Cellular and Molecular Biology™ **44** (5), 721-733
Printed in France.

0145-5680/97
1998 Cell. mol. Biol.™

LASER TWEEZERS ARE SOURCES OF TWO-PHOTON EXCITATION

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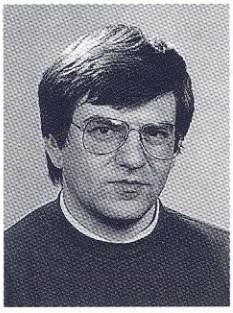
Received May 22, 1998; Accepted July 16, 1998



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Abstract - The most important application of continuous wave (cw) near infrared (NIR) microbeams in cellular and molecular biology are single-beam gradient force optical traps, also called "laser tweezers". Laser tweezers have been used for optical picoNewton force determination as well as for 3D cellular and intracellular micromanipulation, such as optical spermatozoa transportation in laser-assisted *in vitro* fertilization. Light intensities in the MW/cm^2 range are necessary to confine motile spermatozoa in the optical trap. The enormous photon concentration in space and time results in non-resonant two-photon excitation of endogenous and exogenous absorbers with electronic transitions in the ultraviolet and visible spectral range. Trap-induced two-photon excitation of intracellular flurorophores can be used to study metabolism and vitality of motile cells without additional fluorescence excitation sources. Therefore, laser tweezers as sources of two-photon excitation may act as novel non-linear tools in cell diagnostics. The far red/NIR trapping radiation, in particular <800 nm, may also excite endogenous absorbers such as NAD(P)H, flavins, porphyrins and cytochromes. Excitation of these cellular absorbers may result in oxidative stress via type I and type II photooxidation processes. Severe non-linear-induced cell damage in a variety of cells confined in <800 nm traps was found. Two-photon induced destructive effects are enhanced in multimode traps due to longitudinal mode-beating phenomena. Pulsed laser sources are not suitable for safe optical trapping of living cells. The use of single frequency long-wavelength NIR traps (800 nm-1200 nm) for vital cell handling is recommended.

Key words: Laser tweezers, optical trap, two-photon excitation, fluorescence, spermatozoa

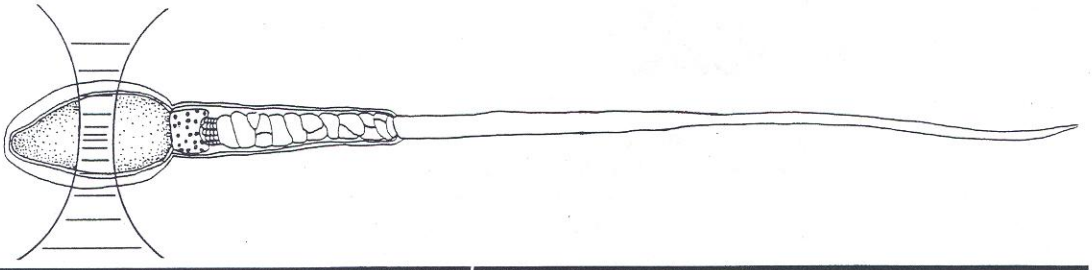
INTRODUCTION

Abbreviations: **cw**: continuous wave; **Hb**: hemoglobin; **IVF**; *in vitro* fertilization; **NA**: numerical aperture; **NIR**: near infrared

Laser tweezers, also called (single beam gradient force) optical traps, are based on force generation during the interaction of focused laser beams with

micrometer-sized and submicrometer-sized particles, such as biological cells and cell organelles. In order to use laser tweezers as optical micromanipulation tools in handling living specimens, the trapping beam should be continuous wave (cw) radiation with a wavelength in the "optical window" of cells and tissues. This spectral window is in the range of 600 nm to 1300 nm (red to near infrared, NIR) where most cells do not contain efficient endogenous molecular absorbers. As a result, the net force in the case of a highly focused NIR laser beam is determined primarily by beam refraction within the sample due to differences in the refractive index between cell ($1.33<n<1.54$) and medium (1.33) (ray optics model). This trapping force F can be sufficient to confine and to manipulate single cells or cell organelles in the focal volume of a high numerical aperture (NA) objective (Ashkin, 1970; Ashkin *et al.*, 1986; Ashkin and Dziedzic, 1987). Major applications of laser tweezers are basic research as well as cell handling, e.g. in pharmacology (e.g. Zahn and Seeger, 1998) and in medical treatment, such as in laser-assisted *in vitro* fertilization (IVF). In the latter case, single sperm cells are optically caught and moved to the oocyte by laser tweezers. In combination with laser microsurgery on oocytes (hole drilling), "optical sperm injection" into the egg by laser traps is possible. For example, 89 patients were treated with 1064 nm traps and 337 nm laser scissors in Germany in 1993 (Wiedemann and Montag, 1994). Meanwhile, the first "laser babies" are born.

In order to confine highly motile cells, such as human spermatozoa, the trapping force has to be larger than the ATP-driven motility force. In order to achieve such trapping forces typical laser powers on the order of 100 mW are required. Note that in the case of immobile cells, such as erythrocytes, lower laser powers of some mW are sufficient for trapping. Typical trapping parameters for human sperm confinement are seen in fig 1. As indicated,



P mW	λ nm	photon energy J	$d=\frac{\lambda}{NA}$ nm	A μm^2	intensity MW/cm^2	photon flux density photon $cm^{-2} s^{-1}$
100	1064	$1{,}88 \times 10^{-19}$	819	0,53	19	$1{,}0 \times 10^{26}$
100	800	$2{,}49 \times 10^{-19}$	615	0,30	34	$1{,}4 \times 10^{26}$
100	780	$2{,}55 \times 10^{-19}$	600	0,28	35	$1{,}4 \times 10^{26}$
100	760	$2{,}62 \times 10^{-19}$	585	0,27	37	$1{,}4 \times 10^{26}$
1,5	365	$5{,}47 \times 10^{-19}$	190000	28350	5×10^{-6}	$1{,}0 \times 10^{19}$

Fig. 1 *Typical trapping parameters.* Powers of about 100 mW are required to confine highly motile cells, such as human spermatozoa, in the focal volume of a high numerical aperture objective. For comparison, parameters of the 365 nm radiation of the 100 W high pressure mercury lamp are depicted.

enormous laser intensities in the MW/cm^2 are induced due to diffraction-limited focusing. In the course of an 1 min. experiment, the cell will experience fluences as high as GJ/cm^2. The question arises concerning the impact of the trapping beam on cellular metabolism and vitality. What about photothermal stress, photomechanical stress and photochemical stress?

