Two-photon near infrared excitation in living cells

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Non-linear effects due to two-photon near infrared (NIR) excitation of endogenous and exogenous cellular chromophores allow novel techniques in tissue, cell and biomolecule diagnostics, as well as in intracellular micromanipulation (e.g. intracellular photochemistry). Two-photon NIR excitation may also result in cell damage effects. The high photon intensities (10^{24} photons cm^{-2} s^{-1}) required for non-resonant two-photon excitation can be achieved by diffraction-limited focusing of continuous wave (cw) laser beams (cw microbeams) with powers in the mW range. For example, NIR traps ("laser tweezers") used as force transducers and micromanipulation tools in cellular and molecular biology are sources of two-photon excitation. NIR traps can induce two-photon excited visible fluorescence and, in the case of <800 nm-traps, UVA-like cell damage. Multimode cw microbeams may enhance non-linear effects due to longitudinal mode-beating. To perform high scan rate two-photon fluorescence imaging, the application of ultrashort laser pulses of moderate peak power but low average power (pulsed microbeams) is required. In NIR femtosecond microscopes, non-destructive imaging of two-photon excited fluorophores in various human and culture cells was demonstrated for <2 mW average powers, <200 mW peak powers and 400 GW cm^{-2} intensities (700–800 nm, ~150 fs, ~100 MHz). However, higher average power levels may result in failed cell reproduction and cell death due to intracellular optical breakdown. In addition, destructive transient local heating and μN force generation may occur.

Keywords: two-photon excitation, optical trap, femtosecond, microbeam, living cell, non-linear effects, laser microscopy, fluorescence imaging, fluorescence spectroscopy, NIR.

Principle of two-photon excitation

Multiphoton excitation of electronic states, based on the simultaneous absorption of photons (in the case of two-photon excitation: two photons), was predicted by Mrs Göppert-Meyer in 1931 and first realised with the availability of lasers by Kaiser and Garrett in 1961.[1,2] They detected the blue fluorescence of $CaF_2 : Eu^{2+}$ crystals with red light excitation of a 500 μs ruby laser.[2] Later on, nano- and picosecond laser sources were used for multiphoton experiments. In the eighties, two-photon excited fluorescence of non-organic materials induced by continuous wave (cw) dye laser beams has been achieved,[3,4] In 1990 Denk, Strickler and Webb reported on non-linear fluorescence scanning microscopy of living cells using a 100 fs near infrared (NIR) dye laser microbeam.[5] Today's two-photon microscopes are based on the application of tunable (670–1000 nm) femtosecond Ti : sapphire lasers with ~100 MHz repetition frequency.

Two-photon excitation requires the simultaneous absorption of two photons either of the same or of different energy (Figure 1). A real intermediate electronic state is not required (non-resonant excitation). The fluorescence intensity depends on the product of the excitation intensities in the case of

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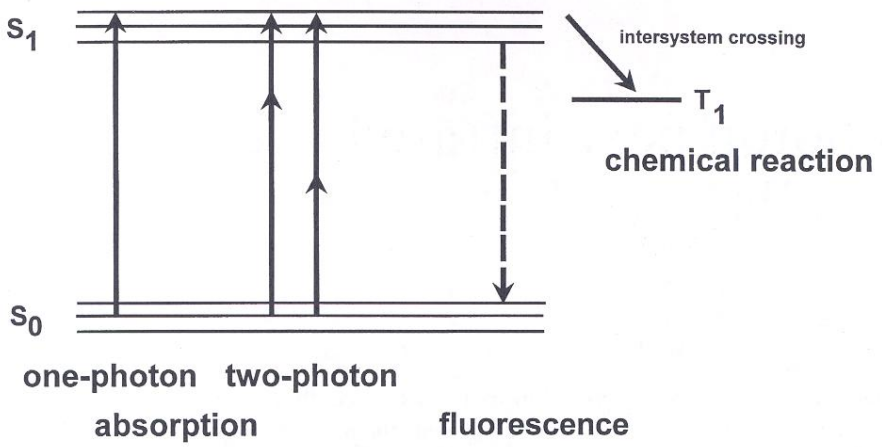

