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Laser tweezers and multiphoton microscopes in life sciences

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Abstract Near infrared (**NIR**) laser microscopy enables optical micromanipulation, piconewton force determination, and sensitive fluorescence studies by laser tweezers. Otherwise, fluorescence images with high spatial and temporal resolution of living cells and tissues can be obtained via non-resonant fluorophore excitation with multiphoton **NIR** laser scanning microscopes. Furthermore, **NIR** femtosecond laser pulses at TW/cm^2 intensities can be used to realize non-invasive contact-free surgery of nanometer-sized structures within living cells and tissues. Applications of these novel versatile **NIR** laser-based tools for the determination of motility forces, coenzyme and chlorophyll imaging, three-dimensional multigene detection, non-invasive optical sectioning of tissues ("optical biopsy"), functional protein imaging, and nanosurgery of chromosomes are described.

Key words FISH · FRET · Laser tweezers · Multiphoton microscopes · Nanosurgery

Introduction

Conventional light microscopy in "Life Sciences" including confocal laser scanning microscopy is based on the use of ultraviolet (UV) and visible **radiation** (Pawley 1995; Greulich 1999). One of the favorite methods in live cell studies is fluorescence imaging. Around 80% of applications of light microscopes in "Life Sciences" involve fluorescence microscopy. Fluorophores include endogenous (intrinsic) and exogenous (applied) dyes. Most of the endogenous fluorophores in cells, such as tryptophan, dopamine, serotonin, NAD(P)H, and flavins, require UV or blue excitation wavelengths. Important exogenous fluorophores, such as the DNA markers Hoechst 33342 and 4′,6-diamidino-2-phenylindole (DAPI) as well as the calcium indicators Fura-2 and Indo-1 possess absorption bands in the UV only. Other exogenous fluorophores are designed to absorb at the visible wavelengths of the laser and mercury lamp excitation **radiation**. UV microscopes have also been employed as microsurgery tools (Greulich 1999).

However, it must be noted that the use of UV and short-wavelength visible radiations in conventional light microscopy have certain disadvantages mainly because of low light **penetration depth** and the potential of severe photodamage to living cells (see Cunningham et al. 1985; Tyrell and Keyse 1990). It has been shown that during high-resolution fluorescence microscopy with the 365-nm mercury lamp **radiation** results within seconds in failed cellular reproduction, modifications in intracellular redox state, and DNA strand breaks (König et al. 1996a).

A versatile innovation in live cell microscopy is based on the application of near infrared (**NIR**) laser **radiation** in the spectral range of 700–1100 nm, the "optical window" of cells and tissues. Unpigmented cells appear as nearly transparent objects in this spectral range. Therefore, these cells can potentially experience extremely high light intensities up to 100 gigawatt per square centimeter (1 GW/cm^2=10^9 W/cm^2) without damage. This enormous intensity corresponds to a 12 orders higher light intensity than sunlight reaching the surface of the earth. When using ultrashort **NIR** laser pulses and the mean intensity remains below 20 MW/cm^2, the cellular

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When using highly focused continuous wave (cw) **NIR** laser **radiation**, cells and organelles can be optically confined in the focal volume of a high numerical aperture (NA) objective by the **radiation** pressure (Ashkin 1970, 1980; Ashkin and Dziedzik 1987, 1989). These tools are called laser tweezers (optical traps) and can be used for optical micromanipulation, cell sorting, piconewton (pN) force measurements, motile cell diagnostics, and as sources for two-photon excited fluorescence.

The other important **NIR** tool is the multiphoton laser scanning microscope for fluorescence imaging with high spatial and temporal resolution, photoinduced uncaging of compounds, and nanoprocessing (Denk et al. 1990; König et al. 1999a). Typically, these microscopes are based on the use of ultrashort laser pulses in the femtosecond range (1 fs=10^{-15} s). Multiphoton excitation based on the simultaneous absorption of photons was predicted in 1931 (Göppert-Meyer 1931) and first realized with the availability of lasers in 1961 (Kaiser and Garret 1961). In 1990, Denk et al. realized two-photon excitation of fluorophores in living cells.

This paper focuses on applications of laser tweezers and multiphoton femtosecond laser microscopes in “Life Sciences” encompassing cell biology, biotechnology, and medicine.

Laser tweezers

Principle

Laser tweezers, also known as (single beam gradient force) optical traps, are based on pN force generation during the interaction of highly focused laser beams with dielectric particles, including cells and organelles.

Light, as a carrier of momentum, exerts pressure which can be used to accelerate particles (Ashkin 1980). This is the reason why the tail of a comet is directed away from the sun. If a light beam of sufficient **radiation** pressure is directed against gravity, particles can be transduced into “gravity-free” conditions. The **radiation** pressure of the sunlight on earth is of the low order of μN/cm^2, however, with the availability of laser sources, laser light pressure can be used to move small objects. As a spin-off of Ashkin’s initial studies on optical particle trapping (Ashkin 1970, 1980), optical cell micromanipulation by laser light became possible.