This review demonstrates that photostress has to be considered in cell micromanipulation by laser tweezers. Photochemical effects have the most significant influence on trapped cells. The origin of this photochemistry is of non-linear nature. Under certain conditions it may result in irreversible cell damage. Otherwise, trap-induced non-linear excitation allows the development of a novel cell diagnostic method suitable even for the investigation of highly motile cells.

MATERIALS AND METHODS

Experimental Set-up

Optical trapping was carried out by using modified invert microscopes (Axiovert 135M, Zeiss, Germany) equipped with scanning units (laser scanning microscopes) and with motorized specimen stages. The trapping beam was provided by a tunable (700 nm-1000 nm) Ar^+-ion laser pumped cw Ti:Sapphire ring laser (899-01, Coherent, CA) or by a cw Nd:YAG laser at 1064 nm (Adlas, Germany). The parallel beam was expanded to fill the back aperture of a 40x, 63x and 100x Zeiss Neofluar brightfield objectives (immersion oil Zeiss 518C with refractive index n=1.51, numerical apertures NA=1.30, 1.25 and 1.30). The laser power at the sample (*in situ* power) as well as the NIR transmission of a variety of objectives was determined using a sandwich set-up consisting of a special medium-filled microchamber between two identical objectives and immersion oil. The objectives were aligned in such a way that both objectives had the same position of the focal spot inside the chamber resulting in a parallel beam leaving the set-up. In contrast to the critical one-objective-measurement of a highly divergent beam in air, the sandwich set-up considers the transmission through oil, coverslip and medium and allows the correct power measurement with conventional power meters. A more detailed description of the method has been reported earlier (König *et al.*, 1996a). A typical NIR transmission value for most objectives of about 50% was found. A high pressure 100 W mercury lamp provided 365 nm radiation for one-photon fluorescence excitation.

Additionally, the highly focused second harmonics (532 nm, 1 mJ pulse energy, SHG generation with KDP crystal) radiation of a Q-switched Nd:YAG laser (4-6 ns pulse length, Surelite 1, Continuum, Santa Clara, CA) was used as laser scissors for flagellum removal from human spermatozoa. Cell manipulations were monitored with "white" radiation of the halogen lamp (brightfield microscopy) and recorded with cooled black/white (bw) and color video cameras (Fig. 2).

Cell Preparation

Chinese hamster ovary (CHO) cells (ATCC no. 61) as well as

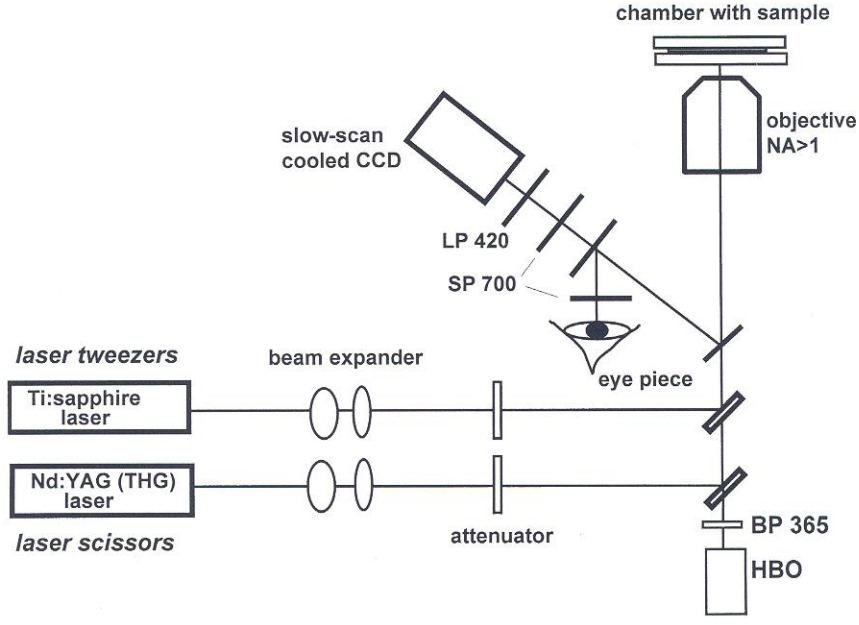


Fig. 2 *Experimental set-up.* A microscope with motorized specimen stage was equipped with trapping- and cutting laser beams for micromanipulation of optically-trapped motile spermatozoa. A cooled CCD-camera was used for fluorescence imaging of trapped specimens.

human spermatozoa of donors with normal semen parameters were studied. For trapping experiments, CHO cells were trypsinized. The typical CHO diameter was determined to be 11.0 ± 1.9 µm. Semen was layered on a discontinuous isotonic percoll gradient (Pharmacia, Sweden). After centrifugation for 15 min. at 200 g, the bottom layer was removed, washed with HEPES buffered fresh human tubal fluid (HTF, Irvine Scientific, Irvine, CA) and centrifuged for 10 min. at 100 g. The pellet containing sperm utilized for experiments was then diluted in HEPES buffered isotonic saline solution containing 1% human serum albumin (HSA). Typical flagellar and head dimensions of spermatozoa were 60 x 0.4 x 0.4 µm and 5 x 3 x 2 µm, respectively. Human erythrocytes, freshly drawn from a finger of a healthy donor, were diluted in PBS with 0.5% glucose. Cells were injected into microchambers consisting of two 0.16 mm thick coverslips separated by a 3 mm thick silicon layer as spacer (Fig. 3). Trapping was performed within the closed sterile cell chamber at 26°C.