Figure 1. Principle of non-resonant two-photon excitation. Electronic transitions of intracellular chromophores, which are normally excited by UV and blue/green photons of energy E in conventional one-photon microscopy, can be excited by simultaneous absorption of two NIR microbeam photons of energy ~E/2 or by photons of different energy E_1 and E_2 ($= E - E_1$). Therefore, NIR photons can induce visible fluorescence and photochemical reactions.

two beams. In the case of single-beam excitation, the fluorescence intensity shows a squared dependence on excitation intensity. Two-photon microscopes for vital cell studies are based on a single NIR microbeam (photons of same energy) due to the lack of efficient endogenous cellular one-photon absorbers in this spectral region. Using appropriate powers, the high concentration of photons in space and time required for intracellular non-resonant two-photon excitation is limited to the sub-femtolitre focal volume of a high numerical aperture microscope objective. Therefore, two-photon scanning microscopy includes inherent three-dimensional resolution and acts as a potent alternative to conventional confocal laser scanning microscopy, where the excitation volume is defined by the intracellular illumination cone and the detection volume by the pinhole size. In two-photon NIR fluorescence microscopes, photobleaching and photodamage is limited to the focal volume. There is no out-of-focus fluorescence. Due to the relatively low typical molecular two-photon absorption cross-sections of about 10^{-48}–10^{-50} cm^4 s,[6,7] non-resonant two-photon excitation requires photon flux densities $>10^{24}$ photons cm^{-2} s^{-1}. Two-photon NIR excitation in the focal volume can be used to induce intracellular visible fluorescence for cell and biomolecule analysis as well as to induce photochemical reactions such as controlled intracellular release of UV-labile "caged" compounds or destructive photooxidations by non-linear excitation of endogenous cellular absorbers (oxidative stress).

Two-photon excitation in living cells by cw NIR microbeams

The most important application of cw NIR microbeams in cellular and molecular biology are single-beam gradient force optical traps, also called "laser tweezers".[8,9] Optical traps have been used to determine cellular forces in the pN range, see References 10–12, or for cellular and intracellular micromanipulation see References 13–15. For example, 1064 nm-traps have been used clinically in laser-assisted *in vitro* fertilisation (IVF) where—in the case of male infertility—single powerless sperm cells are optically caught and transported to a human oocyte.[16] Meanwhile, some laser tweezer producers recommend the use of tweezers <800 nm for vital cell micromanipulation due to the lower absorption coefficient of water compared to 1064 nm.[17] They do not consider possible destructive two-photon effects. NIR traps had been considered as safe micromanipulation tools.

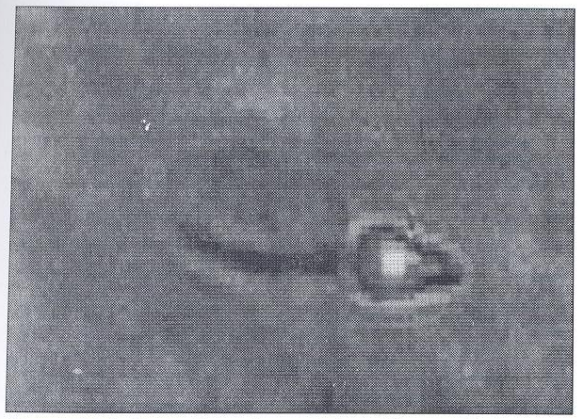

Figure 2(a). Detection of trap-induced intracellular two-photon excited fluorescence. Imaging of the 760 nm excited sub-micrometre fluorescence spot of intracellular SYBR™14.

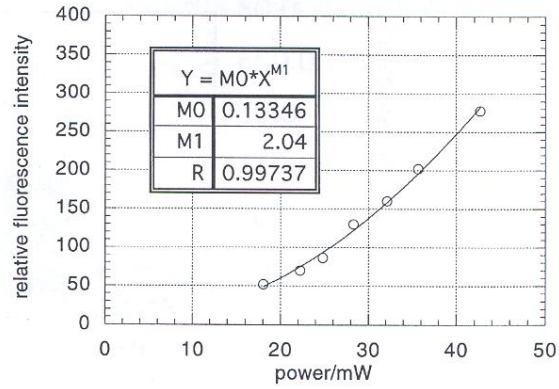

Figure 2(b). Detection of trap-induced intracellular two-photon excited fluorescence. Rhodamine 123 fluorescence intensity vs NIR laser power (intensity).

