How safe is the gamete micromanipulation by laser tweezers?

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1. ABSTRACT

Laser tweezers, used as novel sterile micromanipulation tools of living cells, are employed in laser-assisted *in vitro* fertilization (IVF). For example, controlled spermatozoa transport with 1064 nm tweezers to human egg cells has been performed in European clinics in cases of male infertility.
The interaction of ≈100 mW near infrared (NIR) trapping beams at MW/cm^2 intensity with human gametes results in low mean <2K temperature increases and <100 pN trapping forces. Therefore, photothermal or photomechanical induced destructive effects appear unlikely. However, the high photon flux densities may induce simultaneous absorption of two NIR photons resulting in nonlinear interactions. These nonlinear interactions imply non-resonant two-photon excitation of endogenous cellular chromophores. In the case of <800nm tweezers, UV-like damage effects may occur. The destructive effect is amplified when multimode cw lasers are used as tweezer sources due to longitudinal mode-beating effects and partial mode-locking. Spermatozoa damage within seconds using 760nm traps due to formation of unstable ps pulses in a cw Ti:Sa ring laser is demonstrated. We recommend the use of ≥800 nm traps for optical gamete micromanipulation. To our opinion, further basic studies on the influence of nonlinear effects of laser tweezers on human gamete are necessary.

Key words: cell damage, laser tweezers, gametes, two-photon excitation, NIR

2. INTRODUCTION

The extracorporal sperm-egg fusion in a reaction tube, the in vitro fertilization (IVF), is used to overcome human infertility. Major problems arise in the case of male infertility due to low spermatozoa concentration or disturbed cell function such as impaired motility ("low quality sperm"). The assisted IVF utilizes several techniques, such as the intra-cytoplasmatic sperm injection (ICSI) or the subzonal insemination (SUZI), where sperm cells are microinjected via glass

SPIE Vol. 3260 • 0277-786X/98/$10.00

pipette into the egg cytoplasma or in the layer between zona pellucida and membrane, respectively. An alternative to these mechanical approaches is an optical approach. Optical traps can be used to overcome the problem of impaired motility. In this case, a single sperm cell is confined in the focal volume of high numerical aperture (NA) objectives of a near infrared (NIR) laser microscope. Controlled sperm transport to the egg cell is possible by movement of the sample stage or of the laser beam[1,2]. In addition to the use of laser tweezers, pulsed laser microbeams in the UV or IR can be used to drill holes in the zona pellucida or to induce hatching[3-7]. These laser procedures on human gametes are called laser-assisted IVF.

Optical trapping, introduced by Ashkin et al.[8,9], bases on force generation during intracellular refraction of highly focused continuous wave (cw) laser beams. These trapping forces F are directly proportional to the laser power P and can be represented by F = QP/c, where c is the light speed in medium and Q a trapping efficiency parameter which depends on optical properties of the trapped object and on laser beam quality.
In order to avoid intracellular heating and to minimize the generation of convection-based thermal forces, the trapping wavelength should be in the far red or near infrared (NIR) spectral region, which is characterized by a lack of efficient endogenous absorbers in most cells. Water has a decadic absorption coefficient as low as 0.003 cm^{-1} at 700 nm and about 0.1 cm^{-1} at 1064 nm^2 (10). However, cells with hemoglobin, chlorophyll, or melanin may absorb far red/NIR trapping radiation.
Optical traps can provide trapping forces higher than the ATP-driven intrinsic forces of motile cells. Their main application in biology and medicine is optical micromanipulation where far red/NIR laser tweezers are employed for controlled transport of cells, cell organelles, and biomolecules. In Europe, laser tweezers have been used in optical micromanipulation of human spermatozoa to overcome infertility. For example, 89 persons were treated with 1064nm traps and 337nm laser microsurgery in Germany in 1993[11].
Aim of this paper was to study interactions of high intense radiation of laser tweezers with human gamete and to discuss the safety of laser-assisted IVF.

