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Cell Damage During Multi-Photon Microscopy

Karsten König

INTRODUCTION

Conventional fluorescence microscopes are based on the excitation of exogenous and endogenous fluorophores by ultraviolet (UV) and visible (VIS) radiation. The fluorescence excitation radiation in non-laser microscopes is provided by high-pressure 50 W/100 W mercury lamps with typical emission lines at 365 nm, 505 nm, 436 nm, etc., and broadband 75 W xenon lamps. Most laser-scanning microscopes possess the argon (Ar)-ion laser at 364 nm, 488 nm, and 515 nm emission, the frequency-doubled and -tripled Nd:YAG laser at 532 nm and 355 nm, and the helium–neon (He-Ne) laser at 543 nm and 633 nm.

Typically, the radiation power of these microscopes is on the order of 10 to 100 µW. This corresponds to light intensities in the range of kilowatts per square centimeter when focused to diffraction-limited spots by objectives with a numerical aperture (NA) larger than 1. These light intensities are not sufficient to induce multi-photon effects.

In spite of the low radiation power, photodestructive effects occur. In particular, UVA radiation, including 365 nm lamp radiation, 364 nm argon laser radiation, and 337 nm laser radiation of a nitrogen laser has a relatively high damage potential compared to VIS radiation. Although not directly absorbed by DNA, UVA radiation may also induce DNA strand breaks (König *et al*., 1996a).

In contrast to one-photon fluorescence microscopes, multi-photon microscopes are based on the application of low energy photons in the near infrared (NIR) between 700 nm and 1200 nm. This spectral range is also called the "optical window of cells and tissues" where the one-photon absorption and scattering coefficients of unstained cells and tissues are relatively low and where the light penetration depth is high (Fig. 38.1). In the view of one-photon processes, most cells appear transparent and do not experience destructive effects due to the lack of efficient absorption. Exceptions are pigmented cells (e.g., cells containing melanin, hemoglobin, or chlorophyll) where linear absorption is likely to occur.

In the case of non-resonant two-photon absorption, NIR radiation may induce UVA effects — including photodamage — in particular when using wavelengths below 800 nm. However, the destructive UV effect is, in principle, confined to the tiny focal volume where the nonlinear absorption occurs and does not affect the out-of-focus regions.

When four photons or more are involved in the nonlinear process, ionization of the target combined with the formation of free or quasi-free electrons may occur. This can lead to optical breakdown and plasma formation. These multi-photon processes result in immediate photodamage.

Nonlinear effects such as non-resonant multi-photon absorption require high light intensities in the range from megawatts per square centimeter up to terawatts per square centimeter. In order to obtain such intensities, higher light powers than are used in one-photon microscopy are required. When using continuous wave (CW) NIR radiation, powers of at least 100 mW are necessary (Hänninen *et al*., 1994; König *et al*., 1995, 1996b). That means about 3 orders of magnitude higher power levels or more have to be applied in multi-photon microscopy.

Power levels in the range of 100 mW of NIR light have been used for optical micromanipulation of living cells using laser tweezers. In the case of multi-photon scanning microscopy for fluorescence imaging, such trapping effects may disturb the specimen.

In order to avoid trapping effects and to reduce the mean power, ultrashort laser pulses with kilowatts of peak power but a mean power in the microwatt and milliwatt range are commonly used in multi-photon microscopy.

Almost all multi-photon laser-scanning microscopes are based on the application of ultrashort laser pulses at high repetition rate. Because the multi-photon efficiency of an *n*-photon process depends on the peak power P according to a P^n relation, the use of pulsed laser beams enables efficient fluorophore excitation at a fast scanning rate.

The major application of multi-photon microscopes in life sciences involves the excitation of a variety of intracellular fluorescent probes that lack one-photon absorption bands in the NIR by the simultaneous absorption of two or three NIR photons (Denk *et al*., 1990). Typical two-photon and three-photon absorption cross-sections of these fluorophores are about 10^{-48} to 10^{-50} cm^4 s/photon and 10^{-75} to 10^{-84} cm^6 (s/photon)^2 (Xu *et al*., 1995; Maiti *et al*., 1997).

In a first approximation, the probability of two-photon absorption decreases with the distance d from the focal plane according to a d^{-4} relation. When using high-NA objectives and appropriate power levels, two-photon effects can be limited to a minute sub-femtoliter volume. By contrast, microscopy with UV or VIS lamp/laser light implies possible destructive effects by one-photon absorption in out-of-focus illuminated regions. This problem is often encountered in conventional confocal laser-scanning microscopy as well as in conventional intracellular laser surgery performed with pulsed UV and VIS laser beams (Berns, 1998; Greulich, 1999).

In particular, when studying thick 3D objects such as large cells, multi-cell aggregates, embryos, and tissues by optical sectioning, multi-photon microscopy with its tiny excitation volume can be the superior method compared to one-photon confocal scanning microscopy with its large excitation cones (Fig. 38.2).

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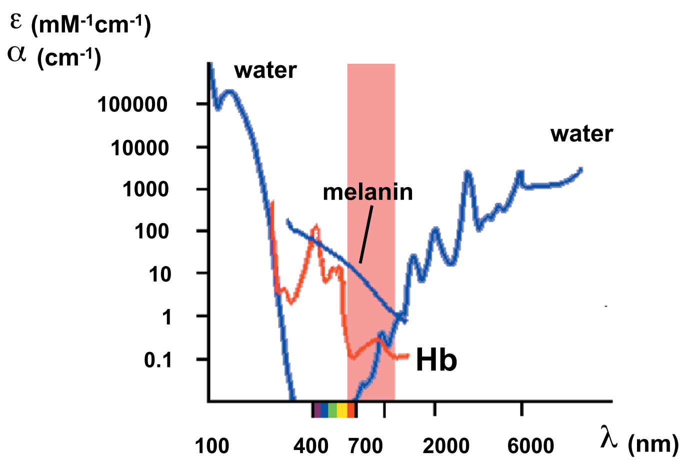

FIGURE 38.1. Absorption spectra of the cellular absorbers water, melanin, and hemoglobin. The range of low absorption and low scattering is from about 600 nm up to 1200 nm (optical window). At higher NIR wavelength, linear absorption of water increases and may induce destructive linear heating effects.