By focusing a single laser beam with a high NA objective, heating at < 100 GW/cm^2 peak intensities is less than 2°C (Liu et al. 1995; Schönle and Hell 1998).

The combination of **NIR** lasers and microscopes provides novel non-contact biomedical tools in “Life Sciences”. These tools include not only imaging devices for damage-free live cell imaging, but also for optical micromanipulation, intracellular photochemistry, and nanosurgery.

Fig. 1 Schematic representation of laser tweezers

When using highly focused cw **NIR** laser beams (“microbeams”), pigment-free cells can be optically trapped and can be manipulated in three dimensions without physically touching them. In particular, contact-free cell transport can be performed by moving the foci of the laser beam in the desired direction. Alternatively, the stage with the sample can be moved without displacement of the trapped target.

Typical laser sources for optical trapping are the Nd:YAG laser at 1064 nm and laser diodes. Often, the laser beam is coupled by light fibers or directly to a video microscope and focused to a diffraction-limited spot by objectives with NA>1. Motor-driven mirrors in combination with joy-sticks allow maneuvering the position of the tweezers.

Optical micromanipulation

In contrast to mechanical manipulation with micromanipulators, laser tweezers enable contact-free micromanipulation even through the glass windows of a closed cell chamber. Such a versatile chamber (JenLab, Jena, Germany) with 170-μm-thick glasses and a silicone gasket is depicted in Fig. 2. Cells and medium can be easily transferred and changed with standard needles and syringes.

We used closed cell chambers to trap human spermatozoa of patients with impaired fertility and to study the influence of the intense **NIR** beam on the cellular metabolism and vitality (König et al. 1995a). The **NIR** beam

jective, a gradient field of light intensity is created with the highest intensity values in the focal volume and the lowest in the periphery. Interaction with objects of a higher refractive index than the surrounding results in the formation of gradient forces. If the interaction is primarily determined by beam refraction and when the absorption is negligible, the net force acts toward the focal volume. The net force (trapping force) of the order of pN can be used to "pull" and to confine micro- and nanometer-sized objects in the focal volume (Fig. 1).

Fig. 2 Photograph of a sterile cell chamber which can be used in optical trapping and multiphoton femtosecond laser microscopy

700–800 nm affected the cellular metabolism and led to cell death within seconds (König et al. 1996a,b). The reason is the phenomenon of trap-induced two-photon excitation followed by harmful UV effects as explained later.

Most optical trapping studies are conducted with the 1064-nm beam of an Nd:YAG laser which appears as a safe micromanipulation tool even if the cell experiences enormous energy densities (fluences) of some GJ/cm ^{2}. When using 100-mW laser tweezers, the trap-induced temperature increase is between 1 and 2°C. Meanwhile, the first clinical applications of laser tweezers for laser-assisted IVF have been reported (see, for example, Wiedemann and Montag 1994). In addition, laser tweezers have been employed as micromanipulation tools in pharmacology, biotechnology, and cell biology. For example, Ashkin and Dziedzic (1989) studied the mechanical properties of the cytoplasm in the interior of living cells, and Chu's group measured relaxation curves of single DNA molecules (Perkins et al. 1994). Zahn and Seeger (1999) used laser tweezers for drug screening. Buican et al. (1987) are referred as pioneers of automated cell sorting by optical traps.

traps spermatozoa by confinement of the head, the DNA-containing region of high refractive index, in the focal volume. The goal of the study was to prove if laser tweezers are an appropriate optical micromanipulation tool to realize laser-assisted in vitro fertilization (IVF) by optical transport of a single sperm cell to the oocyte. This method was suggested by Tadir et al. (1989) as an alternative to mechanical approaches.

Interestingly, laser tweezers at a wavelength of 800 or 1064 nm did not harm the cell even over extended trapping periods of up to 15 min, whereas optical traps at

Q can be calculated theoretically in the case of spherical particles with known refractive index. However, for "real" biological objects with non-uniform morphology and heterogeneous optical density, the trapping parameter and the trapping forces, respectively, have to be determined experimentally. The most common method is the "escape force method" where the trapped object experiences a flow of liquid of increasing velocity. The flow exerts a drag force, F_{drag}, which can be calculated by Stokes' law. This force depends on the velocity, viscosity, and the target morphology and dimension. At a certain velocity, the drag force has the same value as the trapping force and the object can "escape". It is at this point that the drag force can be used to calibrate the trapping force.

We determined the trapping parameter and the trapping forces of human spermatozoa confined in an 800-nm trap (König et al. 1996c). With a mean Q parameter of 0.12, the trapping force was found to be about 50 pN at 100 mW laser power. In the case of healthy spermatozoa, a mean trapping power of about 80 mW was required to confine these highly motile cells. Under the assumption of linear swim motion, the mean intrinsic motility force of human sperm can therefore be estimated to be about 45 pN (König et al. 1996c).