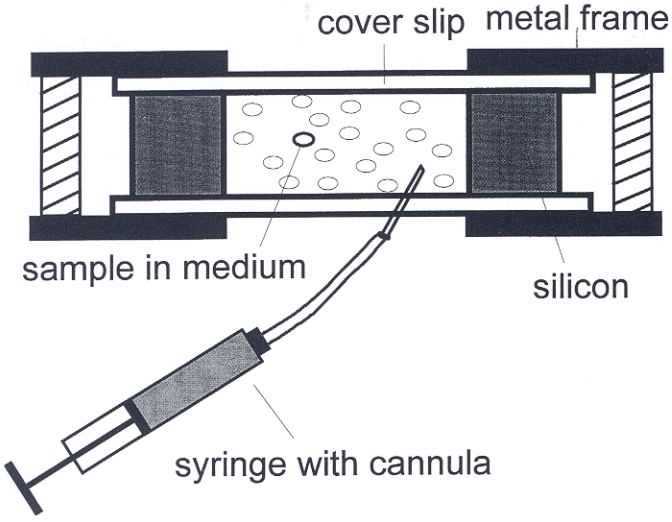


Fig. 3 *The use of appropriate microchambers allows sterile non-contact optical micromanipulation in three dimensions.* Easy medium change, fluorophore injection, pH and temperature measurements can be performed with microneedles injected through the silicon layer.

RESULTS

Photothermal Effects

The enormous GJ/cm^2 fluences during trapping experiments rise the question concerning cell heating. As mentioned, the red and NIR range between 600 and 1200 nm is termed "optical window of cells and tissues" due to the absence of efficient linear absorbers and high penetration depth of light beams. For cells, which do not contain the pigments hemoglobin, chlorophyll or melanin, water is considered to be the major absorber (Fig. 4). The exact intracellular absorption behavior is not known. The absorption coefficient of water, for example at 1064 nm, is as low as 0.1 cm^{-1} (Hale and Querry, 1973). For typical cell dimensions of 10 µm and 70% water content, the intracellular absorption is on the low order of about 10^{-4}. However, the absorption and the heat diffusion into extracellular medium has to be considered.

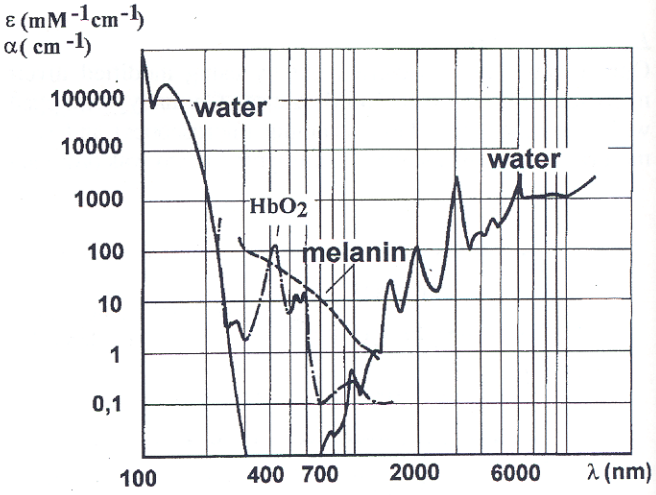


Fig. 4 *NIR absorption spectrum of water, hemoglobin and melanin.*

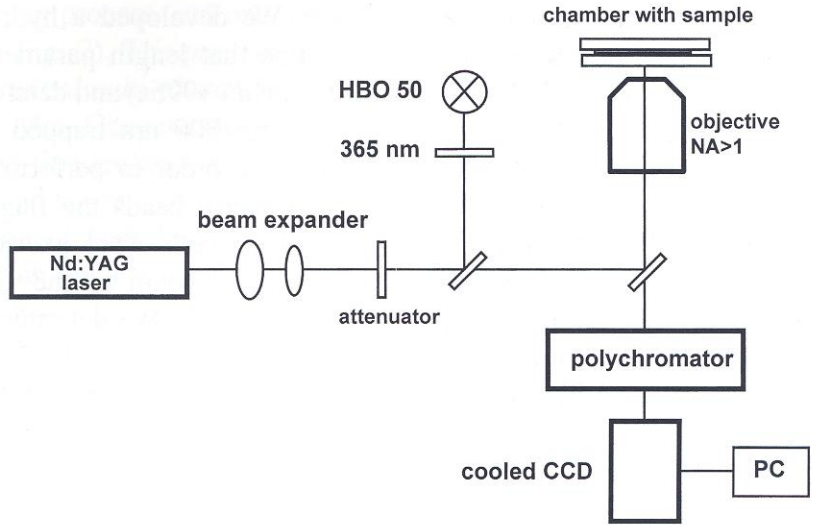


Fig. 5 *Laser tweezers with microspectro-fluorometer for linear and non-linear spectrosopy of intracellular NADH and exogenous fluorophores.*

In order to obtain experimental data of trap-induced intracellular heating, CHO cells in the 1064 nm trap were labelled with the thermosensitive fluorophore Laurdan (Molecular Probes, Eugene, USA) (König *et al.*, 1995a; Liu *et al.*, 1995). The fluorescence spectrum of UVA-excited Laurdan undergoes spectral modifications during heating. Fluorescence spectroscopy was performed with a microspectro- fluorometer in combination with laser tweezers (Fig. 5) as described elsewhere (König *et al.*, 1995b; Liu *et al.*, 1995). The measurements revealed that the thermal equilibrium was obtained after about 1 s trapping. A trap-induced heating rate of (1.15 ± 0.25)K / 100 mW was determined for CHO cells in the 1064 nm trap. For comparison, the temperature increase by the 1 mW UVA excitation source was found to be 1 K and 2-3 K for water and oil immersion objectives, respectively. For human spermatozoa, the heating rate was 1-2 K/100 mW. Therefore, trap-induced photothermal cell damage can be excluded.

