

Femtosecond scalpel-optical tweezers: efficient tool for assisted hatching and trophectoderm biopsy

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Abstract. Ultrashort laser pulses have enabled highly precise and delicate processing of biological specimens. We present the results of using femtosecond (fs) laser pulses for dissection of zona pellucida (ZP) in mouse embryos during assisted hatching procedure and for trophectoderm biopsy as well. We studied the effects of application of fs laser radiation in the infrared (1028 nm) and visible (514 nm) wavelength ranges. Laser irradiation parameters were optimized so as not to compromise the viability of the treated embryos. Embryo biopsy was carried out in late-stage mouse preimplantation embryos. Femtosecond laser pulses were applied to detach the desired amount of trophectoderm cells from the blastocyst, while the optical tweezers trapped the cells and moved them out of the embryo. The parameters of laser radiation were optimized so as to efficiently perform embryo biopsy and preserve the viability of the treated embryos. The thermal effects can be significantly lower when fs lasers are used as compared to CW or long-pulse lasers. It is crucial when dealing with living cells or organisms.

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 on cellular and even subcellular levels with unprecedented accuracy and selectivity. In recent decades, lasers have been used not only for research purposes in the fields of embryology and developmental biology, but also in clinical practice, for example, in various medical centers for reproductive medicine. The most interesting and challenging task is to apply laser sources in assisted reproduction techniques. 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.

Laser technologies have rapidly developed in recent years and a variety of 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. Laser technologies have also

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been used in research laboratories for stem cell derivation or cellular microsurgery (in particular, for oocyte enucleation). In earlier papers it has been shown that nanosecond lasers may be successfully applied for embryo microsurgery [1–2]. At the present time picosecond [3–4] and femtosecond (fs) lasers [5–9] are mainly used. It should be pointed out that femtosecond lasers have several advantages as compared to CW lasers or long-pulsed lasers. The main mechanism of microsurgery with femtosecond laser pulses is based on nonlinear absorption [10]. 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.

2. Materials and Methods

2.1. Embryo collection and culture

All procedures performed in studies involving animals were in accordance with the bioethics standards of Biology department of the M.V. 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 (E0.5).

Zygotes were collected from fresh oviducts according to standard protocol [11]. 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 °C, 5 % CO₂, 98 % humidity).

Embryos at E.2.5–E.3.5 were used in laser-assisted hatching procedure and at E.2.5–E.3.5 — for trophectoderm biopsy. Embryos were transferred to fresh drops of M2 medium in a 35 mm glass-bottom Petri dishes (170 μ m 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.

2.2. Experimental Groups

To examine the influence of laser irradiation parameters on the embryos viability all embryos were divided into 3 groups: 1) a group of intact control (embryos cultured in vitro and not subjected to any manipulations and laser exposure), 2) a group of control embryos that were manipulated in the same way as the embryos from the experimental group except laser treatment, 3) a group of laser-treated embryos (femtosecond laser pulses were applied to dissect ZP). Embryos from both the second and the third groups (after all the manipulations were complete) and the intact control embryos were cultured under similar conditions.

2.3. Experimental set-up

Detailed information about scheme of the combined system “Femtosecond laser scalpel-optical tweezers” is given in [13]. The system consists of the inverted microscope Olympus IX-71 and two laser sources – CW and femtosecond 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). A 20 $\times$ (NA=0.5) microscope lens was used to focus the laser beam on the zona pellucida to carry out assisted hatching procedure; laser spot diameter was $\sim$ 3 microns for the fundamental frequency and about 2 microns for the second harmonic. For trophectoderm biopsy, a 40 $\times$ (NA=0.8) microscope lens was used; laser spot diameter was of the order of 1 micron for the second harmonic.

3. Zona pellucida drilling with femtosecond laser pulses

Assisted hatching can be performed by thinning a large area of the zona pellucida, 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.

Energy threshold value of laser pulse for assisted hatching procedure appeared to be $E_\omega \approx 140$ nJ for the fundamental frequency and $E_{2\omega} \approx 47$ nJ for the second harmonic. Thereafter we gradually increased laser pulse energy. After a certain value further energy increase resulted in an optical breakdown of the medium followed by formation of cavitation bubble. This caused embryo displacement relative to its initial position due to bubble expansion and shockwave formation. Such phenomena appeared at $E_{max}^\omega = 390$ nJ и $E_{max}^{2\omega} = 130$ nJ for the wavelengths of 1028 nm and 514 nm respectively.

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

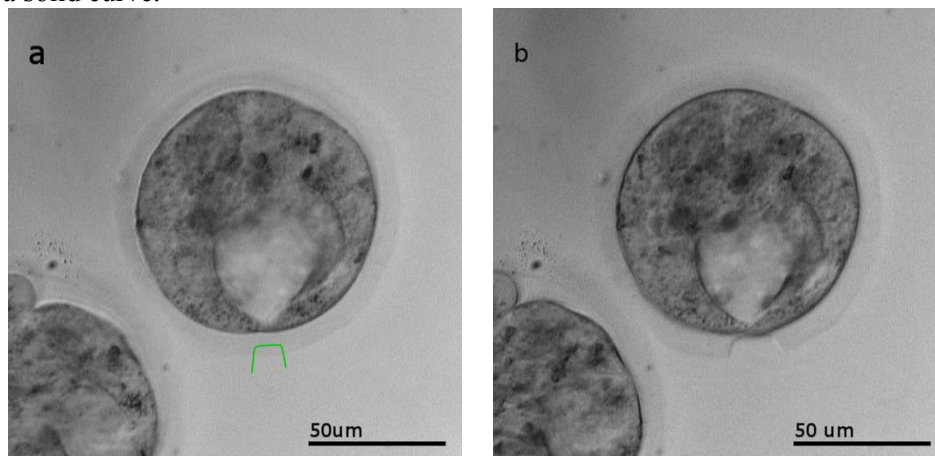


Figure 1. *ZP* dissection mouse embryos by means of femtosecond laser pulses.