3. MATERIALS AND METHODS

Laser Tweezers
NIR optical traps based on highly focused TEM_{00} laser beams of an 1064 nm Nd:YAG laser (Adlas, Germany) or of a tunable (700 nm - 1000 nm) cw Ti:sapphire laser (Coherent, cw ring laser 899-01 or Mira900 in cw mode) have been used. The parallel beam was expanded to fill the back aperture of a 100x Zeiss Neofluar brightfield objective (numerical aperture NA = 1.3) and focused to diffraction limited spots in the submicron range. The NIR beam was introduced in modified Zeiss laser scanning microscopes. The lasers operated as multifrequency cw lasers (several longitudinal modes) but could be transformed into single-frequency cw lasers by adjustment of an intracavity etalon (20 MHz linewidth in the case of the cw ring laser). Laser scanning images of trapped spermatozoa labeled with exogenous fluorophores were obtained with 488 nm microbeams of an Ar^+-laser (power at the sample: 2.2 μW, scanning time: 1s/image. For autofluorescence imaging, the UV excited fluorescence was detected with a slow-scan, cooled CCD camera (Princeton Instruments, model TE576/SET135). A 50 W high-pressure mercury arc lamp equipped with a 365 nm bandpass filter was used as excitation source (1.5 mW, 5.3 W/cm^2) as well as low-power UVA exposure source.
The spot size d of the focused laser beam was assumed to be diffraction-limited and was approximated by $d = \lambda/NA$ (λ: wavelength). The intensities were therefore 20, 35, and 39 MW/cm^2 for 1064 nm, 800 nm, and 760 nm trapping beams at a power of 105 mW, respectively.

Sperm preparation
Semen specimens were obtained from three healthy donors. Semen was layered on a discontinuous isotonic percoll gradient consisting of three layers, 1 ml each, of 95%, 70%, and 50%. After centrifugation for 15 min. at 200g, the bottom

layer was removed, washed with HEPES buffered fresh human tubal fluid (HTF, Irvine Scientific, Irvine, CA) and centrifuged for 10 min at 100g. The pellet containing sperm utilized for experiments was then diluted in HEPES buffered isotonic saline solution containing 1% human serum albumin. Human spermatozoa were kept in sterile cell microchambers consisting of two 0.16 mm coverslips as chamber windows, a silicon gasket with a 2 cm opening as spacer, and metal frames. The cells were trapped inside the closed chamber. Experiments were performed within three hours following ejaculation.

Chemicals

For viability analyzing, the LIVE/DEAD FertiLight^{TM} Sperm Viability Kit (Molecular Probes) was used, which contains the live-cell fluorophore SYBR^{TM}14 (final concentration: 100 nM) with an emission maximum at 515 nm and the dead-cell stain propidium iodide (final concentration: 12 μM) with an emission peak at 636 nm.

4. RESULTS

4.1. Motility and Life/Dead Studies

We applied multimode NIR traps of 105 mW power to confine spermatozoa in the focal volume of a high NA objective. When using 800nm traps and 1064nm traps for micromanipluation, cells remained alive and motile even for trapping periods up to 10 min. No destruction effects were observed.

However, trapping at wavelengths <800 nm resulted in reduced motility. For example, spermatozoa in 760 nm traps provided by the cw Ti:sapphire laser became paralyzed at a mean time of 35 s (n = 30). In order to get more information on the extent of cell damage we imaged the cellular autofluorescence in the blue/green spectral region which is based on the presence of the reduced coenzyme and bioindiactor NAD(P)H. The 365nm-excited autofluorescence of healthy motile cells based on the fluorescent coenzyme NADH was found to arise in the midpiece of the cell, the primary site of mitochondria. The sperm head exhibited no fluorescence. Interestingly, we found a complete different autofluorescence pattern in 760nm trap-paralyzed spermatozoa. In particular, autofluorescence arose now also in the sperm head and the total cellular autofluorescence intensity increased.