Because the water absorption drops by one order for short-wavelength NIR [$\alpha(760\ nm) = 0.01\ cm^{-1}$], laser tweezers with a trapping wavelength <800 nm have been considered to be extremely safe micromanipulation tools. As shown in the following chapters, the opposite case happens. Short-wavelength red/NIR traps are more dangerous for vital cell handling.

Much higher trap-induced temperatures are obtained if hemoglobin (Hb) is the major cell absorber. For example, the molar absorption coefficient of reduced Hb at 730 nm is 1.3 $mM^{-1}\ cm^{-1}$. With an estimated Hb concentration of 23 mM in a human erythrocyte (86 fl mean corpuscular volume, 2 fmol Hb) and a beam propagation along the maximum erythrocyte length of 8 µm, the erythrocyte absorption (2.4×10^{-2}) is about 2 orders higher than for CHO cells and spermatozoa. Fig. 6 shows experimental data on trap-induced hemolysis at 26°C room temperature. Trapping powers of 50 mW

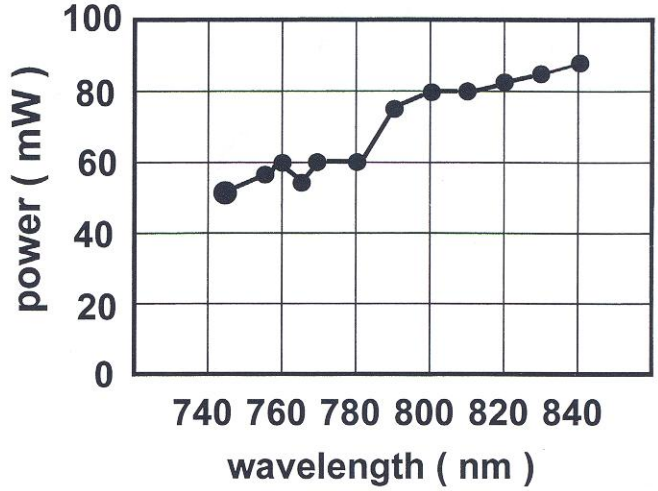


Fig. 6 *Trap-induced hemolysis of human erythrocytes.* Hemolysis occured within 2 min. of trapping.

(740 nm) to 85 mW (840 nm) are sufficient to induce significant cell damage. For comparison, non-trapped erythrocytes were exposed to high medium temperatures in a heating bath. 5% and 100% of cells were hemolyzed at 50°C and 62°C within 2 min., respectively. In a first estimation, the trap-induced heating rate is therefore (62-26)K / 60 mW = 60K / 100 mW. However, it should be mentioned that trapping of erythrocytes do not require power levels as in the case of motile spermatozoa. We were able to trap erythrocytes with a power as low as 1-5 mW.

Photomechanical Effects

In order to study the influence on trap-induced photomechanical effects, the trapping forces within a single human sperm cell was calculated. The net trapping force can be represented by:

$$F = Q\frac{P}{c} \qquad (1)$$

where c is the velocity of light in medium, P the incident laser power at the sample, and Q the trapping efficiency parameter with values 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. It should be mentioned that the parameter Q depends on the optical properties of the trapped sample as well as on the quality of the trap (e.g. beam alignment). The knowledge of the trapping parameter Q would allow to calculate trap-induced forces. For "real" non-homogenous samples, Q has to be determined experimentally. Q can be derived from the Stokes equation by calculating the drag force, F_{drag}, that is exerted when the trapped sample is moved with the maximum (drop off) velocity v_{max} through the medium:

$$F_{drag} = 6\pi R_{eq} \mu v_{max} \qquad (2)$$

where R_{eq} and μ are, respectively, a length parameter of the sample (in the case of a sphere: radius) and the dynamic viscosity of the medium. The normal human sperm head has a geometry that better can be approximated with an ellipsoid than a sphere. We developed a hydrodynamic model to determine that length parameter for sperm heads (König *et al.*, 1996a) and determined the maximum velocity for 800 nm trapped sperm heads in the medium. In order to perform this experiment on isolated sperm heads the flagellum and the midpiece of 10 cells were excised using the highly focused laser beam of the Q-switched Nd:YAG laser at 532 nm. We determined a mean trapping parameter of Q = 0.12 ± 0.02 for human spermatozoa. That means, that according to eq. 1 a mean trapping power of 53 pN is induced in spermatozoa when trapped with 100 mW laser beams at 800 nm.

In the case of healthy spermatozoa (n = 100), a minimum trapping power of 82 ± 38 mW for confining cells in the trap was found (lowest value for a motile sperm: 29 mW, the highest 142 mW). A minimum trapping power for immobile sperm of 0.3 ± 0.1 mW was determined. Under the assumption of linear swim motion, the intrinsic motility forces of motile human spermatozoa can be calculated by use of the Q-value for human sperm heads and the measured 82 ± 38 mW minimum trapping power to be 44 ± 24 pN.

In conclusion, the trap-induced forces are on the order of picoNewton and therefore in the range of typical cellular forces such as the spermatozoa motility forces. Destructive photomechanical effects appear therefore unlikely.

Photochemical Effects

– *Principle of two photon excitation*: From the first point of view one could say: no NIR absorbers, no photochemistry. However, absorption of NIR radiation is possible and can result in the excitation of a variety of endogenous absorbers with electronic transitions in the UV and visible spectral range. Such absorption can occur via a non-linear process in the case of high laser intensities and photon flux densities, respectively. In this case, two or more NIR photons are absorbed simultaneously providing each a part of the energy necessary for molecule excitation. For one-wavelength excitation, the absorbed photons would provide half the

energy in a two-photon process, and one third in a three-photon event (Fig. 7). The process is called non-resonant two-photon/multi-photon absorption. It was predicted by Mrs. Göppert-Meyer, 1931 in her Ph.D. Thesis and first realized with the ability of lasers by Kaiser and Garret (1961). They detected the blue emission of $CaF_2:Eu^{2+}$ crystals with pulsed red light excitation. Two-photon excitation in living cells was first demonstrated by Denk, Strickler and Webb (Denk *et al.*, 1990) who used a femtosecond dye laser source with peak powers in the kW range for intracellular fluorescence imaging.

