproducible three-dimensional (3D) cell system (i.e. spheroid) allows for the maintenance of the viable, functional and physiological state of cells in the absence of exogenous humoral and endocrine factors. Developing a model of spheroid damage using laser microdissection technique opens up the possibilities to study the mechanisms of regeneration and the role of spheroid-forming cells in repair.

2. Materials and methods

2.1. Experimental set-up

Two laser systems were used to perform microsurgery in living biological objects. One of the laser systems used was a combined system “Femtosecond laser scalpel-optical tweezers” designed and built at the Joint Institute for High Temperatures RAS. Detailed information about scheme of the system is given in [17,19]. The system consists of the inverted microscope Olympus IX-71 and two laser sources—cw and fs-lasers applied as optical tweezers and laser scalpel respectively. For laser scalpel, experimental setup allows implementation of both the fundamental frequency of laser radiation (wavelength of 1028 nm) and the second harmonic (wavelength of 514 nm). The system was applied for embryo microsurgery during the procedures of assisted hatching and trophectoderm biopsy. A 20 × NA = 0.5 micro-objective was used to focus the laser beam on the zona pellucida to carry out assisted hatching procedure; laser spot diameter was ≈ 3 µm for the fundamental frequency and about 2 µm for the second harmonic. For trophectoderm biopsy, a 40 × (NA = 0.8) micro-objective was used; laser spot diameter was about 1.5 µm for the second harmonic. The second laser system was a commercial Palm CombiSystem (Zeiss, Germany) which generates 2 ns pulses at a rate 100 Hz (wavelength 355 nm). The system was
employed for precise dissection of surface and inner layers of 3D cell spheroids. The energy of laser pulses was varied in the range of 7–9 µJ. Laser radiation was focused on the objects using micro-objective Zeiss Fluar ($10 \times NA = 0.5$). The focused spot radius was estimated to be $\approx 3.3 \, \mu m$. PALM RoboPro software was used to control laser scalpel.

2.2. Embryo collection and culture
All procedures performed in studies involving animals were in accordance with the bioethics standards of Biology department of Lomonosov Moscow State University. Female mice of the BALB/cJ strain aged 1.5–2 months were paired with males late in the evening. Females were checked the following morning for a presence of a copulation plug. The day of plug detection was counted as embryonic day 0.5 (E.0.5).

Zygotes were collected from fresh oviducts according to standard protocol [20]. Cumulus cells were removed by exposure to hyaluronidase solution (27 ME/ml, Cook). Embryos were then washed several times in fresh M2 (Millipore) medium and kept in culture in M16 (Millipore) medium in 4-well culture dishes under standard incubation conditions ($37 \, ^\circ C, 5\% \, CO_2, 98\% \, humidity$).

Embryos at E.2.5–E.3.5 were used in laser-assisted hatching procedure. Embryos were transferred to fresh drops of M2 medium in a 35 mm glass-bottom Petri dishes (170 µm thick, 5 embryos per dish) under mineral oil balanced with the medium. Experimental dish was placed on the motorized microscope stage and covered with a compact on-stage thermostat to maintain a temperature of 37 °C. After laser-assisted manipulations, embryos were transferred from the Petri dish into 4-well dish and filled with M16 (Millipore) culture medium. Embryos were then cultured under standard incubation conditions for 2 days.

2.3. Spheroid formation
Two primary cell cultures were used in this study: human bone marrow multipotent mesenchymal stromal cells (BM MMSC) and human skin dermal fibroblasts. Samples of the bone marrow were obtained after voluntary written informed consent of patients. Human fibroblasts were isolated from two biopsies of human skin obtained after informed consent of the donors. All the procedures were performed aseptically in compliance with the rules of ethics and the law on health care. Details of 2D culture of BM MMSC and human skin dermal fibroblasts prior to spheroid generation have been given in [21] and [22] respectively.

To obtain spheroids in 3D culture conditions, suspensions of characterized cells at the fourth passage were placed in agarose plates with microwells (Microtissue, USA) at 250.000 cells/ml concentration in complete growth medium. Agarose plates were transferred to wells of 12-well plates and were cultured for seven days under standard conditions (37 °C, 5% CO₂) in complete growth medium. Under non-adhesive 3D conditions, cell suspension successfully formed compact viable spheroids in seven days.

