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9 Laser tweezers are sources of two-photon effects

Abstract: Laser tweezers or optical traps are established laser tools for optical non-contact manipulation of micron/submicron sized objects in liquids such as non-adherent biological cells in medium. Typical laser traps are based on optical gradient forces generated with high numerical aperture near-infrared (NIR) continuous wave (cw) laser microscopes. The laser-cell interaction is determined by a change of the momentum due to the beam direction being altered by refraction. In order to avoid laser absorption, NIR cw lasers such as the Nd:YAG laser at 1064 nm, the frequency doubled erbium:YAG fiber laser at 760 nm, the tunable cw Ti:sapphire ring laser, and laser diodes at wavelengths < 800 nm are employed. They are considered to be safe tweezer sources. However, two-photon absorption effects may occur due to the generation of high MW/cm² laser intensities when using tightly focused cw laser beams at a power of 100 mW or more. These nonlinear effects can be used for two-photon excited fluorescence spectroscopy of trapped objects. However, when using low-wavelength NIR (< 800 nm) traps, potential “UV-like” photodamaging effects have to be considered during cell manipulation. The use of the Nd:YAG laser at 1064 nm is recommended when using laser tweezers for optical sperm transport for laser-assisted in vitro fertilization (IVF).

9.1 Introduction

Light exerts pressure that can be used to accelerate or buoy nanometer-sized and micrometer-sized particles against gravity. Furthermore, a tightly focused laser beam can pull transparent particles into the focal volume of a high NA objective.

If the particle is greater than the wavelength (Mie particle), the trapping phenomenon can be explained by refraction. The laser beam transmits the object and leaves it in another direction, thereby generating a momentum change and subsequently a force. The net force results in the positioning of the particle within the focal volume (Fig. 9.1).

Arthur Ashkin pioneered laser manipulation of micrometer-sized objects, such as microspheres and bacteria, based on optically-induced forces in the piconewton range [1]. His first publication on a stable laser trap based on two opposite beams holding a single microsphere dates back to January 1970. In his abstract he wrote:

Micron-sized particles have been accelerated and trapped in stable optical potential wells using only the force of radiation pressure from a continuous laser. It is hypothesized that similar accelerations and trapping are possible with atoms and molecules using laser light tuned to specific optical transitions. The implications for isotope separation and other applications of physical interest are discussed [2].
In 1986, he published with his new co-worker Steven Chu and others on optical trapping and cooling of atoms [3, 4]. They used a single laser beam instead of two beams and realized the first stable 3D atom trap. Later on, Chu further developed the method of optical manipulation of atoms [5]. In 1997, Steven Chu, Claude Cohen-Tanneudji, and William D. Philip were awarded with the Nobel Prize for their work on the accuracy of atomic clocks and acceleration measurements based on trapped cooled atoms.

Starting from 1987, Ashkin used the single beam optical trap to manipulate biological objects, such as viruses, bacteria, and intracellular organelles [6]. Interestingly, the group faced problems when using the green argon ion laser as trapping source. A laser power of 100 mW damaged trapped bacteria, likely due to absorption and subsequent heating effects. This optical damage limits the use of the green laser trap in biomedical sciences. The problem could be solved when switching to a NIR laser source [7].

Biological objects do not significantly absorb red and NIR light up to 1200 nm. There are some skin cells and erythrocytes that contain the weakly NIR-light-absorbing pigments melanin and hemoglobin, respectively. However, pigment-free biological cells appear nearly NIR transparent. Also, scattering in NIR is negligible compared to UV or visible light. Therefore, the red and NIR spectral range between 600 nm and 1200 nm is termed the “optical window of cells and tissues”. The heating effect in NIR trapped living pigment-free cells such as CHO (Chinese hamster ovary) cells and sperm cells was measured to be less than 2 K at 100 mW laser power [8].

When Ashkin and his coworkers employed an Nd:YAG laser at 1064 nm, bacteria and yeast cells survived the trap for some hours when using a mean power of 80 mW and started to reproduce even during confinement in the laser trap. Also, red blood cells could be trapped without damaging the membrane. However, a significant lower laser power was required [7].
Later on, laser traps (laser tweezers) became a valuable tool in cell biology. Applications include the determination of motor protein forces, DNA molecule stretching, cell sorting, and spectroscopy of motile cells such as algae and sperm [9–20].

