Cellular and Molecular Biology™ **45** (2), 195-201
Printed in France.

0145-5680/99
1999 Cell. mol. Biol.™

INTRACELLULAR NANOSURGERY WITH NEAR INFRARED FEMTOSECOND LASER PULSES

K. KÖNIG✍, I. RIEMANN, P. FISCHER and K.-J. HALBHUBER

✍ Institute of Anatomy II, Friedrich Schiller University Jena, Teichgraben 7, 07743 Jena, Germany
fax: +49 3641 938 552; e-mail: kkoe@mti-n.uni-jena.de

Received December 10, 1998; Accepted January 4, 1999

Abstract - We report on laser-assisted knocking out of genomic nanometer-sized regions within the nucleus of living cells. The intranuclear nanosurgery was possible by application of highly intense near infrared femtosecond laser pulses. The non-contact laser treatment was performed within a closed sterile cell chamber. The destructive multiphoton effect was based on 10^{12} W/cm^2 light intensities and limited to a sub-femtoliter focal volume of a high numerical aperture objective. We used the intracellular nanoscalpel for highly precise non-contact dissection of Hoechst-labelled chromosomes within a nucleus of a living Chinese hamster ovary cell. Following laser treatment, the cell remained alive and did not show any signs of membrane perturbation. The use of near infrared pulses provide the possibility of non-invasive intracellular nanoprocessing also within living tissue in depths of more than 100 microns.

Key words: Femtosecond microscopy, nanosurgery, multiphoton effect, living cell, chromosome, DNA

INTRODUCTION

The current major application of femtosecond (10^{-15} - 10^{-12} s) laser pulses in biotechnology and cell biology is non-destructive 3D multiphoton fluorescence microscopy (Denk *et al.*, 1990; Maiti *et al.*, 1997). Multiphoton fluorescence microscopy is based on non-linear fluorescence excitation by the simultaneous absorption of two or three near infrared (NIR) photons of an intense laser beam in a sub-femtoliter focal volume of a high numerical aperture objective. Each NIR photon provides half or one third, respectively, of the energy which is necessary to excite the fluorophore and to induce fluorescence. Multiphoton excitation requires light intensities in the range of MW/cm^2 - GW/cm^2 which are typically provided by diffraction-limited focusing of 100 - 200 fs radiation of an 80 MHz titanium:sapphire laser at 1-10 mW mean power at the sample. The multiphoton effect is limited to the tiny focal volume. There is no out-of-focus excitation and consequently no out-of-focus photobleaching and no out-of-focus photodamage due to the lack of efficient cellular absorbers in the 700 - 1200 nm spectral region. The combination of an ultrashort pulsed laser with a scanning microscope provides the possibility of non-destructive 3D fluorescence imaging of biomolecules.

However, we found that non-linear-induced destructive effects may occur in the sub-femtoliter excitation volume above certain laser power thresholds. For example, beam scanning (80 µs pixel dwell time) of Chinese hamster ovary (CHO) cells with 150 fs pulses at 760 nm led to impaired cell division in 50% of cells at a mean power of 3 mW. At 10 mW mean power, severe morphological damage occurred. Below 1-2 mW mean power, CHO cells remained unaffected by the laser even for 70 min. exposure times (König *et al.*, 1997, 1999).

The goal was to use these destructive multiphoton effects of near infrared femtosecond pulses and to develop a nanotechnology for highly localized and ultraprecise non-contact laser surgery within living cells, in particular for nanoprocessing of intranuclear structures.

MATERIALS AND METHODS

Cell line, cell culture and chemicals

Peripheral human blood was freshly drawn from a finger of a healthy donor and diluted in PBS with 0.5% bovine serum albumine (BSA). The sample was immediately dropped onto a poly-lysine coated premarked cover glass (18 x 18 mm^2) and subsequently rinsed with PBS. The coverslip containing the monolayer of adherent cells was than carefully transfered onto the microscope stage. Cells were initially visualized with bright field illumination and brought into focus. Specific marked cell areas were irradiated with defined laser power in the scan mode. After laser esposure, the samples were fixed with 4% glutaraldehyde in 0.5% PBS for 30 min. at room temperature and than processed for scanning electron microscopy (REM 260, Cambridge Intruments, at 15 kV).

