Two-photon excited cellular autofluorescence induced by cw and femtosecond NIR microradiation

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1. ABSTRACT

We report the first two-photon excited autofluorescence measurements in single cells induced by continuous wave NIR laser microbeams. In particular we demonstrate NIR-excited NAD(P)H fluorescence of motile spermatozoa in a single-beam gradient force optical trap ("optical tweezers").

In addition, two-photon NIR excited autofluorescence imaging and "τ–mapping" of CHO cells was performed with laser scanning microradiation of a tunable femtosecond-Ti:sapphire laser. Cellular response on autofluorescence to two-photon absorption of NIR radiation was compared with exposure to UVA light (one-photon absorption).

Keywords: optical trapping, NIR, microbeam, NADH, laser scanning microscopy, two-photon excited fluorescence, femtosecond laser pulses, lifetime imaging

2. INTRODUCTION

Recently, we reported on nonlinear effects of continuous wave (cw) near infrared (NIR) laser microbeams. In particular, we found that NIR trapping beams are able to induce cell damage via a two-photon process[1]. Microbeams can create an enormous intracellular photon flux. The intensity and photon flux in a diffraction-limited cw 100 mW trapping beam at 760 nm (NA= 1.3) is ≈40 MW/cm^2 and ≈10^{27} photons cm^{-2} s^{-1}, respectively. Because typical molecular cross sections are 10^{48}-10^{50} cm^{4}s (refs.2, 3), the photon flux of the 100 mW microbeam is sufficient to induce two-photon effects. Therefore, optical traps are sources of nonlinear excitation resulting in visible fluorescence and possible cell damage via oxidative stress.

However, higher photon fluxes are necessary for two-photon fluorescence imaging with a reasonable frame rate. An increase in cw power would induce thermal damage (mean intracellular temperature increase: 2K/100 mW for 1064 nm microbeams[4]). In contrast, efficient multiphoton-excitation can be achieved by application of pulsed laser microbeams of moderate peak power. Appropriate laser sources are mode-locked, tunable Ti:sapphire lasers (700 - 1000 nm) which provide pulses of about 150 fs with high repetition frequency of 80 MHz. In addition to fluorescence intensity measurements, the application of ultrashort excitation pulses allows fluorescence lifetime measurements. As demonstrated by Piston et al.[5] and So et al.[6], time-resolved fluorescence imaging (τ–mapping) in the frequency domain can be performed in microscopes using two-photon excitation by the heterodyning technique in combination with microbeam scanning.

 0-8194-1992-3/96/$6.00

This paper focuses on two-photon excited cellular autofluorescence induced by cw NIR microbeams (optically trapped spermatozoa) as well as by femtosecond NIR laser pulses (CHO cells). In particular, the UVA transition (≈340 nm) of the endogenous fluorophores β-nicotinamide adenine dinucleotide (NADH) and β-nicotinamide adenine dinucleotide phosphate (NADPH) was excited via simultaneous absorption of two photons in the spectral range 730 nm - 800 nm. The reduced pyridine coenzymes act as sensitive bioindicators of metabolic function[7-9]. Free NAD(P)H fluoresces at ≈460 nm (mean lifetime of ≈400 ps), whereas bound NAD(P)H exhibits a blue-shifted fluorescence maximum at ≈440 nm ($\tau \approx 2$ ns)[9]. The oxidized forms NAD(P) show no significant visible fluorescence.

3. MATERIALS AND METHODS

Cells

Chinese hamster ovary cells (CHO, ATCC no. 61) were maintained in GIBCO's minimum essential medium (MEM, 10% fetal bovine serum). Cells were grown in modified Rose cell culture chambers. Semen specimens were obtained from three donors with normal semen parameters according to the World Health Organization guidelines. Semen was diluted in HEPES buffered isotonic saline solution containing 1% human serum albumin. Spermatozoa were injected into similar microchambers.