Trap-induced forces have been extensively used to study motor proteins (Block et al. 1990; Kuo and Scheetz 1993) and binding behavior, such as receptor-mediated interactions of cells with glycoproteins (Zahn and Seeger 1999). A review of biological applications of optical forces was published by Svoboda and Block (1994).

Fluorescence diagnostics of motile cells

Fluorescence studies on single motile cells are difficult due to pN motility force driven sample movement. Certain bacteria, algae, and spermatozoa can achieve velocities higher than 50 μm/s. The problem of sample movement can be overcome when the object of investigation is confined to the focal volume of the objective by laser tweezers. We combined sensitive imaging devices, such as ultrafast time-gated slow-scan CCD cameras, and laser tweezers to be able to image the autofluorescence of

Force measurements

The net force (trapping force, F) depends linearly on the laser power P and can be represented by:

$$F=QP/c$$

where c is the velocity of light in the medium and Q the trapping efficiency parameter with values between 0 and 2. The parameter Q depends on the optical properties of the trapped object, such as the refractive indices, as well as on the beam profile and alignment. The knowledge of the trapping parameter allows the calculation of the trapping force and the subsequent use of the laser tweezers as force measure (picotensometer).

highly motile cells with high spatial (submicron) and temporal (picosecond, 1 ps=10^{-12} s) resolution.

Figure 3 demonstrates an example of autofluorescence imaging of a highly motile sperm cell. The endogenous fluorophores have been excited with the 365-nm **radiation** of a high-pressure mercury lamp and detected in the blue/green spectral range. The only intracellular region of autofluorescence of the motile cell was found to be the midpiece of the cell which is the location of the mitochondria. The fluorescence of mitochondria is mainly based on the reduced coenzyme NADH. Interestingly, the sperm head including the acrosome region became brightly fluorescent following exposure to extended UVA **radiation**. The changes in the autofluorescence pattern were accompanied by reduced motility and finally loss of vitality (König et al. 1996b).

An example of autofluorescence lifetime imaging is depicted in Fig. 4. The fluorescence lifetime is an intrinsic property of the fluorophore and its microenvironment and independent of concentration. Imaging of fluorescence lifetimes (τ mapping) enables spatially resolved fluorescence lifetime determination and fluorophore separation. The figure exhibits the autofluorescence pattern of the biflagellate green microalga *Haematococcus pluvialis* which was trapped by means of multiple traps at 1047 nm. Chlorophyll was excited with a picosecond laser diode at 633 nm and imaged with an ultrafast time-gated camera with a tunable time-delay (0–20 ns) between fluorescence excitation and detection (König et al. 1998). A mean fluorescence lifetime in the picosecond

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Fig. 3 Autofluorescence imaging of an optically trapped single motile sperm cell. The fluorescence emanates from the mitochondria-rich region corresponding to the midpiece of the sperm

Fig. 4 Time-resolved autofluorescence imaging of an optically trapped motile green alga. The time-delay between excitation of chlorophyll and detection of fluorescence was spaced in steps of 500 ps

Fig. 5 Trap-induced visible fluorescence inside the optically confined head of spermatozoa labeled with SYBR-green

Fig. 6 Principle of two-photon excitation. The simultaneous absorption of two near infrared (**NIR**) photons at MW/cm ^{2} and GW/cm ^{2} light intensities induces visible fluorescence. At TW/cm ^{2} intensities multiphoton ionization leads to plasma-induced ablation which can be used for nanoprocessing. S_n, S_1, S_o represent different energy levels, *E* energy

range was determined which increased up to 1.4 ns when exposed to herbicides, indicating disturbed energy transfer.

Laser tweezers as non-linear diagnostic tools

10^{-50} cm ^{4}s). To achieve such a high photon concentration, **NIR** light intensities of at least 20 MW/cm ^{2} are required.

Assuming a 100-mW laser beam at λ=800 nm and an illumination spot of diameter d=λ/NA=615 nm and area 0.30 μm ^{2}, a high intensity of power/area=33 MW/cm ^{2} can be calculated which is sufficient to induce two-photon

Fluorescence diagnostics can also be performed with the trapping beam as fluorescence excitation source. Surprisingly, during trap experiments with dye-labeled spermatozoa in 1994 we found that highly focused cw **NIR** beams at 100 mW power are able to excite the visible fluorescence of the intracellular dyes (König et al. 1995b). Excitation occurred only in a sub-femtoliter focal volume (1 fl=10^{-18} m^3), the region of highest light intensity (Fig. 5). The fluorescence followed a squared relation on laser power indicating a two-photon process. In this case, the fluorescence is excited by the simultaneous absorption of two low-energy (**NIR**) photons. Each of them provides half the energy required for molecule excitation (Fig. 6). As an example, a fluorophore which is normally excited at 400 nm (for example red fluorescent protoporphyrin IX) can therefore be excited with 800-nm **radiation**.

