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Cell viability in optical tweezers: high power red laser diode versus Nd:YAG laser

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Abstract. Viability of cultivated Chinese hamster ovary cells in optical tweezers was measured after exposure to various light doses of red high power laser diodes (λ=670–680 nm) and a Nd:yttrium–aluminum–garnet laser (λ=1064 nm). When using a radiant exposure of 2.4 GJ/cm^2, a reduction of colony formation up to a factor 2 (670–680 nm) or 1.6 (1064 nm) as well as a delay of cell growth were detected in comparison with nonirradiated controls. In contrast, no cell damage was found at an exposure of 340 MJ/cm^2 for both wavelengths, and virtually no lethal damage at 1 GJ/cm^2 applied at 1064 nm. Cell viabilities were correlated with fluorescence excitation spectra and with literature data of wavelength dependent cloning efficiencies. Fluorescence excitation maxima of the coenzymes NAD(P)H and flavins were detected at 365 and 450 nm, respectively. This is half of the wavelengths of the maxima of cell inactivation, suggesting that two-photon absorption by these coenzymes may contribute to cellular damage. Two-photon excitation of NAD(P)H and flavins may also affect cell viability after exposure to 670–680 nm, whereas one-photon excitation of water molecules seems to limit cell viability at 1064 nm.
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1 Introduction

Optical trapping of transparent particles, e.g., cells or organelles, in a collimated light beam is mainly related to deflection of incident photons.[1] Due to radiation pressure a force is generated that moves the particle towards the center of a Gaussian laser beam (lateral displacement) and, furthermore, towards the focus of this beam (axial displacement).[2] This principle of optical tweezers has been applied, e.g., for measuring motility forces of cells,[3,4] macromolecules,[5,6] or organelles,[7] for micromanipulation of cells or chromosomes,[8,9] for cell fusion,[10] or for sperm insertion into oocytes through a previously drilled hole.[11,12] For the latter applications optical tweezers were used in combination with a ultraviolet (UV)-laser microbeam (optical scalpel). Despite high power densities, cell damage due to absorption of radiation has to be excluded or minimized.

Absorption of visible or near ultraviolet light has been reported for several enzymes or coenzymes, in particular the reduced forms of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), or flavin adenine dinuleotide (FAD), and different cytochromes. The main absorption bands are around 310–370 nm (NADH[13] and NADPH), 350–500 nm (flavins[14]), and 380–440 as well as 520–620 nm (cytochromes[15]). Therefore, in order to minimize cell damage after single photon absorption, the near infrared radiation of a Nd:yttrium–aluminum–garnet (YAG) laser (λ=1064 nm) or the radiation of a tunable Ti: sapphire laser (700 nm≤λ≤1000 nm) has so far been used for optical tweezers. Two-photon or multiphoton absorption at high photon flux densities in the near infrared spectral region, however, has also been suggested to cause cell damage,[16–18] involving thermal[18] as well as photochemical reactions.[16]

Cell viability after laser irradiation has previously been determined as a function of wavelength and light dose in the range of 700 nm≤λ≤1064 nm.[19] Cell damage measured in terms of a loss of clonogenicity has been found to be rather low at 800–850 and 930–1000 nm, moderate at 700 and around 1064 nm, and high at 730–760 as well as around 900 nm. So far, no data are reported, for the wavelength range of 650–700 nm. In this spectral region, however, high power laser diodes with almost diffraction limited beam diameters have recently become available. In comparison with near infrared lasers, these laser diodes offer several advantages, e.g., easier adjustment and less chromatic aberrations of microscope optics in the visible part of the spectrum. Therefore, these laser diodes may offer an attractive alternative to Nd:YAG and Ti: sapphire lasers.

In the present article cell viabilities (assessed as colony forming units and colony sizes) are compared after exposure to various light doses of red laser diodes (670 nm≤λ≤680 nm) and a Nd:YAG laser (λ=1064 nm). Data are related to

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experiments on fluorescence excitation spectroscopy and to previous measurements of cell viability.[19]