Chinese hamster (*Cricetulus griseus*) ovary cells (CHO-K1, ATCC no. 61) were maintained in GIBCO's nutrient mixture (HAM F-12) supplied with 10% fetal bovine serum and L-glutamine. For laser exposure, cells were grown in sterile 2 ml cell chambers consisting of two 0.16 mm coverslips as chamber windows, a silicon gasket with a 2 cm opening as spacer, and metal frames. Easy medium change and injection of fluorophores and colcemide was performed with microneedles through the silicon layer. The injection of colcemide (20 µg in PBS, Seromed) resulted in detachment and rounding of CHO cells. For visualization of intranuclear chromosomes the live cell fluorophore Hoechst 30042 (3 µM, Molecular Probe) was introduced into the chamber. Viability was determined with a live/dead fluorescence kit (Molecular Probes) consisting of the green fluoresent live cell stain calcein (2 µM) and the red fluorescent dead cell indicator ethidium homodimer (Eth-D1, 4 µM). The cells were incubated with this viability kit 30 min. after laser surgery.

Giemsa banding

Human metaphase chromosomes extracted from cultured white cells were placed consecutively in 0.025% pepsin/10 mM HCl solution for 5 min. at 37°C, in phosphate-buffered saline (PBS) for 5 min., in 1% formaldehyde/PBS/50 mM MgCl solution for 10 min. at room temperature in humidified chamber, 70%, 95%, 100% ethanol for 5 min. each, PBS (pH 6.8) for 3 s and 10% Giemsa stain (Fisher, Pittburgh, PA) in PBS for 2 min. Slides were air-dried.

Laser microscope

The 780 nm beam of a mode-locked 76 MHz Titanium:Sapphire laser (Mira model 900-F, Coherent, Santa Clara, USA) was expanded by an 1:3 Galileian telescope and coupled to a modified inverted confocal laser scanning microscope (LSM410, Zeiss, Jena, Germany). A pair of galvanometer scanning mirrors were used to deflect the laser beam in x- and y- direction and to realize single-point beam exposure, line beam scanning, and area beam scanning. A high numerical aperture objective (Plan Neofluar 40x, N.A. 1.3 oil) was used to focus the beam to an aperture-diffraction limited submicron spot. The pulse duration at the objective back-focal-plane was measured with an autocorrelator (APE, Berlin, Germany) and found to be 170 fs. The mean laser power was measured in air after transmission through the objective with the power meter Fieldmaster FM (Coherent, Santa Clara, USA). At a mean power of 30 mW, the peak power and the peak intensities reaches values of 2.4 kW and 10^{12} W/cm^2, respectively. Two-photon excited fluorescence was measured with the microsope´s photomultiplier which was equipped with a 700 nm short pass filter to prevent the scattered laser radiation from reaching the detector.

RESULTS

With a view to employ intense femtosecond NIR laser pulses for the precise disruption of specific cellular domains we initially performed experiments with femtosecond laser pulses at 780 nm on the membrane of native human erythrocytes. Line beam scanning on the cell surface at 12 - 15 mW mean laser power led to significant destructive effects, in particular resulting in membrane alterations (furrow formation) and convolutions.

When the mean power was increased up to values of 30 mW and to 10^{12} W/cm^2 peak intensities, respectively, we were able to evoke highly localized multiphoton induced optical breakdown and plasma formation. The destructive effects accompanied with multiphoton-induced plasma enabled ultraprecise disruption of cellular areas. When using laser exposure along one line (line beam scan) we were able to perform significant incisions.

First clear incisions along the scanned line in erythrocytes are shown in fig. 1. The SEM image of such a cell revealed that the cut itself was nearly 200 nm wide. This severe membrane damage seems to be accompanied by the efflux of hemoglobin.

Subsequently, we also tested the efficacy of such laser nanosurgery on individual isolated human chromosomes. Fig. 2 demonstrates a cut through the Giemsa-labelled metaphase chromosome 1 with a resolution below 500 nm.