Multi-photon microscopy on living specimens can be performed without phototoxic effects under certain conditions. This has been demonstrated by long-term scanning experiments on (i) single hamster ovarian cells with a peak intensity of about 200 GW/cm^2, where laser exposure even for hours had no impact on cellular reproduction and vitality (König, 2000) as well as (ii) on living hamster embryos. The embryos were exposed for 24 h with NIR femtosecond laser pulses of a multi-photon fluorescence microscope without impact on embryo development in contrast to control studies performed with a conventional one-photon VIS laser scanning microscope (Squirrel *et al*., 1999).

Nevertheless, NIR multi-photon microscopes can potentially induce undesirable destructive multi-photon effects by the high light intensity within the focal volume (König *et al*., 2000; Diaspro and Robello, 1999). Indeed, an additional optical window exists for the safe, non-destructive observations of the specimens of interest. It is mainly defined by light intensity thresholds but also has a dependence on NIR wavelength. Outside this window, cells may undergo either (i) a photothermal damage in the case of pigmented cells, (ii) a slow destructive effect related to multi-photon

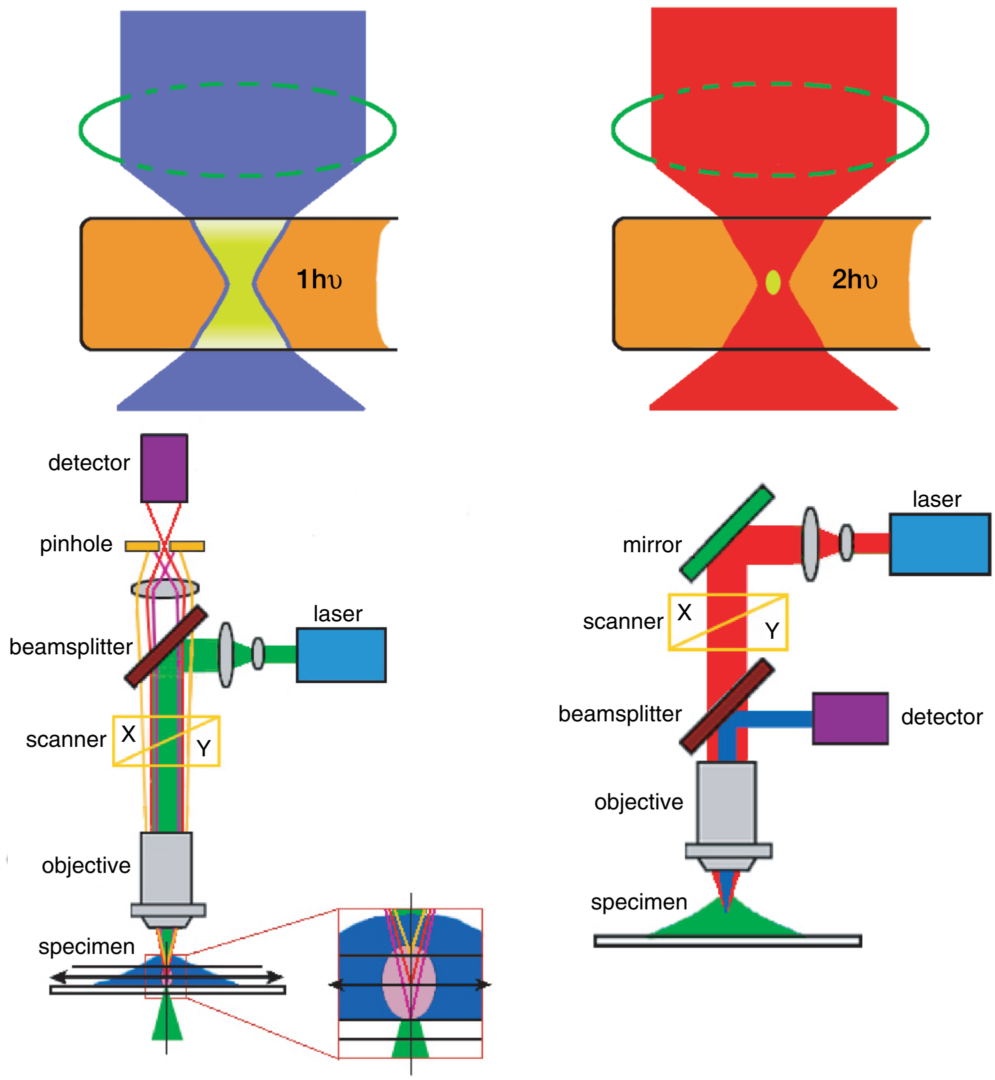

FIGURE 38.2. Scheme of one-photon excitation versus multi-photon excitation and corresponding scanning microscopes. Optical sectioning based on multi-photon excitation of fluorophores does not require spatial pinholes and can avoid out-of-focus photodamage/bleaching.

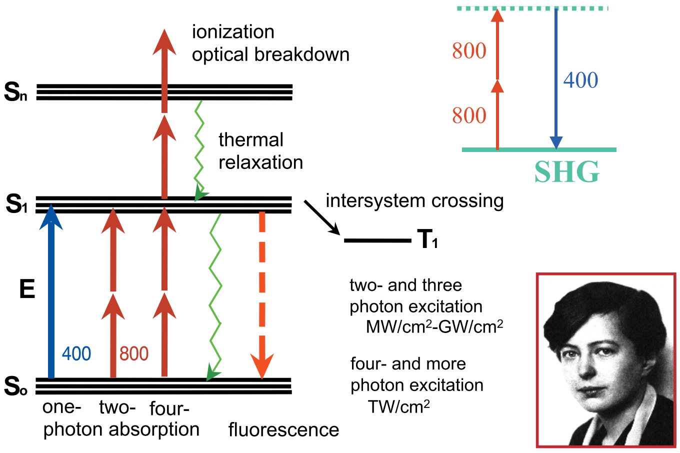

FIGURE 38.3. Two-photon effects with intense near-infrared radiation can induce visible fluorescence, second harmonic generation, and photochemical reactions. Photochemical reactions such as photo-oxidation processes may lead to slow photodestructive effects. When using laser intensities in the range of terawatts/cm^2, multi-photon ionization and plasma formation may occur that can result in immediate cell damage. Maria Göppert-Mayer predicted two-photon effects in her 1930 Ph.D. thesis.

excitation of cellular absorbers and subsequent cytotoxic photochemical reactions, or (iii) an instantaneous destructive opto-mechanical effect by optical breakdown phenomena and plasma formation due to multi-photon–mediated ionization of molecules (Fig. 38.3).