2 Materials and Methods

Chinese hamster ovary cells (CHO-*K*1) (ATTC no. CCL 61) were a gift of Dr. T. M. A. R. Dubbelman (University of Leiden, NL). Cells were routinely cultivated in F-10 HAM nutrient mixture supplemented with 10% fetal calf serum, 7.5% sodium bicarbonate and penicillin-streptomycin at 37°C, and 5% CO_2. For microspectrofluorometric measurements cells were grown for 3 days in cultivation flasks, trypsinated, centrifuged and suspended in 2–3 layers on microscope object slides. Fluorescence excitation spectra were recorded using a 75 W xenon high pressure lamp (XBO) together with an excitation monochromator (bandwidth 5 nm) adapted to the microscope (Axioplan, Carl Zeiss Jena, Germany).[20] Using a 40×/0.75 objective lens an object field of 500 μm diameter was illuminated. Excitation and fluorescence light were separated by a dichroic mirror as well as appropriate long pass and band pass filters inserted into the detection path. A photomultiplier (R928, Hamamatsu Photonics, Ichino-Cho, Japan) was used for photoelectric detection. Fluorescence was detected around the emission maximum of the coenzymes NADH and NADPH (dichroic mirror: 460 nm; long pass filter: 470 nm; band pass filter: 477±25 nm), as well as around the emission maximum of flavin molecules (dichroic mirror: 510 nm; long pass filter: 520 nm; band pass filter: 529±25 nm).[21–24] Excitation spectra of intracellular NAD(P)H were registered in the range of 300–460 nm, whereas excitation spectra of intracellular flavins were recorded in the range of 300–500 nm. All spectra were normalized by division through a reference spectrum obtained with a blank object slide.

For measurements of cell viability about ten single cells were seeded within individual wells of an eight-well chamber slide (Nunc, Wiesbaden, Germany). Cells within four wells were irradiated 18 h after seeding, whereas cells within the other four wells served as controls. For each wavelength and light dose, five 8-well chamber slides were examined. Colony formation was determined for all irradiated cells and controls 66 and 138 h after seeding. In addition, colony size was measured after 66 h (but could not be determined reliably after 138 h when colonies of more than 100 cells were formed). For counting of colonies and cells in the upright microscope (Axioskop, Carl Zeiss Jena, Germany) the eight-well chamber slides were turned upside down within a special mounting to maintain sterile conditions. Colonies were related to originally seeded cells by using a bidirectional scanning table with a step motor (EK32; Merzhäuser, Wetzlar, Germany) together with a purpose-made computer program to regain cell coordinates.

For irradiation of the cells with different light doses the following continuous-wave (cw) lasers were used:

1. a set of two laser diodes (SDL 5762 M-MOPA, SDL Inc., San Jose, USA; λ=670–680 nm; P=500 mW each) together with a polarization beam splitter (Figure 1);
2. a single laser diode of the same type which was attenuated to 140 mW;

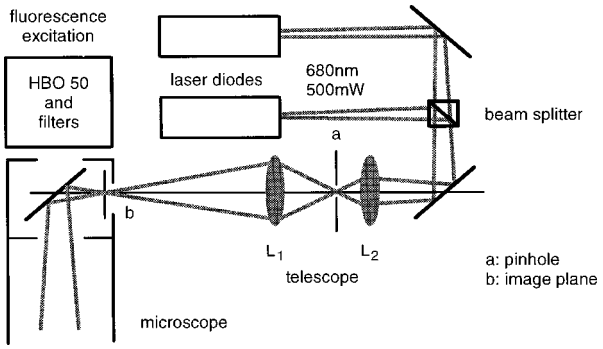

Fig. 1 Optical setup for laser tweezers using two high power laser diodes (500 mW) together with a fluorescence microscope (sample and objective lens are omitted).

3. a Nd:YAG laser (ADLAS Lasertechnik, Lübeck, Germany; λ=1064 nm), operated at 2 W, 850 mW, or 280 mW.

Laser beams were focused in the image plane of the microscope using a telescope and again in its object plane (single cells) using a 40×/0.60 long distance objective lens. Laser radiation was deflected in the microscope by a dichroic mirror (which permitted transmission of UV or blue fluorescence excitation light of a mercury high pressure lamp, HBO 50). Focal lengths of the telescope lenses L_1 and L_2 were selected such that the diameter of the laser beam in the objective lens was identical with its aperture to obtain a maximum of resolution. Laser power on the samples (i.e., after transmission of microscope optics) was determined pyroelectrically (power meter LM-10, Coherent, Auburn, USA), the diameter d of the diffraction limited spot was calculated according to $d=1.22\lambda/\mathrm{NA}$ (NA=numerical aperture). After verification of the calculated spot size by a micrometer line standard, power densities of 20 as well as 2.8 MW/cm^2 were determined for both laser diode and Nd:YAG laser. After exposure times of 120 s, these power densities corresponded to light doses of 2.4 GJ/cm^2 and 340 MJ/cm^2, respectively. An intermediate power density of 8.3 MW/cm^2 (corresponding to a light dose of 1.0 GJ/cm^2 after 120 s) was also used for the Nd:YAG laser. In addition, the power density of 8.3 MW/cm^2 (Nd:YAG laser) was applied during a longer exposure time of 290 s, thus giving again the maximum light dose of 2.4 GJ/cm^2.