This nanodissection of chromosomes was also possible within living cells in metaphase. For which we used, round (d = 20 μm) colcemide-exposed CHO cells . These were maintained in special closed cell chambers consisting of a silicon spacer between two 160 μm thick glass windows. The laser beam was focused through the glass into the cell nucleus. In order to visualize the cut through nuclear nanostructures the DNA was labelled with Hoechst 30042. The DNA marker with a typical blue fluorescence was imaged by a two-photon excitation process using the same 780 nm pulsed laser beam but at 1 mW mean power. For nanosurgery, the scanning mode was stopped and the NIR beam was parked within the desired genomic region using the spot mode (single point exposure). Further, the mean power was increased up to 30 mW and the chromosome was exposed for 500 μs. As indicated in fig. 3 precise dissections of chromosome could be performed within the nucleus of the living CHO cell.

Three hours following laser surgery the morphology and the vitality of the cell was probed with the same 780 nm beam but at 1 mW mean power. Detection of possible membrane defects was performed in transmission mode whereas, the vitality was evaluated by monitoring the intracellular accumulation either of the live-cell fluorophore calcein or of the

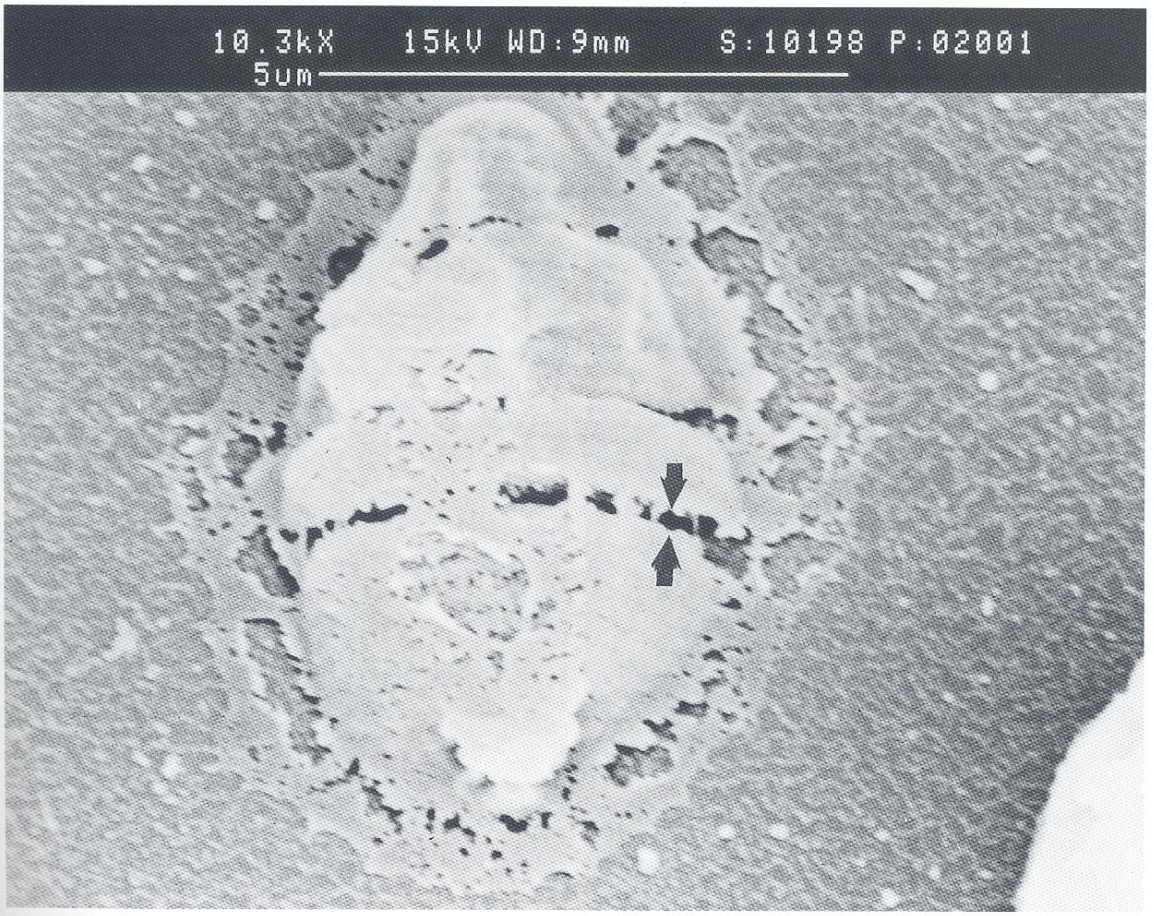


Fig. 1 *SEM image of an erythocyte exposed to 780 nm laser line scans showing incised regions.* A cut size of 200 nm was determined (arrow heads).