2.4. Analysis of cell spheroid reparation after laser microdissection
Analysis of spheroid reparation was initially performed using short-term time-lapse microscopy at the PALM CombiSystem photodetection installation (Zeiss, Germany) with photorecording every 5 seconds for the first 4 min after irradiation. Then long-term live registration for 7 days was performed under standard conditions 37 °C, 5% CO₂) in a chamber of the Cell-IQ time-lapse system (CM Technologies, Finland) with photorecording every 20 min using the Cell-IQ Imagen software. Spheroids of control and experimental groups at different time points after microdissection were fixed, with glutaraldehyde (1.5% solution in 0.1 M cacodylate buffer, pH 7.3, 1–2 h) for subsequent histological analysis, as well as scanning electron microscopy. Detailed description of spheroid preparation can be found elsewhere [21, 22].
Figure 1. Femtosecond laser microsurgery of zona pellucida of E3.5 mouse embryos: (a) embryo prior to laser exposure (solid curve shows the path of the laser beam.); (b) the hole in the zona pellucida formed after laser treatment. Laser wavelength is 514 nm, light microscopy.

3. Femtosecond laser microsurgery of embryos

3.1. Zona pellucida drilling with femtosecond laser pulses for assisted hatching

Assisted hatching can be performed by thinning a large area of the zona pellucida (ZP), partially dissecting (creating an incomplete hole) or drilling completely through the zona (creating a hole 10–25 µm in diameter). A purpose of this work was to find an optimum range of laser irradiation parameters required for efficient ZP dissection that does not compromise viability of the treated embryos and their preimplantation development. One of the most important parameter is the energy (or intensity) of laser pulses. For both wavelengths (1028 nm and 514 nm) we have determined the acceptable range of laser pulse energies required for successful ZP dissection.

Dissection of the zona pellucida was performed by application of laser pulses along a freehand or straight line defined by an operator in the system control software. The microscope was focused at the so-called “equatorial plane” of the embryo. A freehand line was drawn in this plane and exposed to femtosecond laser pulses with 2.5 kHz repetition rate. The movement of X–Y microscope stage resulted in movement of the embryo relative to the focused laser beam with a speed of 0.01 mm/s.

Figure 1 demonstrates a successful dissection of ZP at a stage of development of E.3.5 day by femtosecond laser pulses with the wavelength of 514 nm. A path irradiated with fs-laser pulses is marked by solid curve.

After determining the acceptable energy range of laser pulses causing ZP dissection, an optimal laser pulse energy was found in terms of viability and developmental rates of laser-treated embryos. Four energy values were chosen for second harmonic of laser radiation between $E_{2\omega_{\text{max}}} = 47$ nJ and $E_{2\omega_{\text{max}}} = 112$ nJ. The maximum energy $E_{2\omega_{\text{max}}} = 112$ nJ was chosen to be 15% less than the value of $E_{2\omega_{\text{max}}} = 130$ nJ obtained earlier in [17], which caused embryo displacement during laser treatment.

A total of 100 embryos were divided into 4 groups (20 embryos per each experimental group, and 10 embryos per each of two control group). In the experimental groups of embryos ZP
Table 1. Data on the blastocyst formation rate and the hatching rate in control embryos and zona pellucida-drilled embryos treated with laser pulses.

| Energy (nJ) | λ = 514 nm | λ = 1028 nm |
|------------|------------|-------------|
| 47         | 66         | 83          | 112         |
| No. of embryos for assisted hatching | 20 | 20 | 20 | 20 | 20 | 20 |
| Blastocyst formation rate (%) | 100 | 100 | 100 | 100 |
| Totally hatched embryos (%) | 100 | 100 | 90 | 100 |

Table 2. Survival rates of embryos in the control groups and the experimental group after trophectoderm biopsy with femtosecond laser pulses with the wavelength of 514 nm.

| Energy (nJ) | 100 | 140 | 190 | 220 |
|------------|-----|-----|-----|-----|
| No. of embryos for trophectoderm biopsy | 20 | 20 | 20 | 20 |
| Totally hatched embryos (%) | 100 | 90 | 70 | 10 |

dissection was performed by applying laser pulses with the following energies: group 1, 47 nJ; group 2, 66 nJ; group 3, 83 nJ; and group 4, 112 nJ.

Viability and developmental rates of embryos subjected to laser-assisted ZP drilling with fs-laser pulses at the wavelength of 1028 nm were also investigated. A total of 60 embryos were divided into two experimental groups (20 embryos per experimental group, 10 embryos per each control group). In the first and the second experimental group of embryos zona pellucida was drilled by applying femtosecond laser pulses with energies of 250 nJ and 320 nJ respectively.

At the same time the analysis of embryos development in the groups of intact control (embryos cultured in vitro and not subjected to any manipulations and laser exposure, 10 embryos per group) and parallel control (embryos were manipulated in the same way as the embryos from the experimental group except laser treatment, 10 embryos) was performed. Data on blastocyst formation and hatching rates are presented in table 1.

As can be seen from the table 1, the majority of embryos has developed to the blastocyst stage (> 90%) and has successfully hatched in almost all embryo groups. There are no considerable differences in hatching rate for both experimental and control groups. We suggest that although no differences were found in terms of assisted hatching efficiency for the two wavelengths applied, using laser radiation at the wavelength of 514 nm would minimize thermal influence on embryos, because of the less pulse energy required to perform ZP drilling.