(a) Embryo before the exposure. (b) Dissected *ZP* after laser treatment.

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 the second harmonic of laser radiation between $E_{tr}^{2\omega} = 47$ nJ and $E_{max}^{2\omega} = 112$ nJ. The maximum energy $E_{max}^{2\omega} = 112$ nJ was chosen to be 15% less than the value of $E_{max}^{2\omega} = 130$ nJ obtained above to avoid embryo displacement (due to shock wave formation in the medium) during laser treatment. A total of 100 embryos were divided into 6 groups (10 embryos per control group, 20 embryos per each experimental group): two control and four experimental groups. In the experimental groups of embryos *ZP* 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 40 embryos were divided into two experimental groups. 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 1) and parallel (Control 2) controls was performed. Data on blastocyst formation and hatching rates are presented in table 1.

Table 1. Data on blastocyst formation rate and hatching rate in experimental group treated with laser pulses

	$\lambda = 514 \text{ nm}$				$\lambda = 1028 \text{ nm}$	
	Energy (nJ)				Energy (nJ)	
	47	66	83	112	250	320
No. of embryos for assisted hatching	10	10	10	10	10	10
Blastocyst formation rate, %	100	100	100	100	100	100
Totally hatched embryos, %	100	100	90	100	90	100

As can be seen from the table, the majority of embryos has developed to the blastocyst stage (> 90%) and has successfully hatched in almost all embryo groups. There were 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 cause ZP drilling.

4. Trophectoderm biopsy using femtosecond laser scalpel

Trophectoderm biopsy procedure was performed on embryos at E.4.5 — the natural hatching stage. At this stage an 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 since 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. As a rule, it is required to apply several (2–4) laser actions in order to fully detach trophectoderm cells. We performed this procedure using the second harmonic of laser scalpel radiation.

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 $\pm 5\%$. 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 finished their hatching process, as well as to staining the cells with fluorescent dyes *Hoechst 33258* (Sigma) and *Propidium iodide* (Sigma). At the same time we controlled the survival rates in the first and second control groups. The experimental results are summarized in table 2.

Table 2. Survival rates of embryos in 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

As can be seen, laser-assisted trophectoderm biopsy is a kind of balance between maximum efficiency of cell detachment (at high energy) and maximum survival rate (at low 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. Figure 2 demonstrates the case, when 3 laser actions are applied to detach the trophoctoderm cells. The position of action of laser scalpel is indicated by "+" marker. Dashed line represents the boundaries of the ZP. Laser tweezers was used to hold the dissected cells.

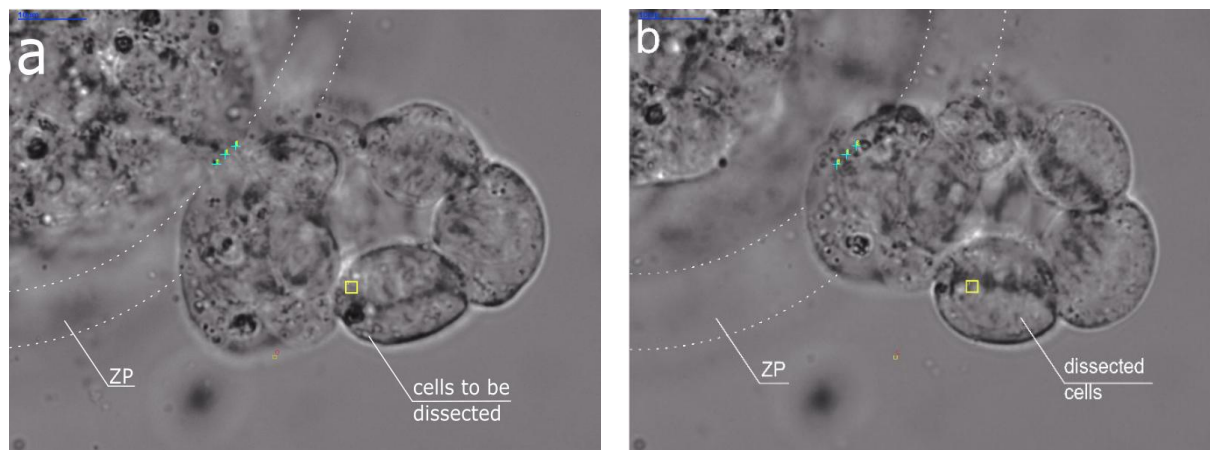


Figure 2. (a) Hatching embryo before a biopsy procedure. (b) Trapped with optical tweezers trophoctoderm cells right after the dissection. + Markers indicate the places where laser shots are applied; □ marker indicates the position of optical trap

Thus we demonstrated efficient assisted hatching and trophoctoderm biopsy procedures performed by the femtosecond scalpel-optical tweezers.

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