– *Trap-induced fluorescence*: Two-photon excitation requires a high photon concentration in space and time due to the low molecular two-photon absorption cross sections α on the order of 10^{-48}-10^{-50} cm^4 s for most endogenous and exogenous absorbers. In fact, the exact two-photon cross sections are not known for most dyes. As already indicated in fig. 1, 100 mW laser tweezers provide intensities and photon flux densities of I >20 MW/cm^2 and Φ >1026 photons cm^{-2} s^{-1}, respectively. This is sufficient to induce two-photon effects. According to equation:

$$dN/dt = \eta N \alpha \Phi^2 \qquad (3)$$

the expected fluorescence photon rate dN/dt of a highly fluorescent 10 µM dye (η = 1, approximately N=600 molecules in the 0.1 fl excitation volume) is about 10^5-10^7 photons per second. In 1994, we were able to detect trap-induced two-photon excited fluorescence within living sperm (König *et al.*, 1995c). To our knowledge this was the first demonstration of non-linear effects in living cells with cw microbeams. CW-induced fluorescence in non-organic materials has been reported earlier (e.g. Foot *et al.*, 1985; Johnson and Lytle, 1980). Hänninen *et al.* (1994) performed two-photon imaging with radiation of a Ti:sapphire laser in the cw mode on single (dead) spermatozoa (scanning rate: 12 min./image).

In order to prove trap-induced two-photon effects in living spermatozoa, the intracellular viability indicators SYBR14 (green-fluorescent live cell indicator) and propidium iodide (red fluorescent dead cell indicator) from Molecular Probes were incubated and excited with NIR trapping beams in the spectral range from 730 nm to 1064 nm. The fluorescence was detected with slow-scan cooled bw and color CCD cameras. A visible fluorescence spot of about 0.5 µm diameter was found in the sperm head, sometimes also in the midpiece. The spot was the resulted of non-resonant two-photon

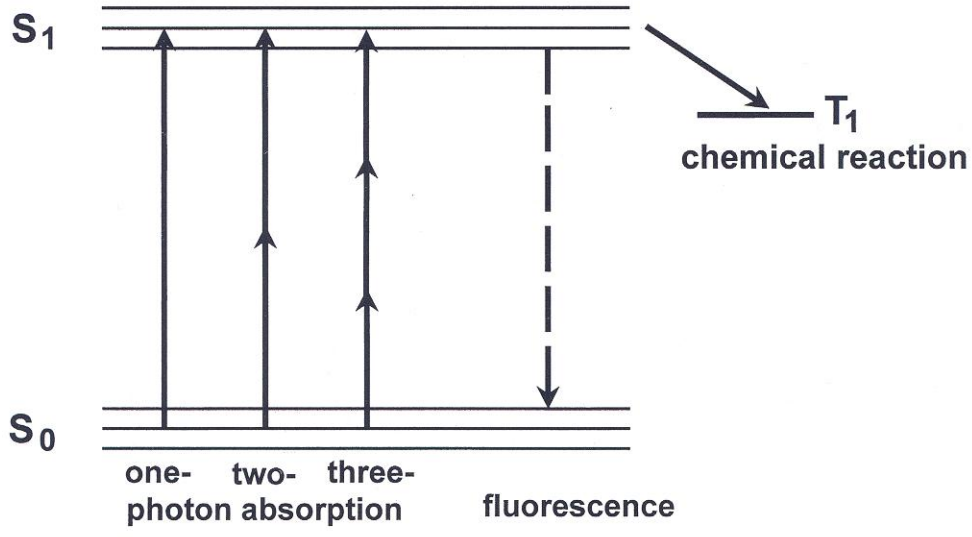


Fig. 7 *Principle of two-photon and three-photon excitation with one NIR beam.* Electronic transitions in the ultraviolet and short-wavelength visible spectral range can be excited by the simultaneous absorption of two NIR photons or three NIR photons. Non-linear excited fluorescence has in general the same spectral and temporal characteristics as in the case of conventional one-photon excitation.

excitation and indicated the exact intracellular trap position. The monitored intracellular fluctuations of the spot localization was found to be in correlation with sperm motility (phases of high flagellar motion changed with "relaxation phases"). Cells in 800 nm and 1064 nm traps remained alive up to 10 minutes as indicated by two-photon excited green DNA fluorescence. However, cells in short-wavelength <800 nm traps died as monitored by color change from green to red.

A large variety of exogenous fluorophores could be excited by the cw laser tweezers, such as the thermosensitive fluorophore Laurdan, the pH indicator SNARF, Rhodamine 123, Acridin Orange and various fluorescent microspheres. It was also possible to measure the trap-excited fluorescence spectrum of intracellular fluorophores using the microspectrometer (Figs. 8a-8c). Interestingly, we were able to image a submicron fluorescent spot in unlabelled sperm and CHO cells within <800 nm