Interestingly, the highly localized destructive multi-photon effects can be used to realize precise, 3D nanoprocessing of biological structures such as cell membranes, intracellular organelles, and human chromosomes, as well as corneal and brain tissues. Therefore, multi-photon microscopes can also be used to knock out single mitochondria, to realize efficient targeted transfection (Tirlapur and König, 2002), and chromosome dissection as well as to perform intrastromal nanosurgery in eyes. Multi-photon–induced minimum cut sizes of 85 nm full-width at half-maximum (FWHM) have been realized in the human chromosome 1 using 80 MHz NIR nanojoule femtosecond laser pulses.

This chapter provides a review (i) of potential photo-induced effects that may cause cellular damage and (ii) the use of destructive multi-photon effects with nanojoule and sub-nanojoule femtosecond laser pulses for nanosurgery and gene technology.

PHOTOCHEMICAL DAMAGE IN MULTI-PHOTON MICROSCOPES

Absorbers and Targets in Biological Specimens

Photochemical damage can be induced by the nonlinear excitation of endogenous and exogenous absorbers followed by phototoxic reactions. These reactions include the formation of reactive oxygen species (ROS), which result in oxidative stress. A wide variety of endogenous cellular absorbers can be excited with multi-photon microscopes. Some of them are fluorescent and can be imaged by autofluorescence detection. Table 38.1 represents important naturally occurring endogenous absorbers in cells and tissues which can be excited with NIR radiation via two- or three-photon excitation processes.

For example, amino acids with a one-photon absorption maximum below 300 nm can be excited via a three-photon process with NIR photons. The reduced coenzymes NAD(P)H absorb intense NIR radiation in the 700 nm to 800 nm range by two-photon excitation. It is known from one-photon studies that excitation of amino acids as well as of NAD(P)H can induce irreversible DNA damage (Cunningham *et al.*, 1985; Tyrell and Keyse, 1990; Bertling *et al.*, 1996). Excitation of flavins, which can also be excited by a two-photon process, may also cause cell damage by the photo-induced production of H_2O_2 and its metabolites, which are hydroxyl radicals (Hockberger *et al.*, 1999).

In addition, the excitation of cellular porphyrins leads to the formation of ROS. Singlet oxygen is produced by a type II photo-oxidation process (photodynamic reaction). Metal-free porphyrins, such as coproporphyrin and protoporphyrin, are such efficient ROS producers that they are termed photosensitizers. As known from photodynamic porphyrin therapy, ROS formation in mitochondria often leads to apoptosis, whereas ROS formation in lysosomes or other organelles may induce either necrosis or apoptosis (Doiron and Gomer, 1985; Moor, 2000). Photodynamic reactions are also the major source of phototoxic response when using fluorescent markers or other exogenous absorbers.

Laser Exposure Parameters

In most experiments we have used compact solid-state 80 MHz titanium : sapphire lasers (Vitesse, Coherent; MaiTai, Spectra Physics) as turn-key laser sources; however, a tunable Ar-ion laser-pumped Mira system (Coherent) was employed for some of this work. The laser was coupled to an adapter module (JenLab GmbH, Jena, Germany) which consisted of a 1 : 5 beam expander, a step-motor driven beam attenuator, a fast photodiode for power measurements, a trigger pulse supply, a laser control unit, and a shutter. The expanded beam at tunable power was introduced into a modified laser-scanning microscope (LSM 410, Carl Zeiss Jena GmbH, Jena, Germany) with baseport detector (König, 2000) as well as into a compact scanning microscope where the galvoscanners were attached to the side port of an inverted Zeiss Axiovert microscope (TauMap, JenLab GmbH) (Ulrich *et al.*, 2004). The beam was focused to a diffraction-limited spot by conventional high-NA objectives. The typical NIR transmission of the objective was found to be 50%, whereas the overall transmission was typically

TABLE 38.1. Spectral Characteristics (One-Photon Maxima) of Important Endogenous Absorbers

Absorber	Absorption Maximum (nm)	Fluorescence Maximum (nm)
Water	1500, 1900, 2900	—
Hemoglobin	410, 540, 580, 760	—
Tryptophan	220, 280–290	320–350
NAD(P)H	340	450–470
Flavins	370, 450	530
Melanin	<300	575
PP IX	405, 505, 540, 575, 630	635

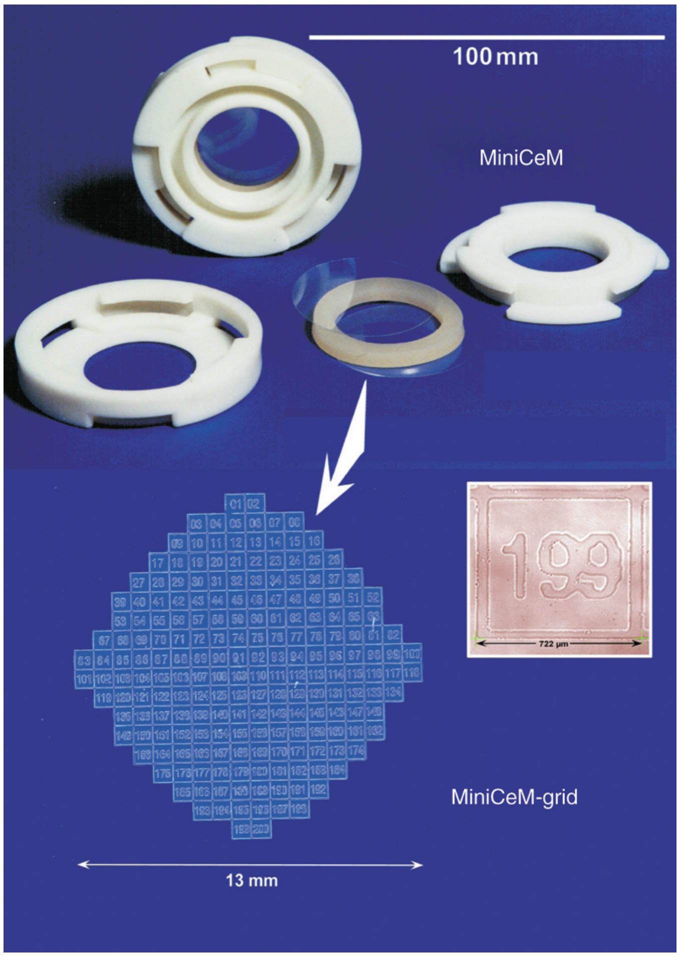

FIGURE 38.4. Cell chamber for high-resolution microscopy with two thin 170 µm thick glass windows with grid. Cells can grow as monolayers on the windows. Media and dyes can be added by injection through the gas-permeable silicone gasket.

in the range of 30%. When studying living cells, a closed microchamber made of two 170 µm thick glass windows (one with an etched grid) was used (MiniCeM-grid, JenLab GmbH) (Fig. 38.4).