The motor proteins kinesin, dynein, and myosin are responsible for the active molecule transport in cells. ATP consumption results in conformation changes that are used to move binding partners along the cytoskeleton. Laser tweezers were used to understand these chemomechanical processes on a single molecule level, to determine the exact pN forces exerted by the motor proteins for organelle transportation within living cells and to calculate the transportation velocity [9–12]. The DNA molecule became a further biological object of interest for laser trapping. By attaching microbeads to a single DNA molecule, the DNA could be stretched and its elasticity determined [13, 20, 21]. Cell sorting by the use of laser tweezers, a microfluidic chip, and a target cell recognition system has been realized, for example, to sort human embryonic stem cells [26].

Laser tweezers are also an interesting tool to study highly motile cells, such as certain bacteria, algae, and sperm. By keeping them in the trap, sensitive spectroscopic measurements, such as optical metabolic imaging by fluorescence lifetime imaging, can be performed [15, 23]. Furthermore, the motility forces can be determined. We performed measurements and calculations to determine the force of a healthy human sperm cell to be 44 pN [18]. Furthermore, our group has demonstrated that the motility force did not decrease significantly after freezing and thawing. The frozen sperm of a healthy donor will still be a “high quality sperm”.

Since NIR laser tweezers were considered to be perfectly safe manipulation tools, the clinical application of optical traps was considered. Trapping of sperm is of special interest for clinicians working in the field of male infertility treatment. Conventional IVF (in vitro fertilization) techniques rely on mechanical intracytoplasmic sperm microinjection (ICSI) directly into the egg cell (oocyte) and subzonal insemination (SUZI) by less invasive injection of sperm between the egg cell membrane and the outer zona pellucida layer. Yona Tadir and other IVF experts [27, 28] came up with the idea to use laser tweezers as a non-contact optical tool to transport single sperm cells of low motility directly to the egg cell. Furthermore, it was suggested to use an additional pulsed laser to drill a hole through the zona pellucida (LZD: laser zona drilling) and the oocytes membrane as well as to support hatching (LAH: laser assisted hatching) by zona opening of the embryo. Furthermore, the microsurgery laser could be used to cut off the flagellum from the sperm in order to maneuver the optically-trapped sperm head more easily (Fig. 9.2).

In order to realize laser-assisted IVF with cw laser tweezers and pulsed lasers for cell surgery, we performed laser safety studies on trapped human sperm using a tunable cw Ti:sapphire ring laser. We were astonished to find that trapping sperm with 760 nm laser tweezers for one minute resulted in a stop of flagellar motion and finally cell death. That means, cw NIR laser microbeams used as laser tweezers may cause cell damage. The reason is nonlinear absorption based on non-resonant absorption
9 Laser tweezers are sources of two-photon effects

Fig. 9.2: Laser-assisted in vitro fertilization. “Bad quality” sperm of low motility can be transported to and into the oocyte by laser tweezers. To assist the transport into the oocyte and the “fusion”, the outer membrane of the egg cell, the zona pellucida, can be perforated by non-invasive intracellular surgery with a second pulsed laser beam (laser zona drilling). Furthermore, the flagellum can be cut off for easier optical trapping. Finally, laser-assisted hatching of the embryo can be performed by a further opening of the surrounding zona. N: nucleus, ZP: zona pellucida.

of two NIR trapping photons [14, 16, 17, 24]. The required high MW/cm² intensity is provided by the cw laser beam of 100 mW or more power when focusing with high NA objectives. This chapter describes the experimental setup and the methods leading to the discovery of laser tweezers as sources of two-photon effects and their possible damage potential.