dead cell fluorophore ethidium homodimer. Fluorescence was induced by a two-photon excitation process. We found that nanosurgery within the nucleus was possible without membrane damage and loss of vitality.

When increasing the mean power of the femtosecond laser beam in the spot mode up to 50 mW destructive effects did not longer remain highly localized. In particular, the position of intranuclear chromosomes changed and the nuclear envelope as well as outer cell membrane ruptured. The cells were not longer able to exclude ethidium homodimer.

DISCUSSION

The study demonstrates femtosecond laser-assisted knocking out of genomic nanometer-sized regions within the nucleus of a living cell. In particular nanodissection of chromosomes was performed with femtosecond pulses in the NIR spectral range. Highly precise nanosurgery was possible within a certain optical window. The lower threshold was determined by the onset of intracellular optical breakdown and plasma formation within a sub-femtoliter focus volume. Optical breakdown requires intensities exceeding 10^{11} W/cm^2 in biological and other materials and about 10^{14} W/cm^2 in air. The high light intensities induce enormous >10 MV/cm electric field strengths which cause highly localized multi-photon ionization of the molecules and atoms (plasma) spatially confined to the focal volume (Niemz, 1996; Stern *et al.*, 1989; Teng *et al.*, 1987). By means of diffraction-limited focusing and plasma-induced ablation, highly precise nanosurgery can be performed without apparent thermal or mechanical damage to surrounding intracellular areas.

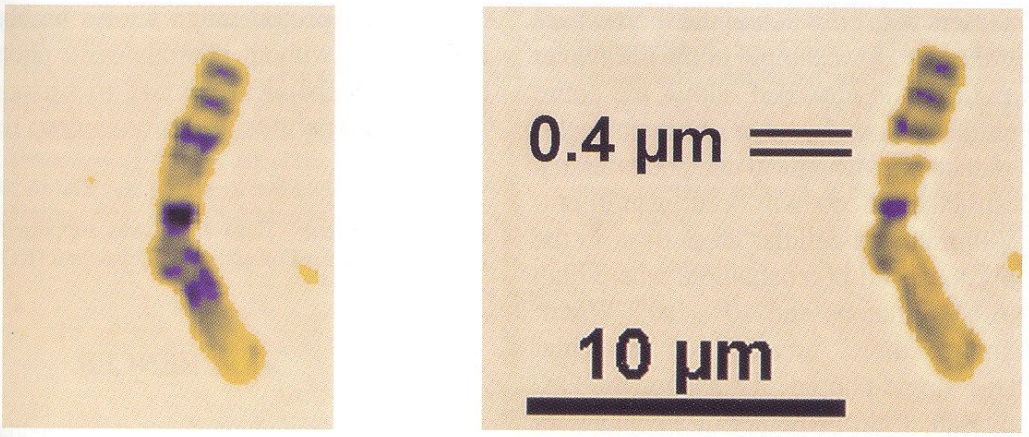
Below the optical breakdown threshold, non-linear fluorescence imaging based on non-resonant two-photon fluorophore excitation can be performed as demonstrated with the fluorophores Hoechst, calcein and ethidium homodimer.