We used three illumination modes: (i) scanning a 512 × 512 pixel area, (ii) scanning a defined region of interest (ROI), or (iii) single point exposure, where the beam is parked at pixels of interest within the living cell.

The high sensitivity of the baseport photomultiplier enabled the detection of two-photon excited intracellular fluorophores with high fluorescence quantum yield (e.g., DAPI and Hoechst) using a low mean power at the sample of from 25 µW to 1 mW at a frame rate of 1 Hz (512 × 512 pixels). More importantly, detection of endogenous fluorophores, such as fluorescent coenzymes, was possible with a mean excitation power of less than 2 mW at the specimen at appropriate NIR wavelengths.

The laser power after transmission through the whole microscope was measured in air using a Fieldmaster power meter with the two detector heads LM-2 and LM-3. In order to correct for the true photon flux through oil and the chamber window into the medium as well as for the limited detection angle of the power detector heads, correction values of 1.1 and 2.0 had been determined using a sandwich system (Fig. 38.5; König *et al.*, 1997a).

Optical dispersion results in pulse broadening during transmission through microscope optics. Typically, the pulse width at the sample was about 150 fs to 250 fs (König, 2000). At 10 mW mean power, the peak power and the peak intensities were 0.8 kW and 1.2×10^{12} W/cm^2 (1.2 TW/cm^2), respectively, assuming a FWHM beam size of $\lambda/2$ NA ≈ 310 nm. A typical beam dwell time (time of exposure per pixel) during one scan was about 30 µs, which resulted in a frame rate of 8 s/frame. At zoom setting 2, 512 × 512 pixels covered an area of 160 µm × 160 µm. For cell damage studies, typically one or more cells of interest were scanned 10 times in the same focal plane.

Evidence for Near Infrared-Induced Reactive Oxygen Species Formation

In order to know if multi-photon NIR laser-scanning microscopy with 80 MHz laser radiation above certain intensity levels is able to induce ROS formation and DNA breaks in living cells, we have performed extensive photodamage studies on PTK2 cells.

Using 170 fs laser pulses at 800 nm, we found that a mean power of ≥7 mW was sufficient to evoke ROS in PTK2 cells within a scanned region of interest. By contrast, no ROS formation was detected in the non-irradiated adjacent cells. As a ROS indicator, we used the fluorescent probe *Jenchrom px blue* (JenLab GmbH). The punctate fluorescence of the *Jenchrom* reaction product was found to be exclusively confined to the irradiated cells (Tirlapur *et al.*, 2001).

In addition, we have used the membrane-permeable dye dihydrofluorescein (DHF) for *in situ* detection of ROS (Hockberger *et al.*, 1999) in PTK2 cells. We exposed one half of the DHF-loaded PTK2 cells to 170 fs pulses at 800 nm using a mean power of 2, 5, 7, 10, 12, or 15 mW. There was virtually no DHF fluorescence in cells exposed to mean laser power of ≤5 mW. However, diffuse DHF fluorescence was exclusively present in cells exposed to a

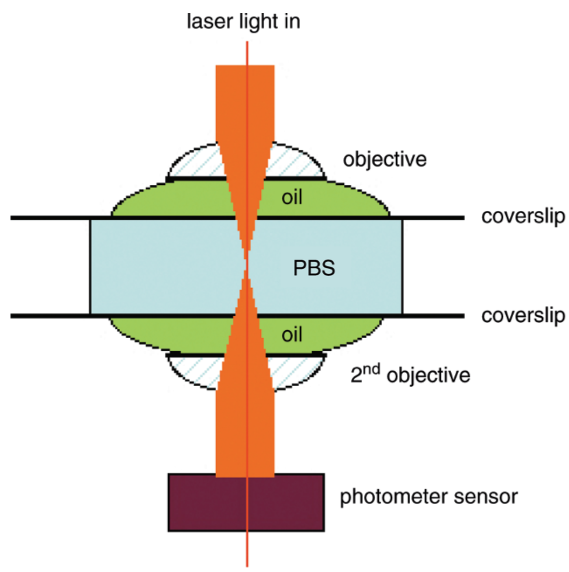

FIGURE 38.5. Diagram of the system used to measure the beam power actually reaching the specimen.

higher mean laser powers and was very intense in cells exposed to a mean laser power of 15 mW.

Evidence for Near Infrared-Induced DNA Strand Breaks

Shafirovich and colleagues (1999) have demonstrated *in vitro* that excitation of pBR322 supercoiled plasmid DNA with intense 810 nm NIR femtosecond laser pulses having a pulse width of 140 fs, a pulse repetition rate of 76 MHz and an average intensity of 1.2 mW/cm^2 (GW/cm^2 peak intensities) resulted in single strand breaks as a result of a simultaneous multi-photon absorption process.

Using the well-established TdT-mediated dUTP-nick end labeling (TUNEL) assay (Gavrieli *et al*., 1992; Negri *et al*., 1997), we have undertaken a more detailed *in situ* analysis of the effect of 800 nm NIR femtosecond laser pulses at different mean laser powers on DNA in unlabelled PTK2 cells. Cells were irradiated with the 800 nm (170 fs pulse duration) NIR scanning beam 10 times with a beam dwell time of ~60 or ~120 µs per pixel that corresponded to 16 or 32 s per frame of 512 × 512 pixels (at zoom setting 2) using mean powers of 2, 5, 10, and 20 mW.

Neither control PTK2 cells (not exposed to the 800 nm laser beam) nor those exposed to 2 to 3 mW mean power showed any TUNEL-positive green fluorescence in their nuclei. This indicates that lower mean laser powers do not induce any DNA strand breaks *in situ* and hence are not deleterious.

However, most of the PTK2 cells exposed to a mean power of 5 mW demonstrated TUNEL-positive green fluorescence, mostly in the nuclear area. When the mean laser power was increased to 10 or 15 mW, almost all of the cells had TUNEL-positive nuclei. When cells were exposed to 20 mW, green fluorescence was seen in all nuclei (Fig. 38.6).

The pattern of this green fluorescence, indicative of DNA strand breaks in cells exposed to high power NIR, is strikingly similar to that reported for apoptosis in other mammalian cells (Negri *et al*., 1997).