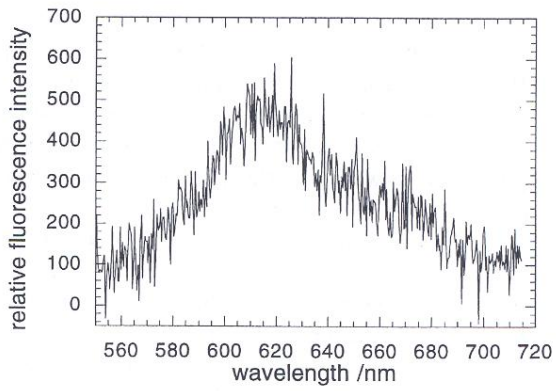

Figure 2(c). Detection of trap-induced intracellular two-photon excited fluorescence. 1064 nm-excited propidium iodide spectrum of a damaged sperm cell.

We found that optical traps are sources of two-photon excitation and demonstrated for the first time two-photon excited fluorescence in living cells by cw beams.[18] For example, the intensity and the photon flux density in a diffraction-limited 100 mW NIR cw microbeam (sub-micrometre spot size) are 20–40 MW cm^{-2} and 10^{26} photons cm^{-2} s^{-1}, respectively. This is sufficient to induce a fluorescence photon rate of ~10^5–10^7 photons s^{-1}, assuming ~600 molecules (10 μM) of a highly fluorescent dye in the typical 0.1 fL two-photon excitation volume. We were able to detect intracellular fluorescent viability indicators (SYBR™14: green-fluorescent live cell indicator, propidium iodide: red-fluorescent dead cell indicator, Molecular Probes) in single trapped spermatozoa by two-photon excited fluorescence using the NIR trapping beam of a cw Ti : sapphire laser / Nd : YAG laser as excitation source. Fluorescence imaging by slow-scan cooled CCD cameras revealed a trap-induced green (~0.5 μm) spot in the head region of motile sperm cells [Figure 2(a)]. The intracellular spot position changed with transient motility force fluctuations. Short interactions of trapping beam with the cell midpiece were possible in some highly motile cells. Cells remained alive in 800 nm-traps or 1064 nm-traps during a 10 min trapping period. In contrast, the intracellular fluorescence spot changed from green into red for <800 nm traps (70 mW), indicating trap-induced cell death. Trap-induced visible fluorescence was detected within a wide range of intracellular fluorophores or fluorophore solutions, such as the temperature indicator Laurdan, the pH indicator SNARF, the mitochondrial marker Rhodamine 123, the viability fluorophores SYBR™14 and propidium iodide, and the reduced coenzyme NADH. The fluorescence intensities showed the typical squared dependence on NIR trapping power [Figure 2(b)]. Intracellular trap-induced fluorescence was also detectable by spectroscopy [Figure 2(c)]. This demonstrates clearly that laser tweezers may act as

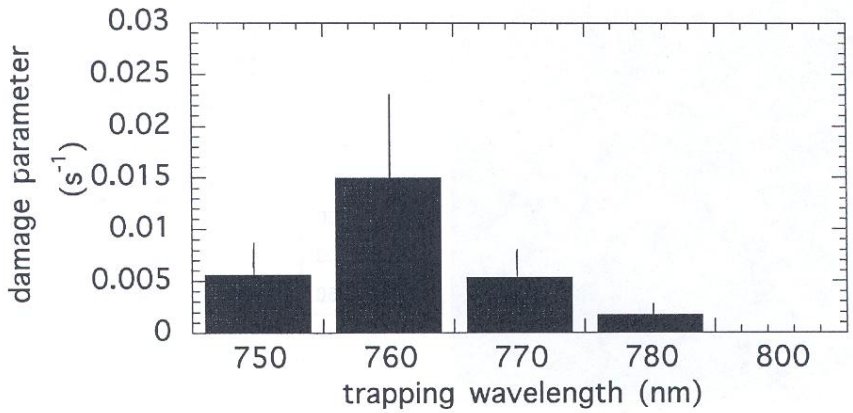

Figure 3. Action spectra of trap-induced cell death (105 mW, human spermatazoa). The damage parameter represents the reciprocal time value of onset of intracellular propidium iodide fluorescence.