Two-photon excitation **radiation** requires a high photon concentration in space and time due to the low molecular two-photon absorption cross-sections (10^{-48} to

effects. Therefore, laser tweezers act as sources of two-photon excitation and can be used as novel tools for nonlinear fluorescence excitation (the fluorescence intensity has a squared, not a linear, dependence on power). One of the interesting features of trap-induced fluorescence is the possibility to localize the intracellular trapping spot with high accuracy. Florin et al. (1998) use this effect to create a novel photonic force microscope.

The two-photon excited fluorescence can also be used to study possible harmful effects of the laser tweezers on the cell trapped with the **NIR** beam. For example, when injecting the viability indicators SYBR14 (green fluorescent live cell indicator) and propidium iodide (red fluorescent dead cell indicator) from Molecular Probes (Eugene, Ore., USA) into the cell chamber with spermatozoa, the possible onset of trap-induced lethal effects can be studied without external fluorescence excitation sources. Using the methods of microspectrofluorometry and spectrally resolved fluorescence imaging, we were able to study the damaging effect of short-wavelength

(<800 nm) laser tweezers (König 1998). **NIR** microbeams in the spectral range of 700–800 nm are able to excite endogenous absorbers with electronic transitions in the UV which may lead to harmful UVA effects (Cunningham et al. 1985; Tyrell and Keyse 1990).

Using the trap-induced fluorescence we also studied the effect of the laser beam quality and found: (1) that the damage process is based on a two-photon process and (2) that certain cw laser beams contain unstable picosecond laser pulses (“spikes”) which may enhance destructive effects (König et al. 1996d).

Multiphoton laser microscopy

Principle and setup of a multiphoton laser scanning microscope

CW laser microbeams in combination with scanning microscopes can be used for three-dimensional (3D) imaging of two-photon excited fluorophores (Hänninen et al. 1994; Booth and Hell 1998). In contrast to conventional confocal laser scanning microscopes, no spatial filter (pinhole) is required to obtain 3D images. This is because of the minute (sub-femtoliter) two-photon excitation volume which can be used to scan the sample (Fig. 7). Due to lack of **NIR** absorption outside the focal volume, there is neither out-of-focus photobleaching nor out-of-focus photodamage.

In two-photon microscopes, the fluorescence intensity increases quadratically with the excitation intensity and the power, respectively. However, trapping effects and photothermal effects limit the use of high power cw sources for fast fluorescence scanning microscopy. Fast multiphoton fluorescence imaging is much more efficient when using high repetition pulsed laser systems

Fig. 7 Under same focusing conditions with high numerical aper-

with moderate peak power in the W and kW range but with low mean μW and mW power. The two-photon excited fluorescence yield, I_F, follows the following relation (Denk et al. 1990):

$$I_F \approx P^2 \alpha/(\tau f^2)\cdot\pi^2 NA^4/(hc\lambda)^2$$

where P is the mean power, α the molecular two-photon absorption coefficient, τ the pulse width, f the repetition frequency, NA the numerical aperture, h Planck's constant, c the velocity of light, and λ the wavelength. Because the fluorescence yield depends on a P^2/τ relation, two-photon microscopy with 1-ps laser pulses requires threefold higher mean powers than 110 fs pulses. Both, picosecond as well as femtosecond laser scanning microscopes are now commercially available from leading microscopy suppliers.

In three-photon fluorescence microscopy (Gryczynski et al. 1995; Wokosin et al. 1995; Hell et al. 1996; Maiti et al. 1997), where three photons are absorbed simultaneously, I_F depends on a P^3/τ^2 relation. In this case, efficient excitation requires the use of femtosecond laser microscopes. Three-photon excitation has been used to image serotonin in living cells (Maiti et al. 1997).

ture objectives, two-photon excitation is confined in the minute focal volume due to the required high intensity. By contrast, one-photon excitation results in fluorescence along the illumination cones. *hv* Energy of a photon

Our multiphoton microscopes are based on the use of femtosecond solid state laser sources. We use turn-key, air-cooled, single-box laser systems. The first one is a titanium:sapphire laser (Vitesse 800-HP; Coherent, Santa Clara, USA) with an output of 1 W mean power, 80 MHz repetition frequency, 800 nm wavelength, 80 fs pulse width, and 55.5·34·18 cm^3 dimensions. We use this laser for two-photon and three-photon fluorescence studies in cells and tissues, and fluorescence in situ hybridization (FISH) studies, as well as for nanosurgery. The second ultracompact one (Femtolite; IMRA, Ann Arbor, Mich., USA) is a frequency doubled erbium-doped fiber laser at 780 nm with 40 mW mean power, 50 MHz repetition frequency, 780 nm wavelength, 180 fs pulse width, and 19.3·10.9·8.2 cm^3 dimensions (electronic controller: 24.9·30.5·7.2 cm^3). The power at the sample of <7 mW is sufficient to excite a variety of fluorophores in cell monolayers and in FISH studies.