9.2 Experimental setup

Optical trapping of sperm and laser surgery of oocytes combined with fluorescence imaging were performed with a modified inverted fluorescence microscope (Axiovert 135M, ZEISS, Germany) equipped with galvoscanners for fast beam scanning and a joystick controlled motorized stage for additional stage scanning. The trapping beam was provided by an argon-ion laser pumped cw Ti:sapphire ring laser (899-01, Coherent Inc., USA, beam waist diameter 0.6 mm, divergence 1.7 mrad). The microsurgery laser was a frequency-doubled (KDP) Q-switched Nd:YAG laser with 4–6 ns pulse length (Surelite I, Continuum, USA). Both beams filled the back aperture of a NA 1.30 ZEISS Neofluor objective (100×, 440480, WD: 0.24 mm, immersion oil 518C with refractive index $n = 1.51$). Power regulation was performed with a polarizing beam attenuator (Karl Lambrecht Corp., USA). A 100 W mercury lamp (HBO) provided 365 nm and 405 nm fluorescence excitation light. The fluorescence was detected with a slow-scan cooled CCD camera (ZVS-47DEC, ZEISS, Germany) (Fig. 9.3). In addition, the microscope was equipped with a halogen lamp as white light source for brightfield microscopy. Further information can be obtained from [24].
To determine the optical forces generated by the optical trap on the confined specimen, accurate \textit{in situ} power measurements are required. Often measurements are performed in air with a power meter equipped with a limited sensor size after laser transmission through the objective. However, this does not allow precise measurements of highly convergent beams as in the case of high NA objectives. The measured values are, therefore, different from the real powers at the trapping sample (\textit{in situ} power). In order to determine the correction factors, a sandwich system consisting of identical opposite ZEISS brightfield objectives and the microchamber (with a thin 0.5 mm spacer and filled with PBS) between, was employed. The objectives were aligned in such a way that both had the same focal spot within the chamber resulting in incoming and leaving parallel beams. In the case of nonexpanded laser beams, a total system transmission of 33.9\% was measured (at 800 nm). That means, that each set consisting of the objective, the drop of oil, the 0.16 mm thick coverslip, and a 0.25 mm PBS layer, has a transmittance of about \((0.339)^{0.5} = 0.58\). Further measurements revealed a correction factor of 1.5 for the particular power meter [18].
9.3 Materials and methods

Human spermatozoa of three donors with normal semen parameters were obtained according to the World Health Organization guidelines. Semen was layered on a discontinuous isotonic Percoll gradient (Pharmacia, Sweden) and centrifuged for 15 min at 200 g. The bottom layer was washed with HEPES buffered fresh human tubal fluid (HTF, Irvine Scientific, USA) and centrifuged again for 10 min at 100 g. Finally, the pellet containing the sperm was diluted in HEPES buffered isotonic saline solution containing 1% human serum albumin (HSA). The sperm was injected into microchambers consisting of two 0.16 mm thick glass windows separated by a typical 3 mm thick silicon layer as spacer (calibration experiments: 0.5 mm thick spacer). The injection was performed through the silicon layer. Laser trapping was performed with the microchamber on the motorized stage through the glass window [24].

9.4 Determining the trapping force

The net trapping force \( F \) of the optical trap depends on the trapped object, the medium, and the quality of the trap (e.g., beam alignment) represented by the trapping parameter \( Q \) as well as on the laser power \( P \) and can be calculated as:

\[
F = \frac{QP}{c},
\]

where \( c \) is the velocity of light in medium. The trapping parameter can be derived from the Stokes equation by calculating the drag force \( F_{\text{drag}} \) that is exerted when the trapped sample is moved with the maximum possible velocity \( v_{\text{max}} \) before dropping off from the trap:

\[
F_{\text{drag}} = 6\pi\mu R_{\text{eq}} v_{\text{max}},
\]

where \( \mu \) and \( R_{\text{eq}} \) are the viscosity of the medium and the length parameter of the sample, respectively. Laser tweezers interact with the sperm head and not with the midpiece nor flagellum. In order to use the Stokes formula also for the ellipsoidal shaped sperm head \( (3 \times 5 \mu m^2) \), we developed a hydrodynamic model and calculated the length parameter \( R_{\text{eq}} \) of human sperm. Then, we excised the 60 \( \mu m \) long flagellum of 10 sperm samples with the 532 nm surgery microbeam and trapped the sperm heads (Fig. 9.3). By changing the stage speed we determined the drop-off velocity \( v_{\text{max}} \). Based on these data, we could finally determine a trapping parameter \( Q = 0.12 \) for human spermatozoa in our NIR Ti:sapphire trap for an 800 nm laser wavelength [18].