Our *in vivo* findings of NIR laser-induced DNA fragmentation is consistent and corroborates those of Shafirovich and colleagues (1999). Nuclear events during programmed cell death (apoptosis) are known to begin with the changes in the chromatin in the nuclear periphery (Tirlapur *et al*., 2000). Because the structural changes and DNA breaks in the nuclei of NIR-exposed cells seem quite similar to those occurring during apoptosis in different cell types (Negri *et al*., 1997; Bedner *et al*., 2000), it is therefore conceivable that NIR laser irradiation of PTK2 cells may under certain exposure conditions lead to apoptosis-like cell death.

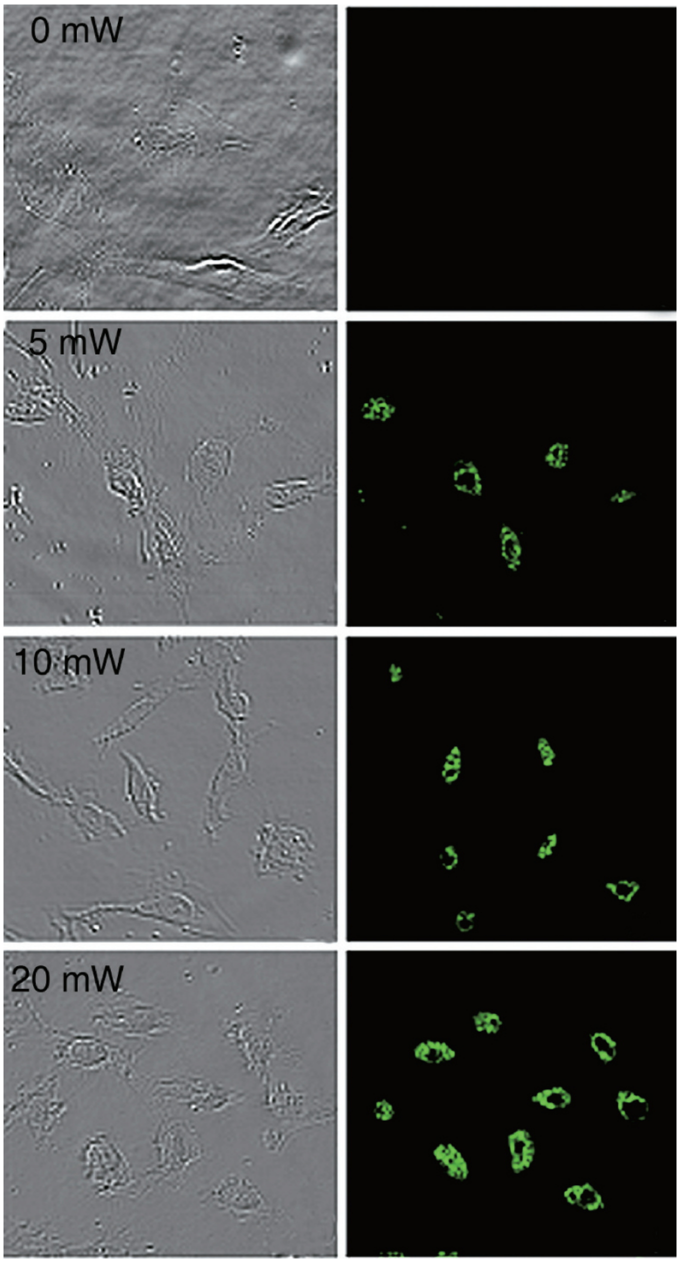

FIGURE 38.6. Evidence of DNA damage in PTK2 cells exposed to intense NIR femtosecond laser radiation. The green-colored fluorescein fluorescence images indicate TUNEL-positive and TUNEL-negative nuclei in dependence on mean laser power.

Photodynamic-Induced Effects

We studied the effect of two-photon excitation of porphyrins and their subsequent interaction with molecular oxygen. Light-excited porphyrin photosensitizers react with oxygen by a type II photo-oxidation process where energy transfer occurs from the metastable triplet state to molecular oxygen. This results in the formation of reactive singlet oxygen which induces cytotoxic effects.

In particular, we studied the effect of 780 nm-excited protoporphyrin IX that was synthesized in the mitochondria of Chinese hamster ovary (CHO) cells after external administration of the porphyrin precursor aminolevulinic acid (ALA, 1.5 mg/mL). Four hours after incubation, cells were exposed to 170 fs pulses at a mean power of 2 mW and a frame rate of 0.0625 s^{-1} (16 s/frame). This corresponds to a typical exposure time of ≈400 ms per scan on a single cell. Live/dead assays revealed lethal effects and fading of the red PP IX fluorescence in most of the cells after 100 scans in the same focal plane. Control cells without ALA could be scanned more than 700 times (up to 3 h) without affecting vitality (König *et al*., 1999b) (Fig. 38.7).

Nonlinear excitation of porphyrins can be used to induce desired photodynamic reactions in tumor tissue. The major advantages of NIR porphyrin photodynamic treatment are the excitation of higher order singlet states (Soret band excitation) and the high light penetration depth. Two-photon excitation can also be used to evoke type I photo-oxidation processes (charge transfer) (Fisher *et al*., 1997).

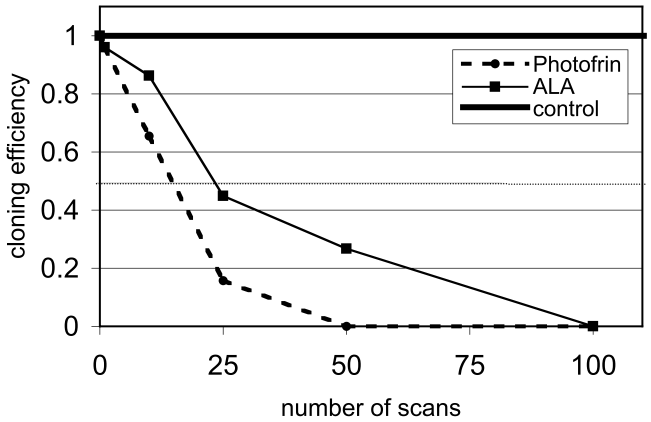

FIGURE 38.7. Impact of exogenous absorbers on reproduction of laser-exposed CHO cells. Two-photon induced photodynamic reactions lead to cell damage.

PHOTOTHERMAL DAMAGE

As we lack detailed knowledge of either the precise intracellular concentration, location, or NIR-absorption of the one-photon or multi-photon endogenous absorbers found in cells, we had to assume that water, with a typical absorption coefficient of less than 0.1 cm^{-1}, is the most important absorber in the optical window of non-pigmented cells and tissues. However, our studies using CHO cells exposed to femtosecond and CW lasers showed that the cell damage at 920 nm was less pronounced than that caused by 780 nm radiation even though water absorbs less at 780 nm. It therefore seems likely that the photodamage caused by intense femtosecond laser radiation is not based on linear water heating.