3 Results

After excitation by near ultraviolet light (355–365 nm), the fluorescence spectrum of CHO cells showed a broad band with a maximum around 465 nm and shoulders around 440 and 515 nm. These emission bands were previously attributed to the coenzyme NADH or NADPH in its folded conformation [465 nm; ''free'' NAD(P)H] or its extended conformation [440 nm; protein-bound NAD(P)H] as well as to flavin molecules (515 nm).[21–24] Fluorescence excitation spectra of CHO cells were registered near the maximum [477±25 nm; free NADH(P)H] and the second shoulder (529±25 nm; flavins). Free NAD(P)H showed a fluorescence excitation maximum at 365 nm (Figure 2, curve a) which was slightly redshifted as compared with the absorption maximum (around 340 nm[13]), whereas flavin spectra exhibited excitation

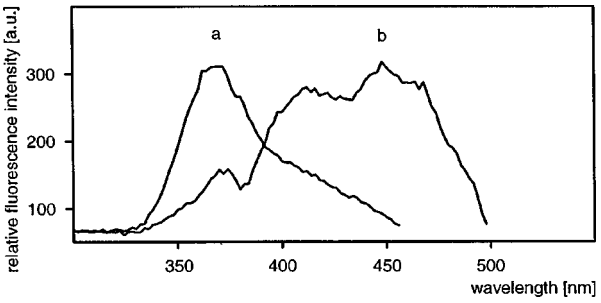

Fig. 2 Fluorescence excitation spectra of CHO cells detected around the emission maximum of free NADH at 477±25 nm (a) or flavins at 529±25 nm (b); spectral resolution: 5 nm.

maxima at 375, 415, and 445 nm (most pronounced; Figure 2, curve b) which roughly corresponded to literature data of flavin absorption.[14] Reference spectra of fluorescence excitation of 10^{-3} M solutions of free NADH and flavin mononucleotide (FMN) were similar to the spectra of the intracellular fluorophores depicted in Figure 2.

Figure 3 (left part) shows mean values and standard deviations of colony formation (% of plated cells) measured 66 or 138 h after seeding of individual cells and after exposure to light doses of 2.4 GJ/cm^2 (a) or 340 MJ/cm^2 (b) of high power laser diodes (λ=670–680 nm) in comparison with nonirradiated controls (exposure time: 120 s). 80%–90% of initially seeded control cells were able to form colonies (plating efficiency). This percentage remained unchanged after exposure to 340 MJ/cm^2 (b), but was reduced to about 45% after exposure to 2.4 GJ/cm^2 (a), thus indicating a reduction of clonogenicity by a factor 2 at this light dose. The right part of Figure 3 shows the average number of cells per colony 66 h after seeding. About five cells per colony were counted after exposure to 2.4 GJ/cm^2, whereas an average of almost 10 cells per colony was obtained from untreated controls. Following an exposure to 340 MJ/cm^2 the number of cells per colony remained almost unchanged as compared with the controls.

Similar results were obtained after irradiation by the Nd:YAG laser (λ=1064 nm, exposure time 120 s; Figure 4). In comparison with nonirradiated controls, colony formation was reduced by a factor 1.6, and the average number of cells per colony by a factor 1.7 after exposure to 2.4 GJ/cm^2 (a). Virtually no reduction was observed after exposure to 340 MJ/cm^2 (c). After exposure to an intermediate light dose of 1.0 GJ/cm^2 (b), colony formation remained almost unchanged, whereas the average number of cells per colony measured 66 h after seeding decreased by a factor 1.4 as compared with the controls. This difference is in the same order of magnitude as the standard deviation of cell number per colony. Additional experiments with a longer exposure time of 290 s and a light dose of 2.4 GJ/cm^2 showed a reduction of colony formation by a factor 1.3 at 66 h and a factor 1.4 at 138 h after seeding as compared with nonirradiated controls. The average number of cells per colony measured at 66 h decreased by a factor 1.7 due to irradiation. The reduction of colony formation was thus slightly smaller, whereas the reduction of cell number per colony was the same as after application of 2.4 GJ/cm^2 during the shorter exposure time of 120 s [Figure 4(a)].