9.5 Determining the motility force

In a next step, 100 healthy spermatozoa were trapped at 800 nm. We started with a laser power of 150 mW and slowly reduced the power. The drop-off power was determined for each individual sperm. The most motile sperm required a minimum power
of 142 mW to confine it in the trap. The lowest power value for a motile sperm was found to be 29 mW.

With a mean drop-off power of (82 ± 38) mW, the calculated trapping parameter $Q = 0.12$, and the assumption of a nearly linear swim motion, the typical motility force of healthy human sperm can be determined to be (44 ± 22) pN.

When switching the laser wavelength from 800 nm down to 760 nm, we noticed an interesting effect on trapped spermatozoa. The motility force dropped within one minute trapping time. The trapped spermatozoa finally became paralyzed. A minimum (drop-off) trapping power as low as 0.3 mW was determined for immobile sperm [18].

### 9.6 Fluorescence imaging of trapped spermatozoa

The trapped cells can be imaged in the visible range above 420 nm with the cooled slow-scan CCD camera. Autofluorescence images were taken with UV exposure at 365 nm using the mercury lamp. As seen in Fig. 9.4, spermatozoa consist of

(i) a head that contains the non-fluorescent DNA,
(ii) a midpiece that contains mitochondria with autofluorescent coenzymes, and
(iii) the flagellum for motion.

Long UVA exposure paralyzed the sperm and sometimes a weak autofluorescence on the front part of the sperm head was monitored.

![Fig. 9.4: White-field image and autofluorescence image of a single trapped sperm][35].

In order to study possible phototoxic effects of NIR trapping beams, a Live/Dead Assay based on intracellular fluorescence was employed. The sperm heads were stained with the green-fluorescent live cell indicator SYBR14 and the red fluorescent dead cell indicator propidium iodide (Molecular Probes, USA). The fluorescence was excited with the mercury lamp for short ms exposure times and detected with the cooled CCD camera. When using 100 mW laser traps at 800 nm or higher wavelength, the spermatozoa remained alive up to 10 minutes in the optical trap. However, when using a shorter wavelength, such as 760 nm, the sperm died as monitored by color change of the fluorescent sperm head from green to red [24].
9.7 Trap-induced two-photon fluorescence

Surprisingly, when switching off the mercury lamp the sensitive cooled CCD camera could still detect a tiny fluorescent spot in the labeled sperm head. The position was identically with the intracellular trapping beam position [14].

In order to prove whether the fluorescence was caused by a two-photon effect, the power was enhanced. Indeed, the fluorescence intensity increased nonlinearly with a \( P^2 \) dependence as indicated in Fig. 9.5. This function is typical for two-photon effects.

Fig. 9.5: Trap-induced two-photon excited fluorescence in the sperm head. The fluorescence intensity depends on the laser power squared [35].

Trapping beams at 760 nm (2.62 \( \times \) 10\(^{-19} \) J photon energy) can be focused down to a spot size with a diameter of 0.61\( \lambda \)/\( \text{NA} = 357 \) nm and an exposure area of 0.1\( \mu \)m\(^2\), respectively, when using an \( \text{NA} = 1.3 \) objective. This results in a laser intensity of 100 MW/cm\(^2\) at 100 mW laser power and a photon flux density of more than 4 \( \times \) 10\(^{26} \) photons cm\(^{-2} \)s\(^{-1}\). This high concentration pf photons in space and time is sufficient to induce nonlinear effects, in particular to excite fluorophores with a high fluorescence quantum yield via non-resonant two-photon absorption.

9.8 Trap-induced nonlinear phototoxic effects

That finally means that cw laser tweezers are sources of two-photon effects. Two-photon absorption may result in the generation of heat, fluorescence, and photochemical reactions as indicated in Fig. 9.6. In principle, 760 nm traps can induce similar photodamage effects as 760 nm/2 = 380 nm UV effects. UV light at 380 nm may induce reactive oxygen species (ROS) that can kill a cell.