3.2. Trophectoderm biopsy using femtosecond laser scalpel

Trophectoderm biopsy was performed on embryos at E.4.5, the natural hatching stage. At this stage, the embryo leaves ZP and a portion of the trophectoderm cells outside the shell can be cut by laser pulses. One needs to monitor constantly the state of embryos starting from the first signs of hatching to succeed in trophectoderm biopsy. The goal is not to miss a moment of hatching, when a specified number of cells is outside the ZP.

Then a series of single femtosecond laser pulses is applied to remove several trophectoderm cells. Generally, it is required to apply several (2–4) fs-laser actions in order to fully detach trophectoderm cells. We performed this procedure using the second harmonic of laser scalpel radiation.
Figure 2. (a) Hatching embryo prior to trophectoderm biopsy. “+” markers indicate the places where laser shots are applied, “□” marker indicates the position of optical trap; (b) trapped with optical tweezers trophectoderm cells right after dissection. (c) Successfully hatched embryo subjected to trophectoderm biopsy before staining, light microscopy. (d, e) PI and Hoechst 33258 staining, fluorescence microscopy.

It is important not only to remove trophectoderm cells from the embryo, but also to preserve the viability of the last one. Thus, our goal was to determine the optimal laser pulse energy in terms of embryos survival. We took four values of laser energy between threshold and maximum energy values: 100 nJ, 140 nJ, 190 nJ, and 220 nJ respectively. The error in energy determination in the experiment was ±5%. A total of 80 embryos were divided into four groups (20 embryos per group). In each group biopsy of trophectoderm cells was performed by application of laser pulses with the prescribed energy. Assessment of the viability of embryos was performed the next day according to the percentage of blastocysts that finished their hatching process. Moreover, embryo staining with fluorescent dyes Hoechst 33258 (Sigma) and Propidium iodide (Sigma) was carried out. At the same time, we controlled the survival rates in the first and second control groups. The experimental results are summarized in table 2.

Figure 2(a) illustrates a result in the case, when 3 laser actions are applied to detach the trophectoderm cells. The position of laser scalpel is indicated by “+” marker. Laser tweezers was used to hold the dissected cells shown in figure 2(b).
As can be seen, laser-assisted trophectoderm biopsy is a kind of balance between maximum efficiency of cell detachment (at high pulse energy) and maximum survival rate (at low pulse energy) in the range of acceptable laser pulse energies. From this point of view, energies of femtosecond laser pulses lying in the range of 140–190 nJ seem to be optimal. Energy value should be selected individually for each embryo. In general, experimental results demonstrated high survivability of embryos subjected to the trophectoderm biopsy: successful hatching was observed in most cases (> 90%) provided that single cells only stained with Propidium Iodide (PI) while positive staining with Hoechst 33258 was observed, figure 2(e).

4. Nanosecond laser microsurgery of cell spheroids: development of a new model for studying reparation in vitro

Modern techniques of laser microsurgery of cell spheroids were used to develop a new simple reproducible model for studying repair and regeneration in vitro. Cellular spheroids are one of the most common options in studies using 3D cell cultures, along with explant cultures, cells on microcarriers and tissue-engineered systems. They represent 3D spherical cell clusters, which self-organize due to natural adhesive properties. In spheroids obtained from single cell suspension, cells not only form intercellular contacts but also contacts with newly synthesized extracellular matrix, thereby forming a structure, the organization of which resembles the organization of tissues [23]. Figure 3 shows an image of an untreated 7-day BM MMSC spheroid obtained with a scanning electron microscopy (CamScan, Japan). As can be seen, two distinct regions in compact spheroids are presented: two to four layers of elongated surface cells and an internal core consisting of polygonal irregular cells. Imbricated cells of the surface region were in close contact with each other, whereas the inner zone was loose, and polygonal cells were separated by the extracellular matrix. The field of application of cellular spheroids is constantly expanding. For example, studying the mechanisms of wound healing is one of the challenging issues nowadays. Using monolayer cultures as a model system allows for studying only specific parameters of cell behavior, but does not allow for evaluating the contribution of intercellular interactions as well as interactions of cells with the extracellular matrix. Hence, similar studies are now performed mainly on organotypic explant cultures [24, 25]. Nevertheless, the search for simple reproducible model...
systems for studying mechanisms of regeneration, as well as problems of the fibrotic and non-fibrotic wound healing continues. Repair of cellular spheroids after damaging effects could be one such model.