Fig. 8 The depicted FISH fluorophores with emission in the blue, green, yellow, and red can be simultaneously excited at 780 and 800 nm. *DAPI* 4′,6-diamidino-2-phenylindole, *DAC* diethylaminocoumarine, *FITC* fluorescein isothiocyanate, Cy cyanine

The laser beam is expanded by an 1:4 Galilean telescope, coupled to a modified inverted confocal laser scanning microscope (LSM410; Zeiss, Jena, Germany) and focused to a diffraction-limited spot by 40·, 63·, or 100· objectives of NA>1.2. Due to optical dispersion which results in pulse broadening during transmission through microscope optics, the pulse width at the sample is about 150–200 fs (König 2000). At 5 mW mean power, the peak power and the peak intensities reach values

different emission wavelengths (König et al. 2000a). Labeling is based on standard FISH procedure (see Speel 1999 for a review).

In contrast to the conventional detection method, where different excitation wavelengths in the UV, blue, and green range are required to induce the visible fluorescence of the most common FISH fluorophores and of the general DNA stain DAPI, the novel approach uses **NIR** light at one excitation wavelength only. Figure 8 shows the emission spectra of FISH fluorophores which can be non-linearly excited at 780 and 800 nm.

In addition to the advantage of using a single excitation wavelength to realize multicolor FISH, multiphoton microscopy enables optical sectioning of thick samples (interphase nuclei, embryos, biopsies) in different

of 0.4 kW and $0.6\cdot10^{12}$ W/cm^2 (0.6 TW/cm^2), respectively, when assuming a full width half maximum beam size of $\lambda/2NA\approx310$ nm. A typical pixel dwell time of the beam during one scan is 4 µs which results in a frame rate of 1 s/frame. At zoom 4, 512·512 pixels cover a sample area of 80·80 µm.

Multiphoton excited fluorescence is typically registered with a detector at the baseport of the modified microscope. A 700-nm short pass filter prevents the scattered laser **radiation** from reaching the detector. We use different camera systems, photomultiplier tubes (PMTs), and a spectrometer as detectors.

The described microscope setup can also be used for conventional one-photon confocal microscopy with an internal He-Ne laser, pinholes, and the internal PMTs of the microscope.

Multiphoton multicolor FISH (MM-FISH)

An interesting feature of multiphoton microscopy is the possibility to excite a variety of fluorophores simultaneously at one **NIR** wavelength due to overlapping two(three)-photon excitation spectra (Xu et al. 1996). We have taken advantage of this to detect various genome regions labeled with multiple fluorescent targets with planes. There is no photobleaching of FISH fluorophores in out-of-focus regions. Reconstruction of 3D fluorescence images from the optical sections provides information on the genome architecture, such as 3D organization of chromosomes and their well-defined domains such as centromeres and telomeres. Such a 3D fluorescence image of centromeric regions of the chromosomes 1, 3, 6, 12, and X in the interphase nucleus of an amniotic fluid cell is seen in Fig. 9. The 3D image has been reconstructed from a stack of 20 images which are spaced by 0.75 µm. The five FISH fluorophores have been excited simultaneously. Also the blue fluorescence of the DNA counterstain DAPI has been induced with **NIR** femtosecond laser pulses. Spatially resolved DAPI fluorescence imaging provides information about the nuclear architecture and enables the determination of the intranuclear loci of the fluorescent centromeric regions.

Besides multifluorophore excitation and optical sectioning, a third advantage of using excitation **radiation** in the 700- to 1200-nm spectral range is the high light penetration **depth**. We used MM-FISH for the detection of centromeric regions in multilayer samples, such as human biopsies. In particular, we imaged two-photon excited spectrum green-labeled C-4 and spectrum orange-labeled C-X probes in thick kidney cryosections with high spatial resolution. The nuclear area was visualized after

Fig. 9 Three-dimensional (dz=0.75 µm) six-color detection of interphase nuclei of amniotic cells with the **NIR**-excited fluorophores spectrum aqua (centromere chromosome X, blue), spectrum orange (centromere chromosome 3, red), rhodamine 110 (centromere chromosome 12, yellow), spectrum green (centromere chromosome 6, green), DAC (centromere chromosome 1, white), and DAPI (light blue). The image is false-color coded and reconstructed from a stack of 20 optical sections

counterstaining with ethidium bromide and optical sectioning with **NIR** laser pulses (König et al. 2000b).