We determined the phototoxic effect of the cw Ti:sapphire ring laser trap (100 mW) in dependency on wavelength. No phototoxic effects could be found for trapping wavelengths of 800 nm or higher. Cells trapped at 780 nm could be held alive for 8 min. The most damaging effect was found for 760 nm traps. In that case, sperm survived only one minute ((65 ± 20) s).
A damage parameter (DP) was defined as the reciprocal value of the mean time when red fluorescence appeared, indicating cell death [24]. The DP values as a function of the trapping wavelength are depicted in Fig. 9.7.

### 9.9 Photodamage effects as a result of mode-beating phenomena

Under normal conditions, the cw Ti:sapphire ring laser operates in the multimode regime, that means, a variety of longitudinal modes is present. Superposition of these modes can cause mode-beating effects resulting in power fluctuations.

In our cw laser trap we measured such power fluctuations based on the occurrence of unstable pulses with a repetition frequency $f$ as a multiple of the 180 MHz base frequency. This base frequency corresponded to the cavity length $L$ of 1.70 m according to the formula $f = c/L$ with $c$ as the velocity of light.
Fig. 9.8: Unstable laser pulses may occur in multimode cw laser due to mode-beating phenomena. The output of the Ti:sapphire ring laser was recorded with a 1 GHz detector [17].

Interestingly, the strongest pulses were found when the tuning birefringent filter with 20 GHz linewidth was optimized for the 760 nm output. Fig. 9.8 shows unstable sub-nanosecond laser pulses at 760 nm and 800 nm laser output. The amplitudes of the 800 nm pulses are about 3 times lower than these for the 760 nm pulses [17].

In order to figure out if the enhanced damaging effect is of biological origin or a result of the enhanced mode-beating effects in the multimode cw laser, modifications on the laser resonator were performed.

By introducing a special etalon into the laser resonator, our multimode laser was transformed into a “perfect” cw single frequency laser. Now mode-beating effects could be avoided and the power output became stable without any occurrence of transient laser pulses. As expected, trapped human sperm now survived longer in the trap. However, photo-killing effects could not be avoided. In fact, trap-induced cell death was now observed after (406 ± 160)s in single-frequency cw traps instead of (65 ± 20)s in multimode cw traps [17].

9.10 Conclusion

Laser traps have become important optical micromanipulation tools for single cell handling and pN force transducers [29]. Typically, tightly focused Nd:YAG laser at 1064 nm, frequency doubled erbium:YAG laser fibers at 760 nm, tunable cw Ti:sapphire ring lasers, and laser diodes are employed as cw NIR trapping sources. Interestingly, these intense NIR laser beams can induce two-photon effects such as two-
photon excited fluorescence and two-photon induced “UV-like” photochemical destructive effects. Therefore, NIR traps can induce cell damage including cell death. Trapping wavelengths higher than 800 nm are safer than short-wavelength NIR. The use of Nd:YAG lasers at 1064 nm is recommended. Also, single-frequency cw lasers are safer than multimode cw lasers due to possible mode-beating effects. This should be considered in particular for clinical applications, such as laser-assisted IVF. Meanwhile, the first “laser babies” have been born. To my knowledge, the first reported laser-assisted IVF with human gametes and successful birth occurred in Germany [30]. A first larger LAZ study on 179 patients was performed in Italy by Antinori et al. [31]. Today, laser-assisted hatching is used in many IVF clinics worldwide [32]. So far, laser tweezers for sperm transportation as well as to test the quality of sperm, in particular of the individual sperm to be used for IVF procedures, are not yet used clinically.

Trap-induced fluorescence can be employed as a novel tool for nonlinear cell diagnostics. In general, two-photon microscopy including two-photon lithography can also be performed with low-cost continuous wave (cw) laser sources [33, 34]. However, the use of femtosecond laser sources is recommended in two-photon microscopes due to the far more efficient process when using transient kW laser peak powers and low sub-mW and mW mean femtosecond laser powers.

References

Laser tweezers are sources of two-photon effects