micromanipulation tools and as novel non-linear diagnostic tools. They provide information on intracellular trap position, on the influence of the trap itself on cellular metabolism/viability and of external influences. Laser tweezers allow sensitive fluorescence measurements of highly motile cells, such as spermatozoa, without external fluorescence excitation sources.

On the other hand, short-wavelength NIR traps may induce cell damage (e.g. paralysis and finally cell death of trapped spermatozoa). An action spectrum of trap-induced spermatozoa damage is seen in Figure 3, indicating the most destructing effect in 760 nm traps. However, the "760 nm-effect" has no biological origin. It arises from the special wavelength-dependent output of the cw multimode Ti : sapphire ring laser (model 899-01, Coherent).[19] Beating of longitudinal modes results in the formation of unstable ps pulses. Interestingly, these pulses showed the highest peak power at exactly 760 nm based on partial mode locking. Because of the squared dependence of two-photon efficiency on power, these transient 760 nm pulses, with instantaneous high peak powers, are responsible for enhanced destruction based on amplified two-photon absorption. Transformation of the multimode cw laser into a "true" single-frequency laser (constant amplitude, non-fluctuating output, instantaneous power = average trapping power) by introduction of a 20 MHz etalon reduced the destructive effect significantly (mean sperm death after 406 ± 160 s, compared to 65 ± 20 s in the multimode laser at 70 mW average trapping power). However, lethal damage could not be avoided. Laser tweezers with <800 nm trapping wavelength are able to excite endogenous absorbers non-linearly with UVA electronic transitions, such as NADH, flavins, porphyrins or cytochromes, which may result in destructive oxidative stress.[20–22] We recommend the use of >800 nm, single frequency traps for optical micromanipulation of living cells.[23] Up to now, most trapping studies have been conducted with multimode cw lasers. Interestingly, the damaging effect (failed cellular reproduction) of 760 nm microbeams on adherent cells has been reported earlier.[24,25] The experiments had been performed with exactly the same cw multimode Ti : sapphire laser as we used in the trapping studies. There is, therefore, a high probability that the reported action spectra (spectral dependence of cell damage) reflect mainly the wavelength-dependent fluctuations in the laser output. In order to obtain true action spectra of biological response to NIR microbeams single-frequency cw beams have to be used.

Two-photon excitation in living cells by femtosecond microbeams

In principle, cw NIR microbeams, in combination with a scanning unit, allow non-linear fluores-

cence imaging. This was demonstrated by Hänninen *et al.* using a multimode Ti : sapphire laser in the cw mode.[26] They imaged the 730 nm excited fluorophores DAPI and rhodamine in a single dead sperm cell. The acquisition time was 12 min (5 mW average cw power). In order to obtain appropriate frame rates of the order of 1 Hz (i.e. microsecond pixel dwell time) higher power levels should be used. However, thermal damage has to be avoided. Today's two-photon scanning microscopes are based on the application of ultrashort laser pulses with peak powers in the W and kW range but low average mW power (high repetition pulse frequency). Most two-photon studies on living cells have been performed with femtosecond scanning microscopes. For example, two-photon three-dimensional fluorescence imaging,[27,28] intracellular release of photolabile "caged" compounds[29,30] and two-photon excited fluorescence lifetime imaging,[31] in single cells or tissues, based on the combination of scanning microscopes with femtosecond Ti : sapphire lasers, have been reported. Pinhole-free non-linear four-dimensional fluorescence microscopy (time and space) is possible by utilisation of the temporal resolution as well as the inherent spatial resolution.