We measured a warming of only 1 °K/100 mW in CHO cells exposed to CW 1064 nm microbeams at mW/cm^2 intensity and GJ/cm^2 fluence (Liu *et al*., 1995). The temperature measurements were performed by microspectrofluorometry. CHO cells were labeled with the thermosensitive, fluorescent membrane stain Laurdan, which manifests a red shift of the fluorescence maximum with increasing temperature. Calibration enabled the determination of a mean intracellular temperature with an accuracy of about 0.1 °K. This is in line with temperature calculations for non-pigmented cells exposed to femtosecond and picosecond pulses, which predict only a slight, non-toxic increase in temperature (0.1 °K/100 mW at 850 nm) (Schönle and Hell, 1998).

Nevertheless, pigmented cells such as erythrocytes and those containing melanin may undergo photothermal destruction. In the optical window of cells and tissues, the one-photon absorption coefficients of these pigments are much higher than that of water. Whereas the absorption of melanin decreases with increasing wavelength, the pigment hemoglobin has an absorption maximum in this spectral region. Reduced hemoglobin possess a 760 nm absorption band with a one-photon molecular absorption coefficient of about 1 $mM^{-1} cm^{-1}$. Our studies have shown that human erythrocytes ($\alpha \approx 30 cm^{-1}$) exposed to 780 nm CW micro-irradiation at room temperature undergo hemolysis within 120 s when the power exceeds the value of 60 mW. A heating rate of about 60 °K/100 mW can be estimated from these experimental data. The heating rate decreased at wavelengths longer than 760 nm.

Interestingly, the photoinduced hemolysis studies revealed that femtosecond laser pulses at high repetition rate cannot simply be considered as quasi-CW beams. Damage may occur at a much lower mean power of pulsed radiation at high repetition frequency compared to power values of CW radiation. Using 130 fs pulses (76 MHz) at the same wavelength, a mean power of less than 10 mW was found to be sufficient to induce hemolysis (König *et al*., 1996c).

DAMAGE BY OPTICAL BREAKDOWN

The high transient powers, in particular in the kilowatt range, combined with submicron exposure spots result in enormous light intensities in the terawatt per square centimeter range. These can cause optical breakdown phenomena resulting in plasma formation and destructive thermomechanical effects. Optical breakdown induces immediate cell damage.

The threshold for optical breakdown decreases with the pulse width. In water, the threshold for 100 fs pulses was found to be 2 orders lower than for 6 ns pulses (Vogel *et al*., 1999). The primary cause for optical breakdown in the case of femtosecond pulses is efficient multi-photon absorption. Inverse Bremsstrahlung and collision processes are not dominant.

The onset of optical breakdown depends on the material. For some materials, femtosecond laser pulses at light intensities of about 0.1 TW/cm^2 are sufficient to induce optical breakdown. Optical breakdown and plasma formation is often accompanied by the occurrence of a nearly white luminescence and a complex sub-nanosecond emission decay (König *et al*., 1996d).

We have observed instantaneous white light luminescence and immediate damage of intracellular structures in CHO cells during scanning with 76 MHz, 170 fs pulses at mean powers higher than 10 mW. Interestingly, the optical breakdown occurred preferentially in the mitochondrial region and did not start in either the extracellular medium or the nucleus. Evaluation with the fluorescent live/dead fluorophore kit (calcein and ethidium homodimer from Molecular Probes, Inc., Eugene, OR) confirmed immediate death in scanned cells with extended plasma formation. However, when the phenomenon of optical breakdown was highly confined to a tiny perinuclear region, the cell often appeared to survive. At even higher laser powers, shockwave-induced morphological damage was manifested by total cell fragmentation (König *et al*., 1996d).

MODIFICATIONS OF ULTRASTRUCTURE

Our ultrastructural studies on CHO cells reveal that mitochondria are the major targets of NIR irradiation. Femtosecond laser exposure induced swollen mitochondria accompanied by loss of cristae and the formation of electron dense bodies in the mitochondrial matrix (Oehring *et al*., 2000). In addition, the lumen of the endoplasmatic reticulum was enlarged. For a mean laser power of more than 12 mW at 800 nm, the complete destruction of mitochondria and their transformation to electron dense structures was observed (Fig. 38.8). At higher power levels, the outer membranes ruptured.

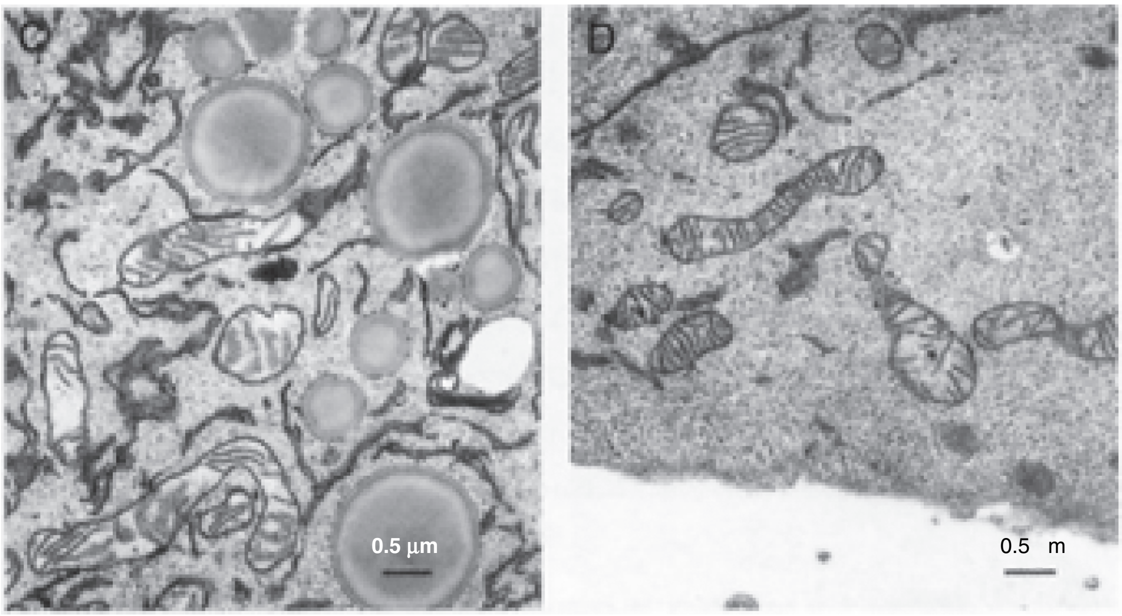

FIGURE 38.8. Electron microscopy reveals significant damage to mitochondria (left) as the major target of multi-photon microscopy. (Right) Control image of non-exposed cell.