We employed nanosecond laser dissector to simulate spheroids damage. The accuracy of nanosecond laser microsurgery was enough to make local incisions of spheroids. The energy of the laser pulses was optimized so that the dissection was carried out only in the specified area of the spheroid and, therefore, would not compromise the viability of the spheroid in general. The surface and inner spheroid layers were dissected by applying laser pulses along the straight-line path (from spheroid periphery to the centre) as defined by the operator in the PALM RoboPro software. The length of laser irradiated path was set to be equal to its radius (typically, 75–100 µm) and was chosen for each spheroid individually. The laser processing of the defined straight-line path was repeated 5–8 times. Every next cycle was characterized by the axial (along z-axis) laser beam focus shift to provide the dissection of spheroid in three dimensions.

The main “surgical” mechanism causing tissue dissection after application of nanosecond laser pulses is the formation of plasma and optical-induced breakdown [26–28]. In the case when energy of pulses is slightly higher than the threshold energy, the optical-induced breakdown is accompanied by the formation of cavitation bubbles the size of a few micrometers. Their emergence has led to visible damage to the irradiated tissue; but, due to the small size of cavitation bubbles, it became possible to perform localized microsurgery of the selected object.

In our experiments we determined, that the threshold energy of laser pulses, required for dissection to start, lay in the range of 6.2–6.6 µJ (the speed of motorized stage movement...
along axis was selected to be 120 \( \mu m/s \). However, the quality of dissection was very sensitive to spheroid’s size and heterogeneity of agarose plate thickness, where spheroids were placed. Application of laser pulses with higher energies led to more efficient spheroid dissection and reproducible results. Further increase in pulse energies (> 9.1–9.2 \( \mu J \)) caused the formation of a great number of cavitation bubbles and the chaotic displacement of the object from its initial position during laser exposure. As a result, the dissection of the spheroid chosen area could not be performed efficiently. Moreover, uncontrolled damage to the surrounding areas or spheroid might have occurred. Taking into account all the listed factors, the optimal energy of laser pulses was set in the range of 7–9 \( \mu J \), which corresponds to laser intensity of \((1–1.2) \times 10^{10} W/cm^2\).

The process of laser microdissection of spheroid and formation of cavitation bubbles, leading to visible damage in spheroids is demonstrated in figure 4. As a result of exposure to laser radiation, a spontaneous opening of the wound edges was observed.

Dynamic changes in the angular opening of the wound surface during first 200 s after laser impact were evaluated on 14 single spheroids. Immediately after microdissection, the wound edges opened up to 118 ± 18° and, in the subsequent four minutes, increased up to 197 ± 25°. The further observed growth of angular opening was negligible. The increase of angular opening of the wound edges was accompanied by a change in morphology of the cells at the wound surface, with their shape changing from elongated and flattened to the round one.

The results of histological analysis showed that within the first 60 min after microdissection wound surface contained fragments of dead cells and rounded cells (figure 5). Directly in the area of damage, there were only single cells that maintained their original morphology; they were surrounded by cellular debris, cell surface was rough and cytoplasmic integrity was compromised. The structure of the surface layer in the intact area remained virtually unchanged and retained its integrity, the cells remained flattened.

Long-term live registration of spheroid reparation was performed for seven days after laser microsurgery (figure 6). The first steps of spheroid structure restoration were observed six hours after laser microsurgery. Full restoration of the initial structure of the spheroids, with a few surface layers of flattened imbricated cells and polygonal cells of the inner zone, occurred seven days after microdissection.
Figure 6. Long live time-lapse microscopy snapshots during seven days after BM MMSC spheroid microdissection with nanosecond laser scalpel: (a) 6, (b) 12, (c) 24 h and (d) 7 days after nanosecond laser scalpel impact, light phase contrast microscopy.

The described dynamics of repair were also confirmed by histological analysis of damaged spheroids from both BM MMSC and human fibroblasts.

5. Conclusions
We have demonstrated that femtosecond laser pulses can be efficiently used for assisted hatching and trophectoderm biopsy of preimplantation embryos. Low energy level of a single femtosecond laser pulse makes it possible to minimize embryo damage and maximize their survival rate as well. Using optimized laser pulse energies for each of two wavelengths (1028 and 514 nm) we
achieved efficient ZP drilling and high rate of successful embryo hatching (> 90%). High survival rates and efficient cell detachment were also observed when laser pulses with the energies in the range of 140–190 nJ (for the wavelength 514 nm) were applied for trophectoderm biopsy.

We have proposed a new simple reproducible model based on precise laser microsurgery for studying regeneration in 3D cell spheroid. The developed model of laser microdissection and optimally chosen conditions of laser exposure allowed us to damage the surface and the inner area in a selected region of spheroid without disturbing the viability of the object in general. The internal structure of spheroids recovered gradually and, within seven days, cells in the irradiated area gradually acquired the initial morphology, the dense layered structure of the surface zone with flattened cells was restored and the repair processes were complete. We assume that the repair of spheroids after microdissection is mainly performed by remodeling of existing viable cells. The proposed model opens up opportunities for searching and exploring new ways to stimulate repair.

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