We expanded the capabilities of a conventional Zeiss confocal laser scanning microscope based on an Ar^+ laser and He–Ne microbeams to two-photon NIR microscopy by additional application of a femtosecond 76 MHz Ti : sapphire laser (Mira 900-F, Coherent).[31] Short-pass 685 nm filters were adjusted in front of the two PMT fluorescence detectors to avoid detection of scattered NIR excitation light. Switching from one-photon visible microscopy to two-photon NIR microscopy was possible within 1 s just by moving a high precision mirror. Two-photon excited fluorescence images were obtained with (sub-micrometre axial resolution) or without (~1.1 axial resolution at 780 nm) pinholes. Figures 4(a)–(c) demonstrate pinhole-free 780 nm excited fluorescence images (frame rate: 1 s^{-1}) of Rhodamine 123 stained Chinese hamster ovar (CHO) cells, DAPI-stained chromosomes and of a macrophage during endocytosis of fluorescent 2.8 μm microbeads (Fluoresbrite carboxy YG beads with 458 nm one-photon excitation maximum, unknown molecular two-photon absorption cross-section, Polysciences). The beads were detectable with an average NIR power as low as 200 μW (150 fs).

Mean powers, <2 mW (730–800 nm, 150 fs) and peak powers <200 W, as well as intensities <400 GW cm^{-2}, respectively, did not induce changes in morphology and viability of erythrocytes nor changes in the reproduction behaviour and viability of CHO cells. In that power range, two-photon NIR femtosecond microscopes act as useful novel tools in non-invasive cell analysis.

However, average powers >2 mW resulted in discocytic–echinocytic shape transformation and lysis of erythrocytes.[32] Switching the fs laser into the cw mode (same average power, same exposure time in the range of seconds, 780 nm) avoided cell damage. Step-wise increase of the cw power revealed haemolysis >60 mW within 2 min. Therefore, the damage process depends on the presence of ultrashort pulses. CHO cells started uncontrolled cell growth and became giant cells They failed cell division, died as shrinked cells or exploded (cell fragmentation).[33] Mechanical damage was accompanied by the onset of intense ultrashort (we measured mean lifetimes <500 ps) luminescence with non-exponential decay in the mitochondrial region. It was possible to watch this luminescence as "white flashes" (whole visible spectrum) through the microscope ocular by eye. The damage in CHO cells showed a dependence on power (intensity) and not on fluence. The damage can be explained by intracellular optical breakdown due to GW cm^{-2} intensities (e.g. 5 mW average power corresponds to ~300 W peak power, 0.3 μm spot size). The "white" luminescence reflects intracellular plasma formation.

Conclusions

Pulsed and cw microbeams are able to induce two-photon effects. In the case of NIR microbeams, non-resonant two-photon absorption of endogenous and exogenous cellular chromophores can be used to induce visible fluorescence or to induce photochemical reactions (such as oxidative stress and release of UV-labile "caged" compounds) in a sub-femtolitre focal volume of high numerical aperture objectives. Therefore, optical traps may act as force transducers, micromanipulation tools and, even si-