INFLUENCE OF ULTRASHORT NEAR INFRARED PULSES ON REPRODUCTIVE BEHAVIOR

Squirrel and colleagues (1999) intermittently exposed Chinese hamster embryos for 24 h with femtosecond 1047 nm laser pulses of gigawatt per square centimeter peak intensity without impact on embryo development, in contrast to visible light exposure which the embryos did not survive. We have studied the influence of intense NIR exposure on the reproductive behavior of more than 3000 CHO cells. For that purpose, adherent CHO cells were maintained in a sterile cell chamber and exposed through the glass window. Following laser scanning, the cell division of the exposed cell as well as of its daughter cells was monitored up to 3 days. If the cell was not affected by the NIR light, division occurred every 12 h and the cell produced a clone of up to 64 cells within 3 days.

We found that CHO cells can be scanned with an 800 nm 80 MHz femtosecond laser beam at 2 mW mean power for hours without damage to the exposed cells and their derivatives. In particular we irradiated single CHO cells for a time period of 700 scans and 3 h, respectively (16 s/frame). However, at higher mean powers and intensities, a significant decrease in cloning efficiency occurred. In order to study the dependence on power, wavelength, and pulse width we used tunable laser beams which passed pulse stretching units based on prisms pairs and grating pairs. Typically, single CHO cells were scanned at microsecond pixel dwell times, 10 times in the same focal plane (König *et al*., 1997b, 1999a).

We found less damage at wavelengths in the 800 nm to 900 nm range compared to the 700 nm to 800 nm range. Interestingly, at the same average excitation power photodamage was found to be more pronounced in the case of femtosecond laser pulses compared to picosecond laser pulses (1–5 ps, same energy per pulse). At 780 nm, impaired cell division was found for 50% of the cells at 11 mW mean power (P_{50}) in the case of 2 ps pulses and at 3 mW for 240 fs pulses. CW scanning beams did not impair cellular reproduction up to 35 mW power. Using mean laser powers of <2 mW, CHO cells could be scanned with picosecond and femtosecond pulses for hours without impact on reproduction. This implies that below certain power (intensity) thresholds, "safe" multi-photon microscopy is possible.

When the P_{50} value is plotted against the square root of the pulse broadening, a nearly linear function is obtained (König *et al*., 1999a). This indicates that the photodamage process is likely based on a two-photon excitation process rather than on one-photon or three-photon events. This relationship was confirmed by Koester and colleagues (1999) based on calcium measurements in neocortical neurons in rat brain slices.

In order to compare photodamage in NIR multi-photon microscopes versus UV one-photon microscopes, the same cloning assay was performed on CHO cells using a 364 nm beam from a conventional Ar^+ laser scanning microscope. The 50% cloning efficiency was obtained at the low power of 4 µW. Therefore, UV-induced damage occurs at about 3 orders of magnitude lower power (4 µW) compared to the mean values of NIR femtosecond laser pulses (2–8 mW, 750 nm–800 nm).

NANOSURGERY

As pointed out in the section on photodamage by optical breakdown phenomena, the cell can survive if the damage induced by the high-intensity beam is confined to a minute subcellular volume. Exposure of a small region of interest including single spot exposure may therefore be useful for intracellular processing of biological material.

We increased the mean power in femtosecond laser microscopes with high numerical aperture objectives to values of 30 mW to 50 mW and light intensities of terawatts per square centimeter, respectively, to knockout intracellular material, to perform nanodissections (König *et al*., 1999c; König, 2000), and to "drill" holes.

By fine-tuning the laser power, only the central part of the beam spot provided sufficient intensity for material removal. Therefore, we were able to drill holes and to cut structures with a precision below the diffraction limit, that is, the cut size was below the size of the submicron beam spot.

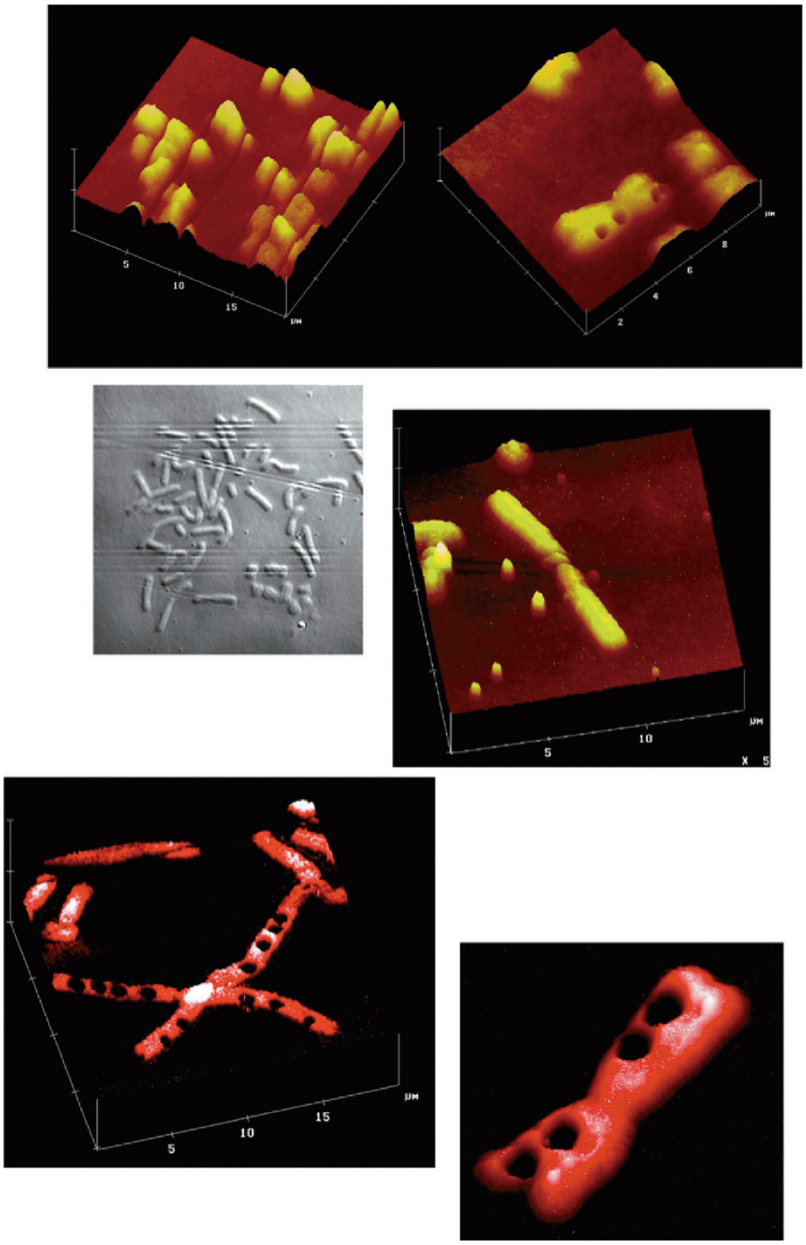

FIGURE 38.9. Nanoprocessing of human chromosomes with 80 MHz NIR femtosecond laser pulses at 800 nm.