4 Discussion

Similar viabilities of CHO cells were observed after exposure to radiation of 670–680 (laser diode) or 1064 nm (Nd:YAG laser). No damage as measured in terms of a loss of clonogenicity and delay in cell growth occurred at a light dose of about 340 MJ/cm^2 applied during 120 s. This light dose was high enough for moving transparent Latex particles (d = 0.5–2 μm), fluorescent microspheres (d = 0.52 μm; Duke Scientific Corporation, Palo Alto, USA) or *Saccharomyces cerevisiae* (d = 5–7 μm) on a microscope object slide over several millimeters. Lethal damages resulting in a reduction of colony formation by a factor 2 (670–680 nm) or 1.6 (1064

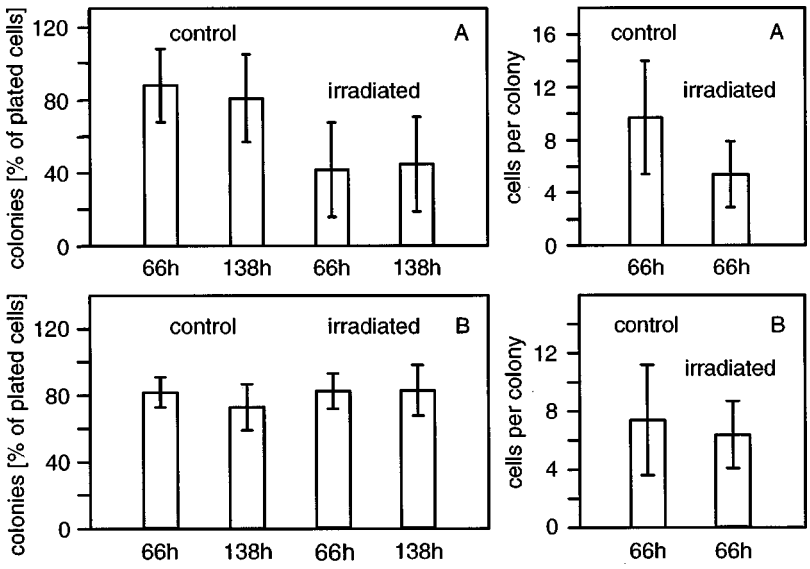

Fig. 3 Colony formation (% of plated cells 66 and 138 h after seeding; left) and number of cells per colony (66 h after seeding; right) of CHO cells irradiated at 670–680 nm and nonirradiated controls. Light doses of 2.4 GJ/cm^2 (a) or 340 MJ/cm^2 (b); irradiation time 120 s. Mean values and standard deviations of 20 measurements (left) or up to 160 measurements (right) in each case.