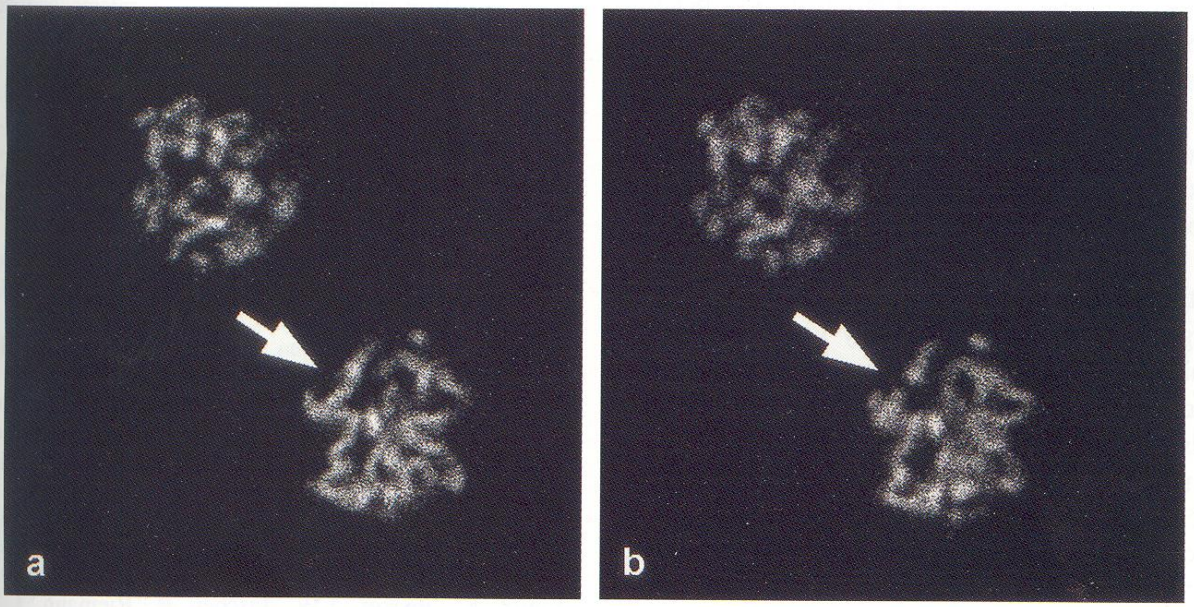
The upper intensity threshold for highly localized nanosurgery was determined by the onset of out-of-focus destructive effects. With increased laser intensities, secondary destructive effects of optical breakdown occur and may expand the range of destruction to a micron scale. The process is called photodisruption. Photodisruption is based on the formation of strong mechanical forces by vapor-induced mechanical stress ("cavitation") and shock wave generation. Mechanical stress waves propagate to adjacent out-of-focus regions. As seen in our 50 mW experiments the optically induced mechanical forces caused displacements of surrounding chromosomes and extranuclear structures, membrane ruptures and immediate cell death.

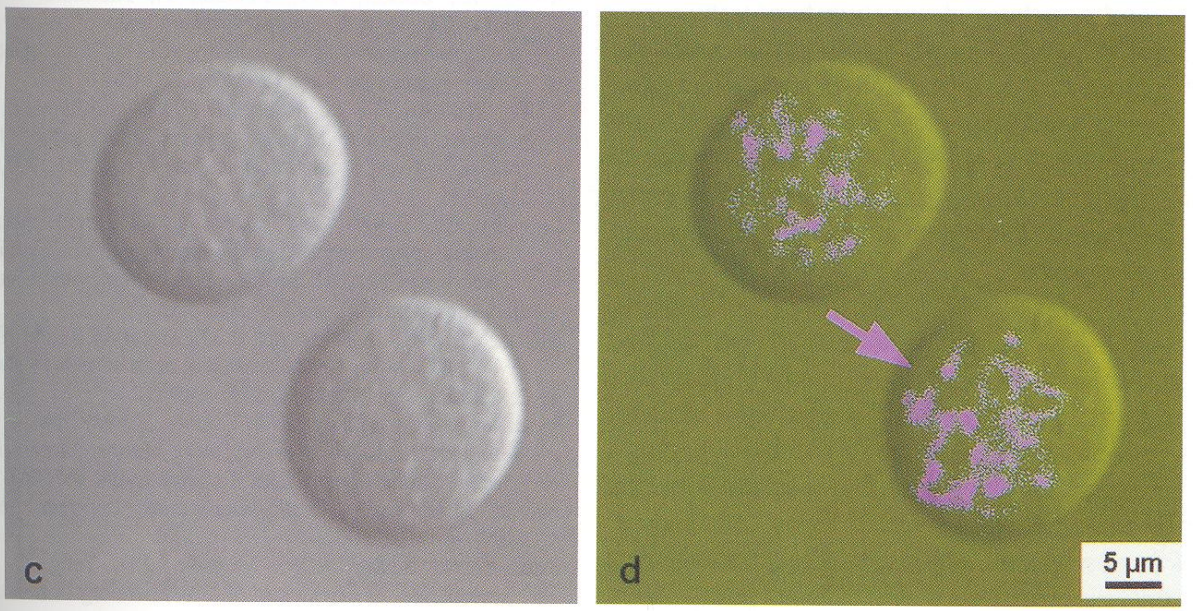
Dissection of chromosomes has been performed earlier with mechanical and optical tools, for example with glass needles (Fisher *et al.*, 1985; Lüdecke *et al.*, 1989; Scalenghe *et al.*, 1981). Also nanoprocessing of single macromolecules with the tip of an atomic force microscope has been studied (Rees *et al.*, 1993). Disadvantages are for example the limitation to the surface and fixation or other severe damage to the sample. Also laser surgery, such as selective destruction of cellular organelles, has been carried out earlier (Amy and Storb, 1965; Berns *et al.*, 1969, 1981; Endlich *et al.*, 1994; Monajembashi *et al.*, 1986). However, these studies