For example, we used the multi-photon–induced ablation process to cut human chromosomes. Measurements with the atomic force scanning microscope reveal chromosome dissection with a FWHM cut size below 200 nm under conditions where the calculated Abbe diameter of the beam was ~600 nm. In addition, partial removal (incisions) of chromosome material with a precision of 110 nm and a FWHM of 85 nm was observed, which is likely the smallest laser cut ever performed in biological material. Figure 38.9 demonstrates examples of hole drilling and line cutting in human chromosomes.

Multi-photon laser microscopes can therefore be used as a non-contact nanoscalpel for surgery inside the cell, inside the cell nucleus, or even inside an organelle without affecting other cellular compartments. Indeed, we were able to cut chromosomes within a living PTK cell without loss of vitality. The cells finished cell division after laser surgery (Fig. 38.10) (König *et al.*, 2005).

CONCLUSION

Multi-photon microscopes can be used in live-cell fluorescence microscopy at moderate laser powers apparently without damaging the target. Repeated scanning of the biological object of investigation over extended time periods is possible without affecting reproductive behavior or producing lethal effects.

Above certain intensity thresholds, which depend on laser wavelength and pulse width, apoptosis-like gradual cell damage by two-photon photochemistry may occur. When studying pigmented cells and tissues, such as erythrocytes or skin with a high amount of melanin, photothermal damage has to be considered. Immediate damage occurs at high light intensities in the range of a terawatt per square centimeter by optical breakdown phenomena. Plasma-induced ablation can be used for intracellular nanosurgery. Femtosecond lasers at high repetition rate and low

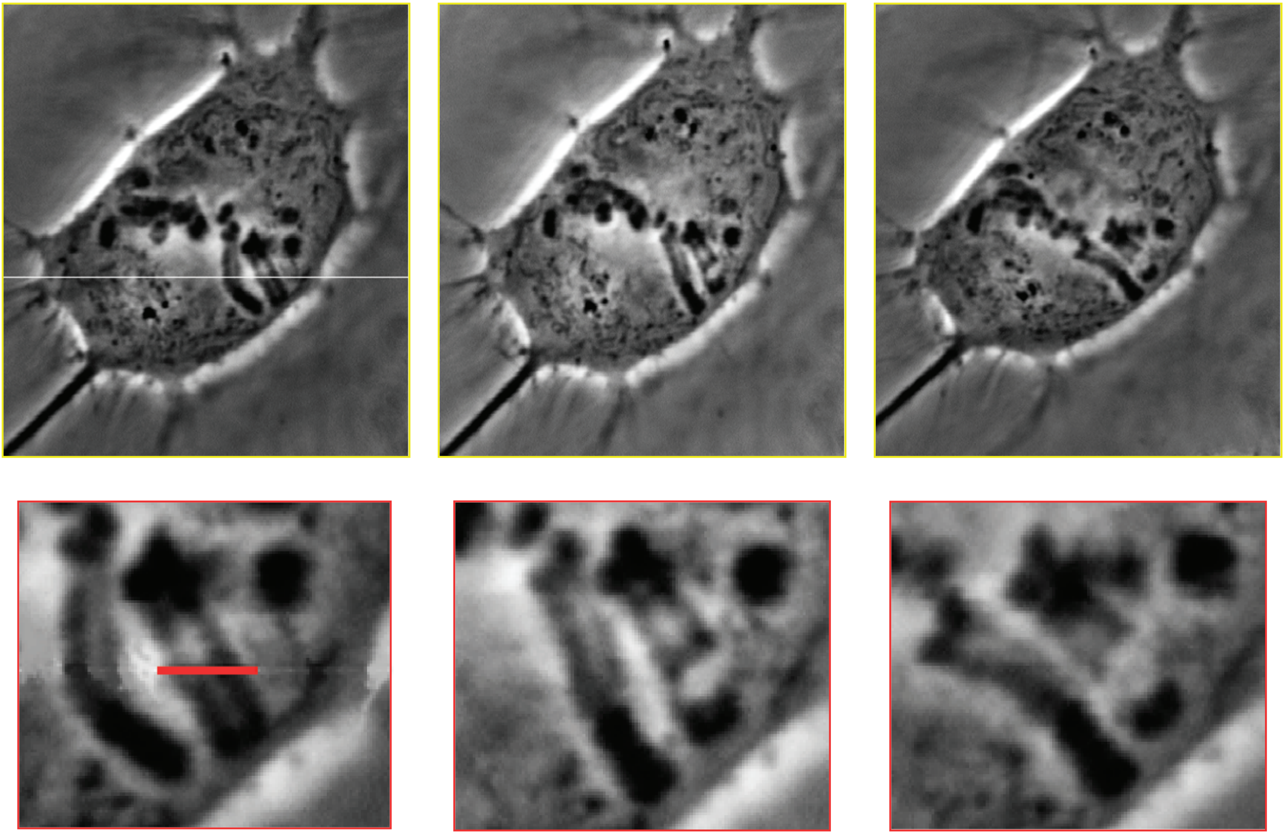

FIGURE 38.10. Intracellular chromosome dissection within a living PTK cell.

pulse energies of 0.1–10 nJ provide novel tools for laser nanomedicine (König *et al.*, 2005).

Meanwhile, the first femtosecond clinical laser imaging device, DermaInspect (JenLab GmbH) is in clinical use. The system is based on a tunable 80/90 MHz Ti : sapphire laser and operates in the safe optical window with low <10 mW laser power on the human skin for cancer diagnosis and *in situ* screening of pharmaceutical and cosmetic compounds (König and Riemann, 2003; König *et al.*, 2006).

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