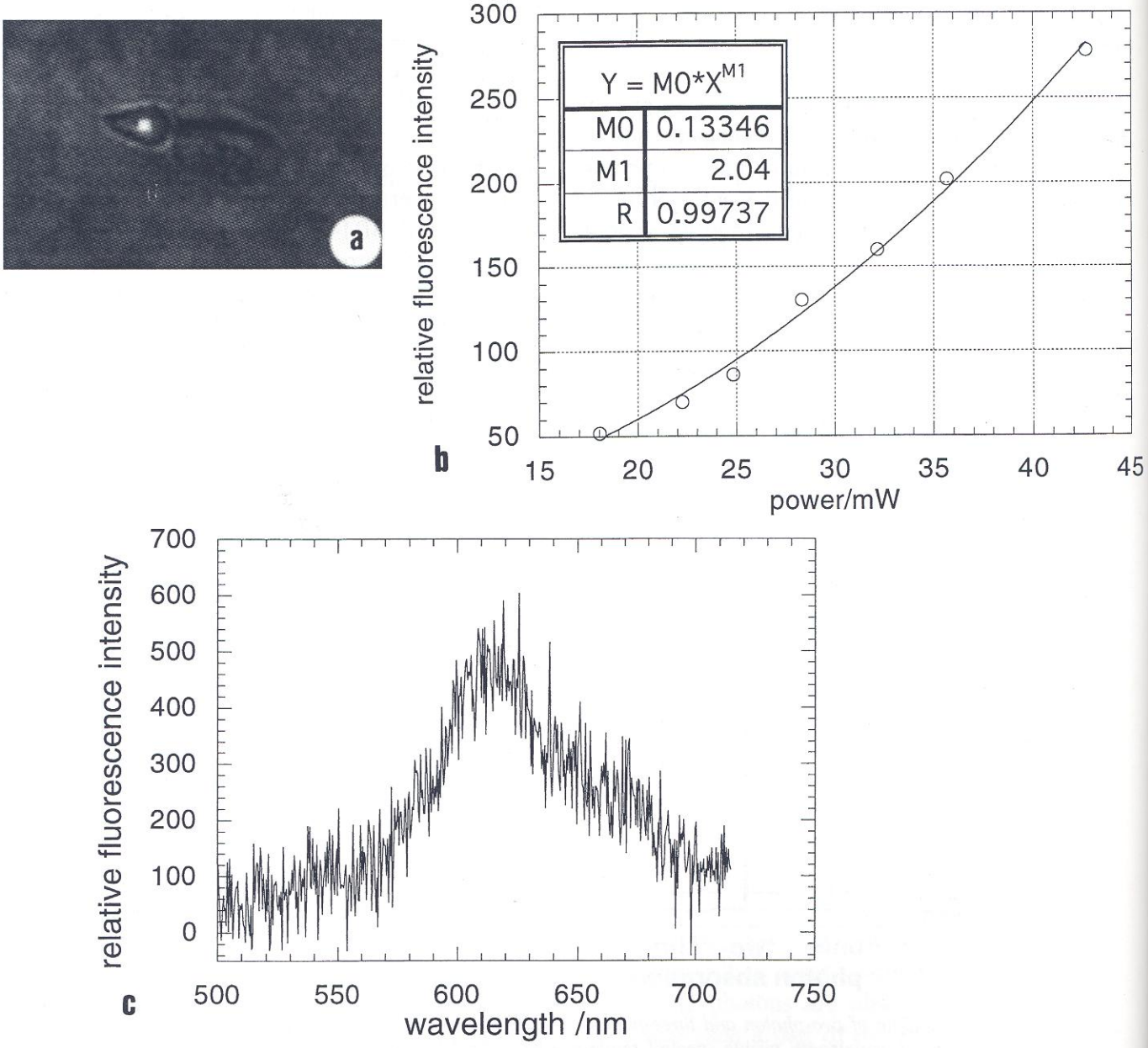


Fig. 8 *Transmission image with two-photon excited fluorescence spot* (**a**), *fluorescence signal vs. laser power* (**b**), *and fluorescence spectrum of trap-induced visible fluorescence in the sperm head* (**c**).

traps with a fluorescence intensity which depended on trapping time. This two-photon excited autofluorescence phenomena is explained in the next chapter.

The results demonstrate that NIR laser tweezers - and cw microbeams in general- are powerful fluorescence excitation sources for endogenous as well as exogenous cellular fluorophores.

– *Trap-induced cell damage*: As mentioned, laser tweezers are under certain conditions able to induce cell death. When tuning the Ti:sapphire laser to wavelengths <800 nm, spermatozoa slowed down flagella movement and got finally paralyzed. In addition, the autofluorescence pattern changed. Before and at the beginning of trapping, the autofluorescence in the blue/green spectral range with a maximum at 450 nm-460 nm was localized in the midpiece of the sperm cell (site of mitochondria in spermatozoa) and in the extranuclear region of CHO cells (mainly in mitochondria). The nucleus of intact cells exhibited no visible autofluorescence. The spectral behavior as well as the fluorescence lifetime (So *et al.*, 1998) were typically for the emission of the reduced coenzymes NADH and NADPH. We investigated autofluorescence modifications during trapping. For that purpose, trap-excited autofluorescence as well 365 nm-excited autofluorescence (1 s exposure time, 1 mW, cell imaging) was detected with a cooled slow-scan CCD camera. In the case of short-wavelength traps, the autofluorescence intensity increased with trapping time and the nucleus becomes highly fluorescent. This is likely due to NAD(P)H efflux from photoinduced damaged mitochondria and diffusion through damaged nuclear membranes. Traps at 800 nm or 1064 nm did not induce such autofluorescence modifications. In fact, CHO cells in an 1064 nm trap did not change the autofluorescence behavior even for long trapping times of 1 hr. and fluences >20 GJ/cm^2 (Fig. 9).

Exposure of adherent CHO cells (no trapping) to 730 nm-780 nm multimode microbeam radiation (90 mW) resulted in reduced cloning efficiency, formation of giant cells and cell death. At 760 nm, no CHO cell was able to divide after 1 min. exposure.

Following changes in flagellar motion and autofluorescence, sperm cells finally died in short-wavelength traps. Interestingly, the most destructive effect was found for traps based on 760 nm multimode cw laser radiation. Trapping of only 65 ± 20 s at 760 nm resulted in onset of propidium iodide fluorescence, that means in cell death. Fig. 10 demonstrates the trap-induced spermatozoa damage. For comparison, the effect of low-power UVA radiation (1 mW, 365 nm) was studied.