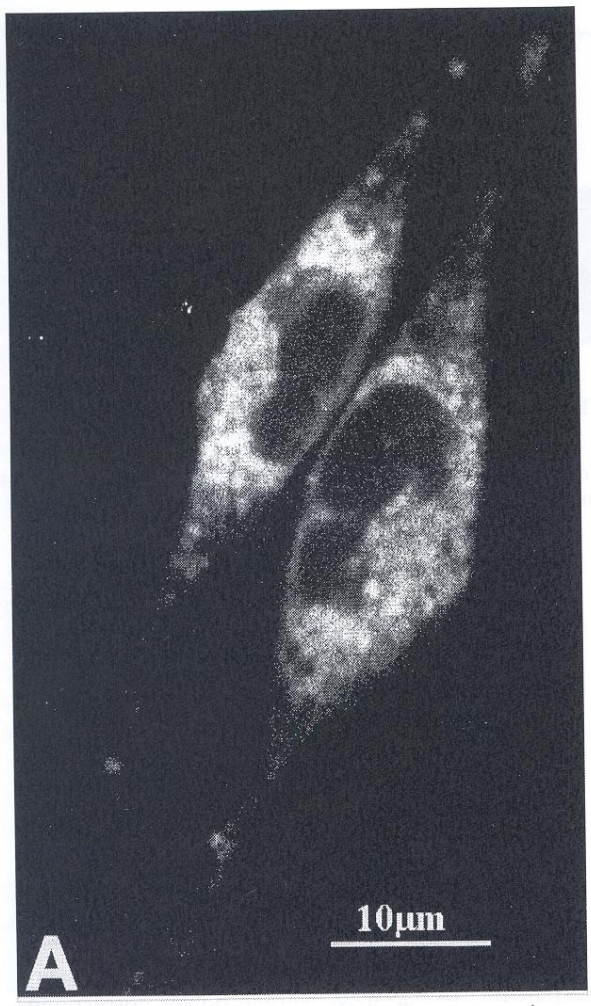

Figure 4(a). Femtosecond two-photon fluorescence imaging at 780 nm. Intracellular Rhodamine 123 in two Chinese hamster ovary cells.

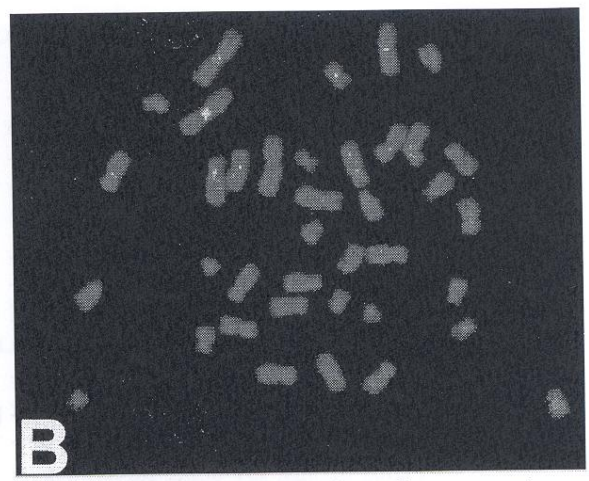

Figure 4(b). Femtosecond two-photon fluorescence imaging at 780 nm. DAPI-labelled human chromosomes.

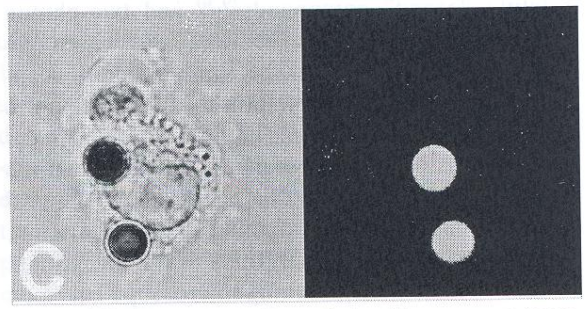

Figure 4(c). Femtosecond two-photon fluorescence imaging at 780 nm. Macrophage during phagocytosis of fluorescent microbeads.

multaneously, as novel non-linear diagnostic tools. Attention should be paid in the case of <800 nm traps, in particular when used in laser-assisted *in vitro* fertilisation, due to possible destructive excitation of cellular one-photon UV absorbers.

Appropriate sources for non-linear fluorescence imaging with high frame rates (0.1–10 Hz) are high repetition NIR femtosecond lasers. Non-destructive non-linear excitation by femtosecond microbeams is possible within a small laser intensity window: the lower limit is given by the molecular two-photon absorption cross-section and the detector sensitivity, the upper limit by the onset of intracellular optical breakdown. Within that window, two-photon NIR microscopy may be used to induce fluorescence of a variety of intracellular chromophores with one-photon UVA excitation maxima (e.g. DAPI, fura-2, indo-1, NADH, NADPH) without cell damage. Advantages of non-linear microscopy, with its sub-femtolitre excitation volume compared to one-photon UV microscopy with its large excitation volume, are, in particular, given in the case of three-dimensional imaging due to reduced photo-stress and reduced photobleaching.

Three-photon NIR microscopy, introduced recently to excite UV-emitting proteins,[34] requires higher peak powers than two-photon microscopy. The chance for non-destructive imaging is, therefore, lower. Shortening of femtosecond pulses

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