In order to yield information on viability, trapped cells were incubated with the Life/Dead viability kit. Simultaneously, the intracellular fluorophores in the single trapped sperm cell were detected. Fluorescence was excited with the high-pressure mercury lamp or with 488nm radiation of an Ar^{+} laser. Cell death in 760 nm traps was found to occur at a mean time of 65 s. The trapping wavelength was tuned to measure cell destruction in dependence on wavelengths. The figure shows an action spectrum of trap-induced spermatozoa killing. As seen the destructive effect was less pronounced at other <800 nm wavelengths.

These results demonstrate those 100mW traps at 800 nm or 1064nm do not induce significant changes in motility, intracellular redox state (autofluorescence), and viability. Although we did not check the particular influence on DNA these laser tweezers appear as relatively safe micromanipulation tools. However, shortwavelength NIR traps =800 nm caused severe damage with a pronounced destructive effect at 760 nm. Such destructive effects at 760 nm with the microradiation (no trap) of the same Ti:sapphire laser were found earlier[12]. What is the possible damage mechanism?

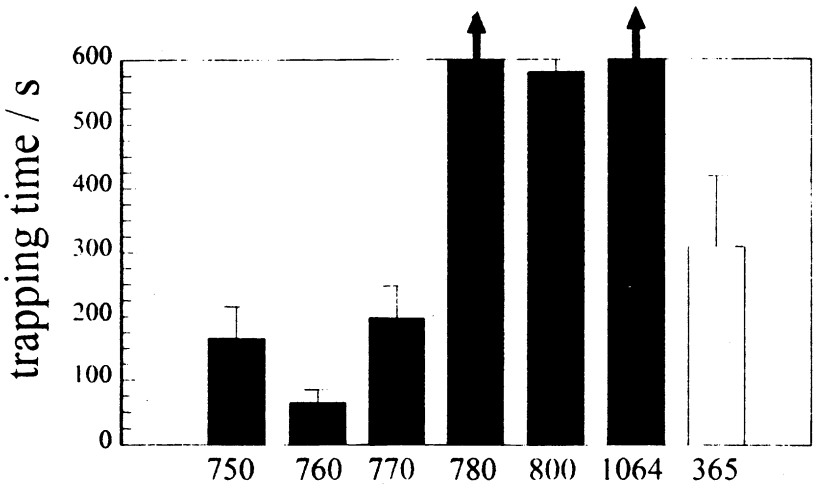

Figure Action spectrum of trap-induced cell killing. The mean time of onset of propidium iodide fluorescence vs. wavelength is shown. For comparison, data on cell death induced by 1 mW 365 nm mercury lamp radiation are included.

4.2. Photothermal and photomechanical stress

The NIR absorption spectrum of sperm heads is not exactly known. The major NIR absorber of human spermatozoa is considered to be water. As mentioned in the introduction, water has a decadic absorption coefficient α as low as 0.003 cm^{-1} at 700 nm and about 0.1 cm^{-1} at 1064 nm[10]. With a typical sperm head thickness of 3 μm, the water absorption is on the order of 10^{-6} and $3x10^{-5}$, respectively. Considering this poor absorption the cell appears as nearly transparent at these NIR wavelengths. The surrounding medium absorbs more than the cell. Due to the fact that spermatozoa can be confined in 800nm and 1064 nm traps up to 10 min without any destructive effect, photothermal damage can be excluded. Although water has a minor absorption band at 760 nm, the observed highly destructive effect at 760 nm has no explanation in the water absorption spectrum (α(760nm) < α(1064nm)). Liu et al.[13] Found a low <2K temperature increase in 1064nm trapped spermatozoa by spectrally-resolved detection of the thermosensitive intracellular exogenous fluorophore Laurdan which undergoes significant spectral changes during heating.