– *Laser output effects*: Many cw lasers operate in the multimode regime, that means, a variety of longitudinal modes is present. Superposition of these modes can produce so-called mode-beating effects which result in power fluctuations. Therefore, the output of multimode cw lasers can consist of unstable pulses with transient powers higher than the average power instead of a constant output power. Because of the squared dependence of the two-photon effect on power, multimode laser traps are sources of enhanced two-photon excitation.

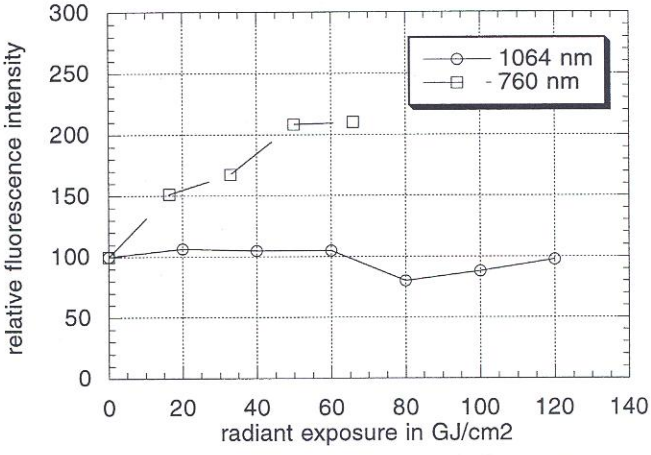


Fig. 9 *Trap-induced changes of CHO autofluorescence.* In contrast to 760 nm tweezers, 1064 nm traps did not induce autofluorescence modifications.

λ (nm)	P_t /mW (2 min)	t_M/s	t_{PI}/s
control	82 ±38	> 600	> 600
365	10 ±18	109± 30	310± 110
760	0	35± 20	65 ±20
800	62 ±21	> 600	> 600
1064	80 ± 30	> 600	> 600

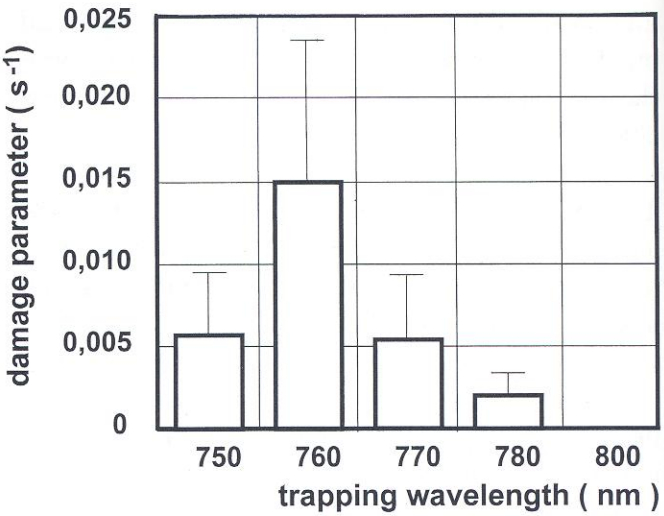


Fig. 10 *Trap-induced spermatozoa damage. 760 nm multimode trapping resulted in cell death within 1 min.*

In fact, we measured in our cw Ti:sapphire laser the presence of unstable ps pulses with a pulse duration <500 ps (König *et al.*, 1996c). The repetition frequency f was multiple of f_o=180 MHz. The base frequency f_o corresponds to c/p, where c is the velocity of light and p=1.7 m the cavity length of the ring laser. Interestingly we found in our cw Ti:sapphire laser a dependence of pulse amplitude on laser wavelength. As indicated in fig. 11, the highest peak power was obtained when the birefringent filter position was optimized for the 760 nm output. Two-photon effects were therefore most efficient at 760 nm.

The shortest possible pulse width τ is obtained in the case of mode-locking and can be calculated by estimation of the number of allowed modes N and the round-trip cavity time T with T=1/f and τ=T/N. The number N is determined by the birefringent filter which had in our case a linewidth of 20 GHz. For 180 MHz pulses, 111 longitudinal modes can, in principle, contribute to the pulse formation with a theoretical shortest pulse length of 50 ps. The direct exact measurement of pulse length is difficult due to the pulse unstability. The fig. 11 0 demonstrates the oscilloscope recording made with a 1 GHz detector. As indicated the peak amplitudes of 760 nm and 800 nm differ by a factor of 3 which would result in a estimated factor 9 difference in two-photon effects in the case of no wavelength dependence.

A multimode cw laser can be transferred into a true cw laser (single frequency laser) by introduction of a special etalon, e.g. of 20 MHz linewidth, into the laser resonator. The output of such a single frequency laser does not contain any power fluctuations due to mode-beating effects. We compared the biological effects of the multimode laser output vs. single frequency laser output at 760 nm. Trap-induced cell death of spermatozoa was monitored at 406 ± 160 s in true cw traps compared to 65 ±20 s in multimode traps. Therefore, cell destruction was 6-7 times more pronounced in multimode traps.

The "760 nm effect" as seen in fig. 10 is based on the output effect of our laser in multimode operation. It has no biological origin. Nevertheless, 760 nm single frequency traps were found to be more dangerous to cell viability than 800 nm multimode traps. A variety of experiments has been conducted at 760 nm using the same cw multimode Ti:sapphire ring laser. Vorobjev *et al.* (1993) reported on enhanced DNA damage at this wavelength and Liang *et al.* (1996) found the strongest microbeam-induced reduction in CHO cloning efficiency at 760 nm. However their action spectra (cell damage