Potential applications of this new MM-FISH tech-

nique are in the field of molecular cytogenetics, prenatal and preimplantation diagnosis, and molecular pathology.

Live cell imaging

In addition to the in vitro studies, imaging of DNA in living cells and tissues can be performed by two-photon excited imaging of the DNA fluorophore Hoechst 33342. The high **penetration depth** of **NIR radiation** also allows the spatially resolved detection of the fluorophore in deep tissue. **NIR radiation** at 800 nm has a typical **penetration depth** in tissue of several millimeters in contrast to some micrometers when using one-photon fluorescence excitation in the UV (Chong et al. 1990). Figure 10 shows DNA images of tumor tissue in living mice after the topical application of a Hoechst–DMSO mixture. The Hoechst-labeled chromatin can be clearly visualized in the different cell layers. Considering a $120 \cdot 120 \cdot 30\ \mu m^3 = 0.4 \cdot 10^{-12}\ m^3$ tumor volume, a mean number of 100 nuclei in such a volume can be counted from the stack of images. Interestingly, scattering in turbid media such as tissues does not decrease the excellent lateral resolution of <0.4 µm and the ca. 1 µm axial resolution significantly within the first 100 µm tissue.

Multiphoton microscopy has been widely used in imaging of single living cells, cell monolayers, and embryos (see König 2000 for a review) including mapping of ion channels by two-photon photochemical microscopy (Denk 1994) and neuron imaging (Denk and Svoboda 1997).

Fig. 10 Non-invasive optical sectioning in a living anesthetized mouse. Depicted are Hoechst 33342-labeled tumor tissue layers

An interesting feature of **NIR** multiphoton microscopy is the possibility to excite endogenous fluorophores such as the reduced coenzymes NADH and NADPH as well as flavin coenzymes with **NIR radiation** in the range of 700–800 nm (Piston et al. 1995; König et al. 1996d). Because the oxidized forms NAD(P) do not exhibit fluorescence, the NAD(P)H attributed autofluorescence can be used to obtain information on the intracellular redox state, modifications in the respiratory chain, and cellular metabolism (König and Schneckenburger 1994; König et al. 1995b).

The two-photon excitation of tissue autofluorescence may become a useful non-invasive technology to obtain "optical biopsies" without physical removal of tissue. Similar to tomography with X-rays, intense **NIR** microbeams can be employed to perform optical sectioning of the tissue.

Endogenous fluorophores and structures which can be excited via a two-photon or a three-photon excitation process include serotonin, dopamine, and tryptophan with emission in the UV, NAD(P)H, collagen, elastin, melanin, and flavins with blue/green fluorescence, lipofuscin, Zn-coproporphyrin, and Zn-protoporphyrin with yellow emission, and coproporphyrin, protoporphyrin, and chlorophyll with red fluorescence.

The first two-photon excited autofluorescence images of in vivo human **skin** have been performed (Masters et

Fig. 11 Non-invasive optical biopsy of high spatial resolution with near infrared femtosecond laser pulses. Depicted is an in vivo autofluorescence image of human **skin** showing an epidermal layer

al. 1997; König 2000). Figure 11 depicts a single in vivo autofluorescence image from a stack of high-resolution fluorescence images. The image was acquired at a **depth** of 30 μm from the palmar surface of the forearm. The stack was obtained up to 150 μm into the **skin** with a **depth** increment of 5 μm. The stratum corneum with a typical thickness of 15 μm at the investigated loci was found to be highly fluorescent when excited at 800 nm. In particular, the border of the hexagonal-shaped tissue structures exhibited fluorescence. Below this tissue layer, individual cells could clearly be visualized by the autofluorescence of intracellular structures in the cytoplasm. Cell nuclei and cell membranes did not fluoresce. The autofluorescence was stronger in the innermost layer of the epidermis, the basal layer, than in the surroundings. Epidermis and dermis could be differentiated, and elastin and collagen fibers could be visualized.

Photodamage due to multiphoton microscopy

Due to the lack of out-of-focus photodamage and photobleaching, multiphoton **NIR** microscopy appears as a safe novel tool without impact on cellular metabolism, reproduction, and vitality. In fact, Chinese hamster ovary cells can be scanned with an 800-nm femtosecond laser beam at 2 mW for hours without damage to the exposed cells and their derivatives (König 2000). Squirrel et al. (1999) intermittently exposed hamster embryos for 24 h with ultrashort laser pulses of GW/cm ^{2} peak intensity without impact on embryo development in contrast to one-photon laser scanning microscopy where the embryos did not survive.

at a **depth** of 30 μm. Note that individual cells as well as cytoplasmic structures are clearly visible

However, above certain thresholds photodamage may occur. As pointed out in the section on laser tweezers, intense cw **NIR** beams can induce cell damage via a two-photon effect. The same destructive effects would therefore occur when using ultrashort laser pulses at even higher light intensities.