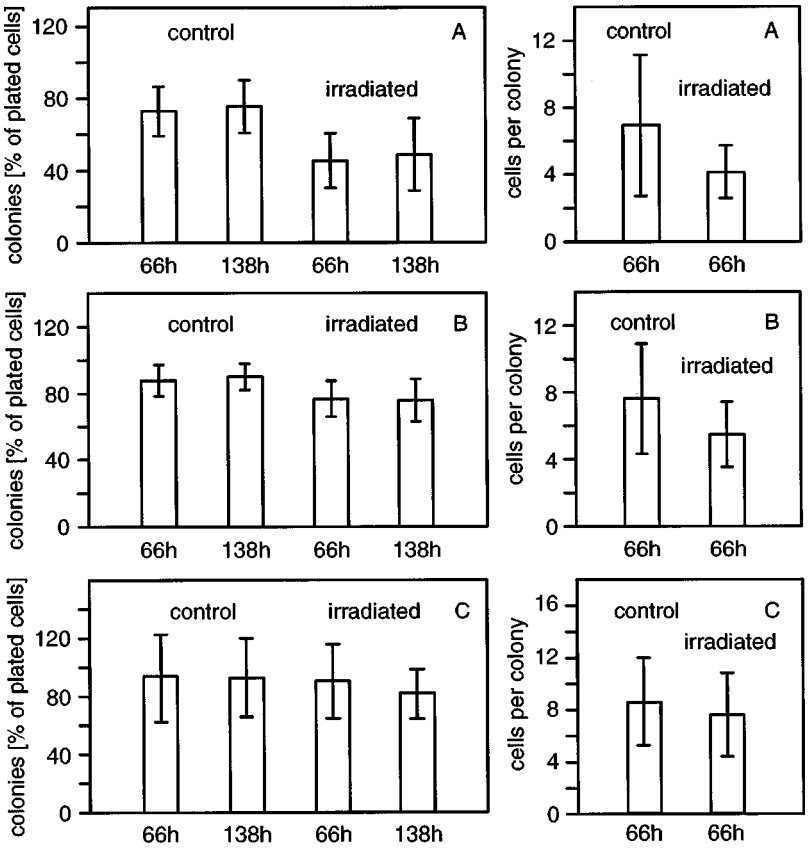

Fig. 4 Colony formation (% of plated cells 66 and 138 h after seeding; left) and number of cells per colony (66 h after seeding; right) of CHO cells irradiated at 1064 nm and nonirradiated controls. Light doses of 2.4 GJ/cm^2 (a), 1.0 GJ/cm^2 (b) and 340 MJ/cm^2 (c); irradiation time 120 s. Mean values and standard deviations of 20 measurements (left) or up to 160 measurements (right) in each case.

nm), as well as pronounced delay of cell growth resulting in smaller cell numbers per colony, were observed after irradiation with a light dose of 2.4 GJ/cm^2. At a light dose around 1 GJ/cm^2 (applied at 1064 nm during 120 s) virtually no lethal damage, but some delay of cell growth occurred.

After exposure to 2.4 GJ/cm^2 of 1064 nm radiation, colony formation was reduced by a factor 1.6 at a power density of 20 MW/cm^2 applied during 120 s and by a factor 1.3–1.4 at a power density of 8.3 MW/cm^2 applied during 290 s. Reduction of the cell number per colony was the same in both cases (factor 1.7). Despite the small difference in colony formation, integral light dose seems to be a key parameter for cellular damages at this wavelength. In the literature,[19] a reduction of colony formation of CHO cells by a factor 1.6 after irradiation at 1064 nm was reported for a power density of 26 MW/cm^2 and an integral light dose of 4.7 GJ/cm^2, i.e., twice the light dose used in the present experiments. This indicates that in addition to integral light dose other parameters, e.g., size of the illuminated spot (Ref. 19: 0.7 μm, present experiments: 2.1 μm) or transient enhancements of power density, may have some impact on cell damage. Transient power enhancement of multimode lasers was shown to reduce cloning efficiency as compared with single frequency lasers of the same average power.[17] Differences in transient intensity maxima of various Nd:YAG lasers may possibly account for different light doses resulting in the same loss of clonogenicity.

Absorption of 670–680 and 1064 nm radiation is likely to occur from different molecular species. In the latter case (one-photon) absorption by water may affect cell viability. As reported in the literature,[25] the absorption coefficient of water is below 10^{-2} cm^{-1} at wavelengths between 300 and 700 nm, but increases considerably in the near infrared region. At 1064 nm the absorption coefficient is about 0.15 cm^{-1}, corresponding to an optical density of 3×10^{-4} for a cell of 20 μm diameter. Therefore, about 0.07% of incident photons, i.e., about 3×10^{15} photons per second of a 100 mW laser beam are absorbed by water. Cell viabilities in the wavelength range of 700–1064 nm are reported in the literature.[19] Lowest cell survival was found at 730–760 nm as well as around 900 nm. These wavelengths are twice the wavelengths of the fluorescence excitation maxima of free NAD(P)H and flavins, respectively (Figure 2). Cell damage due to two-photon absorption at wavelengths between 700 and 800 nm was recently discussed by König et al.[16,17] According to Figure 2, two-photon absorption by NAD(P)H and flavins may also occur at 670–680 nm (corresponding to a wavelength of 335–340 nm for one-photon absorption). Two-photon fluorescence excitation spectra of NADH and flavin mononucleotide (FMN) as reported by Xu et al.[26] were rather monotonic functions, which do not show preferential excitation of these fluorophores at 730–760 or 900 nm. It should, however, be empha-

sized that these spectra were obtained from NADH and FMN solutions, not from intracellular fluorophores. In addition, no two-photon fluorescence excitation spectra at wavelengths below 700 nm have so far been reported.

As reported in Ref. 19, highest cell viabilities were obtained after irradiation around 800–850 or 930–1000 nm. According to the present experiments, laser diodes at 670–680 nm or Nd:YAG lasers at 1064 nm can be used for optical tweezers without affecting cell survival, if radiant exposures are kept below 1 GJ/cm^2. The main advantage of red emitting laser diodes over near infrared Nd:YAG lasers are an easier adjustment of a visible light source, smaller diameters of diffraction limited beams, less chromatic aberrations, as well as possible miniaturization and lower costs in the near future.

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