page 199 (top)
Fig. 2 *Nanodissection of a Giemsa labelled human chromosome 1 with 170 fs laser pulses* (780 nm) *at 30 mW mean power.* Line scan time was 700 µs. The absence of the Giemsa bands in the lower part of the chromosome is likely to be due to slight displacement following laser exposure.

(middle and down)
Fig. 3 *Intranuclear nanosurgery of a chromosome within a living Hoechst-labelled CHO cell in metaphase.* **a**) Two-photon excited Hoechst fluorescence imaging before nanosurgery; **b**) after chromosome dissection; **c**) NIR transmission image 3 hrs. after nanosurgery; **d**) false color overlay of b and c.


0.4 µm
10 µm


a
b


c
d
5 µm

are based on lasers with emission in the short-wavelength spectral range, for example in the ultraviolet (UV). UV radiation is absorbed within the entire exposure volume by a wide range of biomolecules which may induce photochemical and photothermal damage. Destructive effects will occur also in out-of-focus UV-illuminated cellular areas even in the case of highly-localized photoablation, where high energy (>3.6 eV) photons and 10^7 - 10^{10} W/cm^2 intensities induce direct breaking of molecular bonds such as single-photon dissociation of C-C bonds (Srinivasan, 1986). Another disadvantage of former intracellular laser surgery is the low penetration depth of the laser radiation. In contrast, IR radiation in the 700 nm - 1200 nm spectral range is not absorbed in out-of-focus regions and has a high penetration depth which results in the advantage of possible nanosurgery within thick samples, even in depths of more than 100 µm.

A third disadvantage of former intracellular laser surgery is the use of relatively long laser pulses, typically in the nanosecond range. In spite of diffraction-limited focusing, application of these pulses result always in thermal and mechanical damage to surrounding areas. Material removal occurs during the light-matter interaction and the laser-induced plasma is able to absorb and scatter further incident photons of the same laser pulse. This results in huge destructive temperature gradients in the micron range due to plasma shielding and plasma heating effects. In contrast, the pulse duration in femtosecond laser surgery is too short for significant heat transfer and precise cutting of nanostructures without micrometer-sized thermal and mechanical damage to surrounding biomaterial becomes possible.

In conclusion, we demonstrate that near infrared femtosecond laser microscopes can be employed as novel sterile, non-contact and non-invasive tools for nanoprocessing in cell biology and biotechnology. In particular, highly precise intranuclear knocking out of genomic regions within living cells and tissues is possible. In contrast to known mechanical and optical technologies, femtosecond laser surgery in the NIR spectral range allows highly localized nanosurgery without photothermal, photomechanical or photochemical damage to surrounding biomaterial and permits operation in deep tissue.

Acknowledgment – This study was supported by a Grant from the Deutsche Forschungsgemeinschaft (KO 1361/10-1).

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