What about photomechanical-induced damage? The trapping forces can be represented by:

$$F = \frac{QP}{c}$$

where c is the velocity of light in medium, P the incident laser power at the sample, and Q the trapping efficiency parameter. The values of Q will be between 0 and 2. Values of Q equal to 0, 1, and 2 represent, respectively, no interaction, total absorption and total back-reflection of the beam. Recently[14] we developed a model to calculate the parameter Q in the case of human spermatozoa. The model is based on the assumption of an ellipsoidal geometry of the sperm head. Using hydrodynamic theories of ellipsoidal specimens we calculated the trapping efficiency Q in the case of an 800nm trap to be ≈0.12. That implies trapping forces of ≈50 pN induced by 100 mW traps. A mean drop-off power (minimum power to confine a motile cell in the trap) of healthy sperm of ≈82 mW was determined. This value corresponds to a mean linear motility force of ≈40 pN of healthy human spermatozoa. Therefore, the induced mechanical forces are <100 pN and on the order of cellular motility forces.

4.3. Photochemical stress

Considering a typical trapping power of 100 mW and a diffraction-limited spot size of 585 nm for 760 nm radiation ($d \approx \lambda/1.3$), the trapping intensity is about 37 MW/cm^2. This corresponds to NIR photon flux densities $\Phi = 10^{26}$ photons cm^{-2}s^{-1}. With typical molecular absorption cross sections α of 10^{-48} photons cm^{4}s, such high photon flux density is sufficient to induce two-photon excitation. Assuming a typical number of 1000 fluorophore molecules within the trapping volume and a high fluorescence quantum yield of ≈ 1, a fluorescence photon rate of:

$$dN/dt = N\, a\, \Phi^2 = 10^7 \text{ fluorescence photons } s^{-1}$$

can be estimated which can be detected with photomultipliers or CCD cameras. We imaged in SYBR14/propidium iodide labeled spermatozoa an 0.5 μm green spot in the cell head[15]. This fluorescent spot reflected a two-photon excitation process induced by the cw NIR trapping beam. In the case of 800nm or 1064 nm traps the spot remained green during trapping. However, in the case of 760 nm traps the color changed into red after ≈1 min indicating the intracellular presence of the dead-cell indicator propidium iodide. The fluorescence intensity showed a clear squared dependence on laser power. The trap-induced visible fluorescence provided information on intracellular localization of the trapping beam. The beam was found to be localized mainly in the sperm head. However, the spot position changed in dependence on the transient ATP-driven motility force of the sperm (several attempts to escape the trap). Also short interactions with the midpiece have been monitored. According to these results the NIR trapping beam is able to excite cellular absorbers via a non-resonant two-photon excitation process where one NIR photon provides half the energy needed to excite the electronic transition. Using traps at ≥800 nm wavelengths, cellular absorbers with transitions in the visible range may be excited. Obviously, there are no efficient excited chromophores that could induce destructive photochemical reactions.

In contrast, an onset of red two-photon excited fluorescence in <800 nm traps confirmed their destructive effect. Non-labeled spermatozoa, paralyzed with these short-wavelength NIR traps, exhibited a blue sub-micrometer spot in the sperm head which can be explained by diffusion of the endogenous fluorophore NADH from the midpiece in the head after damage. Using <800 nm traps, cellular absorbers with UVA transitions can be excited. Such potential absorbers are e.g. NAD(P)H, flavins, porphyrins, and cytochromes. Excitation may result in destructive photochemical reactions, such as type I and type II photooxidation with oxygen radical and singlet oxygen formation, respectively (oxidative stress). DNA damage by NAD(P)H excitation and the potential of UVA radiation to harm cells has been shown[e.g. 16-17]. Therefore, trapping at <800 nm may induce destructive oxidative stress.

The most destructive effect was found at 760 nm. Endogenous chromophores with a pronounced 760 nm or 380 nm transition are not known. Has this 760 nm effect biological origin? Interestingly, we found in wavelength dependent studies the most efficient fluorescence excitation of rhodamine 123 solutions also at 760 nm. Rhodamine has no special one-photon or two-photon absorption band at this wavelength. The origin of the 760nm effect was found to be a special laser output effect.

4.4. Influence of the laser output

In multi-frequency cw lasers, the photon flux can reach higher values as the estimated 10^{26} s^{-1}cm^{-2} value for the case of a single-frequency laser. The reason is constructive superposition of different longitudinal laser modes ("mode beating") which can result in laser pulse formation. We measured in our "cw" Ti:Sapphire laser unstable light pulses with a repetition frequency of multiples of 180 MHz and a pulse duration of less than 500 picoseconds. These laser pulses were most intense at a laser wavelength of 760 nm. The higher the pulse peak (pulse power) the more efficient the two-photon absorption due to the squared dependence. We observed therefore the most efficient cell killing rate with a multi-frequency laser at 760 nm.