We found mainly two types of photodamage during **NIR** microscopy. The first is a slow process probably based on two-photon excitation of endogenous absorbers and subsequent photo-oxidation processes resulting in the formation of destructive reactive oxygen species (ROS). This process appears at MW/cm ^{2} and GW/cm ^{2} intensities and depends strongly on wavelength. The second damage process is of immediate effect and requires high intensities in the TW/cm ^{2} range and is based on multiphoton ionization, optical breakdown phenomena, and intracellular plasma formation. It results in material ablation and disruption including complete cell fragmentation.

Studying the first photochemical attributed photodamage process we found that the mitochondria and the Golgi apparatus are the major targets of **NIR** microbeams (Oehring et al. 2000). Above certain light intensities, laser-exposed cells either fail to divide, become giant cells, or die. Due to the P^2/τ dependence of the slow damage process, photodamage was found to be more pronounced at shorter pulses (König et al. 1999b).

More recently it has been demonstrated that **NIR** laser irradiation at certain higher power levels evoke genera-

Fig. 12A–C Nanosurgery with **NIR** femtosecond laser pulses. Force microscopy image of cuts through human chromosomes (**A**). A minimum cut size of 110 nm was achieved. Transmission and fluorescence image of living cells labeled with rhodamine 123 before (**B**) and after (**C**) knocking out of a single mitochondrion (*asterisks*)

tion of ROS that can be cytochemically visualized in vivo using Ni-3,3′-diaminobenzidine (Ni-DAB) as well as with a recently developed fluorescent probe Jenchrom px blue from JenLab (Tirlapur et al. submitted). In addition, the irradiated cells manifest membrane-barrier dysfunction, drastic alterations in the morphology of the nuclear envelope, and fragmentation of the DNA. Because the cytological changes observed in **NIR**-irradiated cells bear striking similarities to those seen in cells undergoing programmed cell death (Li and Darzynkiewicz 1999), it has been inferred that intense **NIR** femtosecond laser pulses can induce apoptosis-like death (Tirlapur and König submitted).

Femtosecond laser surgery of cellular nanostructures

When the plasma-mediated photodamage process can be confined to a tiny intracellular volume by parking the beam at pixels of interest, the destructive effects of intense **NIR** laser pulses can be used to cut cellular structures and holes can be generated in the target with a size below the diffraction-limited spot size. We realized a minimum cut-size of 110 nm into the human chromosome 1 which is to date likely be the smallest laser cut in biological material. The topography of this laser cut was analyzed and measured with a scanning force microscope. We were also able to perform chromosome dissections within round living cells (König et al. 1999a). The use of femtosecond **NIR** pulses at TW/cm ^{2} light intensities therefore provide novel non-invasive tools to perform nanosurgery without destructive influence to the surroundings and enable nanoprocessing within living cells and tissues.

Further potential applications of **NIR** microscopes

Four dimensional (4D) microscopy in space and time

An interesting feature of multiphoton laser scanning microscopes with picosecond and femtosecond pulsed la-

tures or to "drill" holes in the target (König et al. 1999a). Material processing with femtosecond laser pulses has the advantages of minimal ablation threshold, low transfer of optical energy into destructive mechanical energy, and the absence of thermal damage to surrounding structures compared to nanosecond pulses used in conventional microsurgery.

We use the novel surgery tool to perform dissections of chromosomes and to knock out intracellular structures at mean powers of 30–50 mW with microsecond and millisecond beam dwell times (Fig. 12A,B). Because only the central part of the illumination spot provides sufficient intensity for plasma-induced ablation, laser cuts

sers is the possibility of simultaneously performing 4D imaging in space and time. Of particular relevance is microscopic imaging with ultrafast temporal resolution in the range of picoseconds and nanoseconds corresponding to the range of fluorescence lifetimes (see, for example, Lakowicz 1983; So et al. 1998). Hence with a pulsed laser excitation source it is possible to upgrade a 3D multiphoton microscope to a versatile 4D imaging device.

Fig. 13 Time-resolved multiphoton microscopy. Fluorescence decay curves after two-photon excitation of intracellular fluorophores along lines of 128 pixels

In order to realize 4D two-photon microscopy we have incorporated a fast PMT in combination with a single photon counting unit (SPC 730; Becker and Hickl, Berlin, Germany). The unit enables the fast registration of fluorescence decay curves following excitation with the 80-MHz femtosecond laser at a pixel of interest during single-point illumination as well as the registration of 128·128=16384 decay curves during scanning. Examples of fluorescence decay curves of a variety of intracellular fluorophores along line scans and an example of a τ image are presented in Figs. 13 and 14.

Two-photon fluorescence resonance energy transfer (FRET)

As yet an unexplored interesting potential feature of multiphoton microscopes with high NA objectives is the

studying single molecules and intramolecular interactions. In particular the combination of two-photon **NIR** excitation and FRET allows monitoring of the spatially resolved relationship between two macromolecules within living cells (Fig. 15).