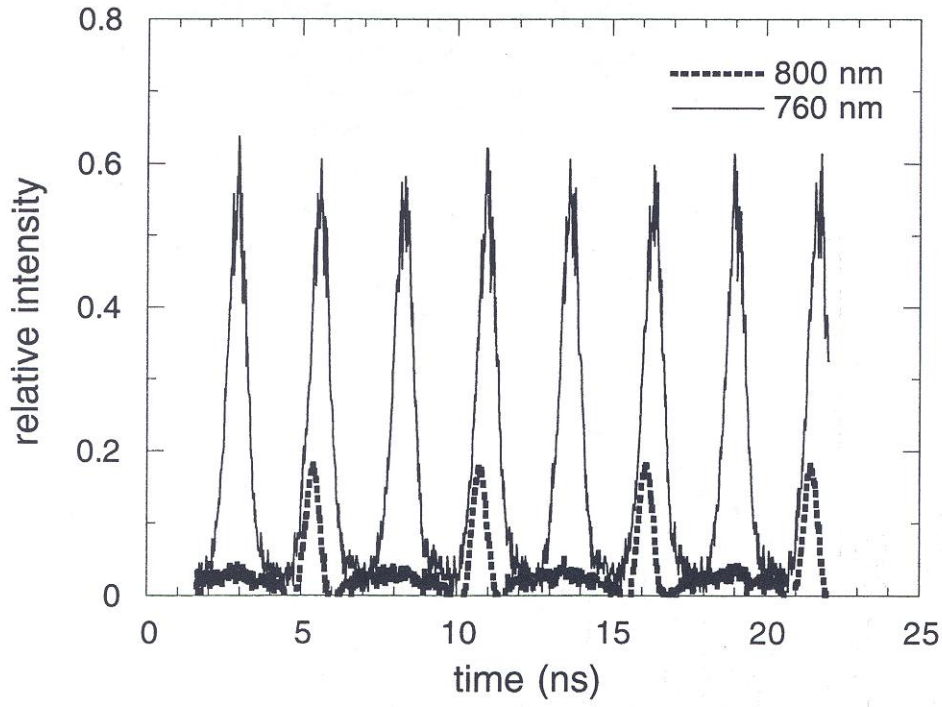


Fig. 11 *Mode-beating phenomena in multimode cw lasers.* The amplitudes of the picosecond pulses amplify two-photon effects

vs. wavelength in microbeam studies, no trapping) did not consider output effects. It is therefore very likely that the obtained "safe" and "unsafe" wavelengths are due to the absence and presence of mode-beating effects in addition to biological effects (e.g. linear and multimode-enhanced nonlinear absorption by endogenous absorbers).

– *Pulsed laser traps*: As shown in the preliminary chapter picosecond pulses may enhance trap-induced two-photon effects due to the squared dependence on peak power. The use of laser pulses with a moderate average power but high peak power are therefore no appropriate tools for safe optical micromanipulation. For example, the interaction of spermatozoa with femtosecond pulses at an average power of 100 mW and a 7 kW peak power (160 fs, 80 MHz), respectively, resulted in immediate cell damage. Hit by the beam, sperm heads "exploded" and the tail ruptured.

Even the average power decrease to <10 mW resulted in severe damage. Because it was not possible to confine motile spermatozoa with this low power, we scanned a single non-motile sperm cell of a donor with infertility and found with the second scan NAD(P)H relocalization (see So *et al.*, 1998).

Trapping of human erythrocytes with femtosecond pulses (160 fs, 80 MHz) was possible with <5 mW average power without hemolysis. However, mean powers in the range 2 -5 mW resulted in discocytic-echinocytic shape transformation (König *et al.*, 1997).

DISCUSSION

Laser tweezers may act as force transducers (e.g. Ashkin *et al.*, 1990; Block *et al.*, 1989; Chu, 1991; Svoboda *et al.*, 1993; Svoboda and Block, 1994), optical micromanipulation tools (e.g. Tadir *et al.*, 1990) and, even simultaneously, as novel nonlinear cell diagnostic tools. They provide the possibility to detect fluorescence in cells and cell organelles including highly motile specimens without external fluorescence excitation sources. The tiny sub-femtoliter excitation volume provides

information on the exact localization of the trapping beam. There is no out-of-focus photodamage and photobleaching, nor background fluorescence excitation. Trap-induced fluorescence can be used to monitor the influence of the trapping beam on metabolism and vitality of the trapped specimens. In addition, traps -in particular longwavelength tweezers- can be used to study the influence of the microenvironment, drugs, external light exposure etc. Traps are therefore novel exciting tools for non-linear cell diagnostics.

Trapping at 800 nm or higher wavelengths seems not to harm cells even for trapping times in the range of minutes and GJ/cm^2 fluences whereas short-wavelength traps (100 mW) are able to induce irreversible damage within seconds. The damage has no linear photothermal origin (less water absorption in the short-wavelength range) and is not based on linear photomechanical effects. The trap-induced damage seems to be based on non-linear photochemistry due to two-photon excitation processes. Potential two-photon targets are the reduced pyridine coenzymes NADH and NADPH, flavins, porphyrins and cytochromes. All these endogenous chromophores have electronic transitions in the UVA range which can be two-photon excited with <800 nm microbeams. It is known that severe cell damage may occur due to photo-induced oxidative stress (formation of singlet oxygen and oxygen radicals) by excitation of these cellular absorbers even though there is no direct absorption by nucleic acids (e.g. Cunningham *et al.*, 1985; König *et al.*, 1996b; Tyrell and Keyse, 1990).

Picosecond pulses as found in multimode traps of a "cw" ring laser amplify the non-linear effects compared to a true cw laser without transient high power amplitudes. In fact, our very recent studies on the interaction of NIR femtosecond and picosecond pulses with CHO cells demonstrate that damage follows a two-photon excitation process rather than a one-photon or a three-photon event (König *et al.*, submitted). Therefore, cell damage by laser traps can be reduced by the use of single-frequency lasers. More detailed studies on the exact non-linear interaction of laser tweezers and gametes including the measurement of true action spectra using tunable single frequency traps and more detailed studies on the possible influence on DNA (major intracellular trap target of spermatozoa) should be performed before using laser tweezers as micromanipulation tools in laser-assisted *in vitro* fertilization.

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