In contrast, transformation of the multifrequency cw laser into a "pure" cw laser (single-frequency laser) reduced the damaging effect significantly. However, two-photon induced lethal cell damage still occurred within 7 minutes, Table.
When using ultrashort laser pulses of a regular pulsed laser source at 50 mW mean power, such as 150 fs pulses from a stabilized mode-locked 80 MHz Ti:sapphire laser, cells were killed immediately by head disruption. The damage behavior was different. Considering the peak powers of ≈5 kW (mean power still 50 mW), transient intensities of ≈1.5 TW/cm^2 occur which may result in optical breakdown, destructive plasma and shock wave formation.

Table
Effect of NIR (105 mW) photostress on spermatozoa confined in multifrequency vs. single frequency cw traps. The mean time (in seconds) t_M where loss of motility occurred (n = 20) and the mean time (in seconds) t_{PI} where onset of intranuclear propidium iodide fluorescence (indicative for cell death) are shown (n = 20). Errors indicate standard deviation.

λ/nm	t_M /s	t_{PI} /s
760 (no etalon)	35± 20	65±20
760 (with etalon)	132±50	406±160

SUMMARY AND DISCUSSION

NIR lasers have been proclaimed to be harmless tools in laser-assisted IVF. Babies have been born after laser-assisted IVF using 1064 nm Nd:YAG lasers. No complication has been reported so far. Other potential laser sources for laser-assisted IVF are laser diodes and Ti:sapphire lasers which provide radiation at shorter wavelengths where water -considered as the major NIR absorber- absorbs less. From a first point of view, damage should appear more unlikely with these trapping sources. However, our results demonstrate that possible cytotoxic effects have to be considered when using short-wavelength NIR traps. We probed the effect of photostress on human motile spermatozoa by motility measurements, by sensitive autofluorescence imaging, and by viability analyzing. Cell damage, in particular paralysis and cell death, was found for short-wavelength NIR trapping beams <800 nm. In contrast, no cytotoxic effects were detected for 800 nm and 1064 nm traps (100 mW) even for radiant exposures as high as 10^{10} J/cm^2.
Due to the high photon flux density within the trapping volume, laser tweezers are sources of two-photon excitation. Two-photon NIR excitation of cellular absorbers can induce photochemical reactions as well as visible fluorescence in a highly-localized sub-femtoliter volume. In principle, <800 nm traps can excite one-photon absorbers with UVA electronic transitions, such as NAD(P)H, flavins, porphyrins, and cytochromes. Some of these excited cellular absorbers are known to be photodynamically active. For example, excitation of NAD(P)H induces singlet oxygen and oxygen radicals, e.g. oxidative stress, via photooxidation processes. These reactive species may induce DNA strand breaks[16,17]. Therefore, short-wavelength NIR traps may induce UVA-like destructive effects.
These destructive photochemical processes are enhanced if transient high powers are present as in the case of pulsed light sources. Two-photon excitation shows a squared dependence on laser power. We found that multimode cw lasers such as cw Ti:sapphire ring lasers are able to form unstable ps pulses due to mode-beating effects. At 760 nm even partial mode-locking occurred resulting in highly efficient two-photon excitation of cellular absorbers and enhanced cell destruction at exactly that wavelength. Using higher peak powers such as in the case of femtosecond pulsed laser sources results in immediate damage due to optical breakdown effects.
In conclusion, we recommend the use of cw trapping laser sources with emission between 800 nm and 1100 nm and single-frequency operation. The laser output should be controlled. The trapping time as well as the trapping power should be

minimized. Further basic studies on nonlinear interactions between gametes and laser tweezers have to be performed, including the measurement of the exact action damage spectrum using tunable NIR single-frequency lasers.

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