The non-radiative energy transfer (Foerster mechanism) occurs in close proximity of a fluorescence donor (D) in the excited state and a fluorescence acceptor (A). The efficiency of the energy transfer decreases with R^{-6}, where R is the distance between D and A. Therefore, FRET occurs mainly within intermolecular distances with R<10 nm. It provides a variety of information on molecular interactions such as binding behavior, intermolecular distance, diffusion kinetics, and association reactions (Pollak and Heim 1999). In order to realize efficient two-photon FRET the following conditions are essential:

use of the minute sub-femtoliter excitation volume for

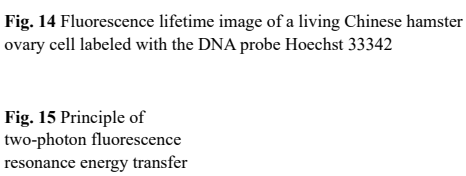

Fig. 14 Fluorescence lifetime image of a living Chinese hamster ovary cell labeled with the DNA probe Hoechst 33342

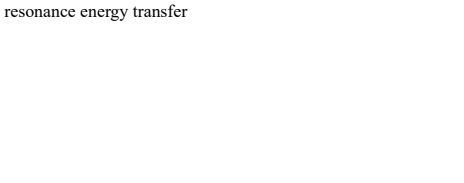

Fig. 15 Principle of two-photon fluorescence resonance energy transfer

1. Efficient two-photon excitation of the donor molecule
2. Spectral overlap between the emission spectrum of the donor and that of the excitation spectrum of the one-photon acceptor as well as appropriate relative orientation of both molecules
3. Separation between acceptor and donor emission
4. Distance less than 10 nm.

Due to the broad two-photon excitation spectrum, a variety of fluorophores preferably with UV and blue one-photon absorption maxima can be used as donors. In the case of probing protein–protein interactions, GFP mutants such as B(lue)FP with 445 nm emission, C(yan)FP (505 nm), Sapphire (511 nm), eGFP (511 nm) and Y(ellow)FP (540 nm) can be used. FRET can occur between two GFPs or one GFP and a second fluorophore. According to the spectral overlap, potential FRET pairs include BFP–GFP, CFP–YFP, Sapphire–fluorescein, and GFP–fluorescein molecules.

4D microscopy provides the possibility to perform time-resolved energy transfer imaging which enables studies of the static and dynamic mobility of macromole-

cules. Often, the fluorescence decays are relatively sensitive to the acceptor-donor distances (Lakowicz 1983).

Non-invasive nanosurgery in tissues

As pointed out earlier, nanosurgery can be performed within a single living cell. In principle, knocking out of cellular structures can be performed deep in living tissue without disturbing surface layers. Such highly precise **NIR** laser-based nanoprocessing of cellular structures without compromising the vitality of cells has numerous potential applications in cell and developmental biology particularly in studies addressing spatial-temporal control of developmental events and functional interactions

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between organelles, as well as cell–cell communication.

Initial **NIR** femtosecond laser experiments have been performed in living plant tissue. Using 800-nm laser pulses, laser-mediated dye loading (Tirlapur and König 1999) and intratissue nanosurgery of the cell wall in a living leaf of *Elodea densa* has also been successfully accomplished. Following treatment, the ultrastructural analysis of the corresponding area revealed a clean non-staggering cut across the cell wall that measured less than 350 nm. More interestingly, using the same **NIR** femtosecond laser pulses, a single plastid or a part of the organelle could be completely knocked out without affecting the adjacent organelles or the viability of the cell. The vitality of the cells after nanoprocessing has been ascertained by exclusion of propidium iodide from the cells as well as by the presence of cytoplasmic streaming (Tirlapur and König submitted).

Potential medical applications include the use of femtosecond laser microscopes in eye- and neurosurgery, tissue engineering, laser-assisted IVF, and gene therapy.

Universal **NIR** laser-based optical workstations

Currently available laser microscope systems utilize Nd:YAG lasers or laser diodes for optical trapping, nitrogen lasers for microsurgery, and titanium:sapphire lasers for multiphoton microscopy (Greulich 1999). In contrast to such an elaborate systems using varied kinds of lasers, **NIR** laser workstations in future can be based on a single laser that combines the possibility of optical trapping, non-contact cell transport, non-linear fluorescence imaging with high spatial and temporal resolution, and photochemical microscopy, as well as nanosurgery.

Conclusions

NIR laser microscopes can be used as versatile non-invasive biomedical tools for optical micromanipulation, diagnostics, photochemistry, and surgery. It is therefore conceivable that in the following years **NIR** microscopy has enormous potential to become a method of choice in biotechnology, cell biology, and medicine.

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