Experimental set-up for cw studies

The NIR radiation of an Ar^+-ion laser-pumped tunable Ti:Sapphire ring laser (Coherent, 899-01) was introduced into a modified inverted confocal laser scanning microscope (CLSM, Axiovert 135M, Zeiss). The parallel beam was expanded to fill the back aperture of a 100x Zeiss Neofluar brightfield objective (NA=1.3). The microscope allowed trapping of single cells and simultaneous fluorescence imaging. Fluorescence was detected with a slow-scan, cooled CCD camera (TE576/SET135, Princeton Instruments). According to previously measured spot sizes using the knife-edge technique[11], we assumed a diffraction-limited NIR spot diameter in our microscope of $d = \lambda/1.3$.
Fluorescence spectra were obtained using another experimental set-up with a 1064 nm trapping beam and a polychromator combined with a cooled optical multichannel analyzer for spectra recording. Each spectrum was acquired in 5 s. This set-up is described in detail in refs.(4, 12).

Two-photon laser scanning microscope

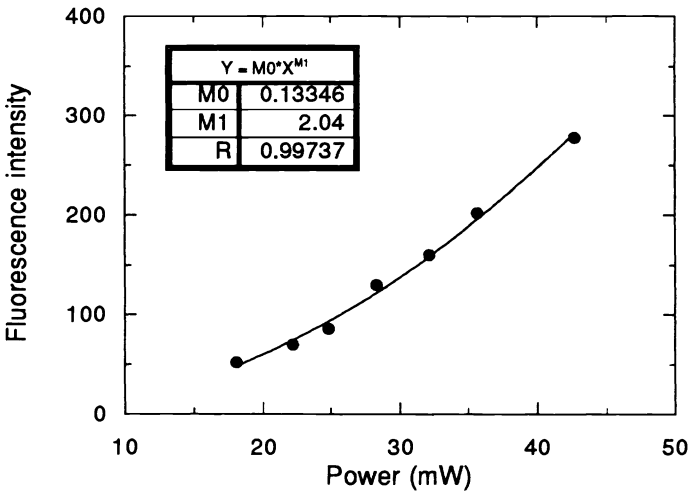
The NIR fluorescence excitation pulses (pulse width: ≈150 fs) for the two-photon laser scanning microscope (Axiovert 35, Zeiss, modified) were provided by a passively mode-locked Ti:Sapphire laser (Mira 900, Coherent) operating at 80 MHz repetition frequency. The attenuated and expanded excitation beam was directed by 1 kHz x,y-scanner-mirrors and focused to a diffraction-limited spot by a 63x Plan-Neofluar objective (NA: 1.25). Autofluorescence was collected by the same objective, transmitted through a dichroic mirror FT 650 and re-focused on a modified, 80.025 MHz modulated R928 PMT (Hamamatsu) equipped with SP 550 filters. POPOP with a decay time of 1.3 ns served as reference in frequency-domain heterodyne measurements. The spatial resolution was determined to be 0.3 μm radial and 0.5 μm axial, the temporal resolution was 500 ps. The mean laser power was measured after the objective near the sample plane (Newport power meter, model 1825-C). The two-photon laser scanning microscope including the imaging processing software is described in detail in ref. 6. We used in all experiments a frame rate of 10 s. The pixel dwell time was 80 μs, the read-out time between two consecutive frames 6 s. Fluorescence was detected in a field of 35 x 35 μm^2 (63x, 256 x 256 pixels). A

peak power of 0.8 kW was estimated for 10 mW mean power (150 fs pulse width at the sample assumed, 80 MHz repetition frequency). We determined experimentally a FWHM beam size at the focal spot of 0.25 μm (730 nm, for experiment details see ref. 10). The intensity at the sample of a 0.8 kW pulse (10 mW) can therefore estimated to be 0.8 kW/(π x 0.125 μm)^2 ≈ 1.6 TW/cm^2 (1.6x10^{12} W/cm^2). The radiant exposure for each scanning point at the sample (0.14 x 0.14 μm^2) during one scan is therefore 0.13 GJ/cm^2.
One-photon UVA radiation (1.5 mW after the 100x objective, wide field illumination, 5 W/cm^2) was provided by a 50 W high pressure mercury lamp (Oriel) equipped with a 365±25 nm bandpass and a heat protection filter.

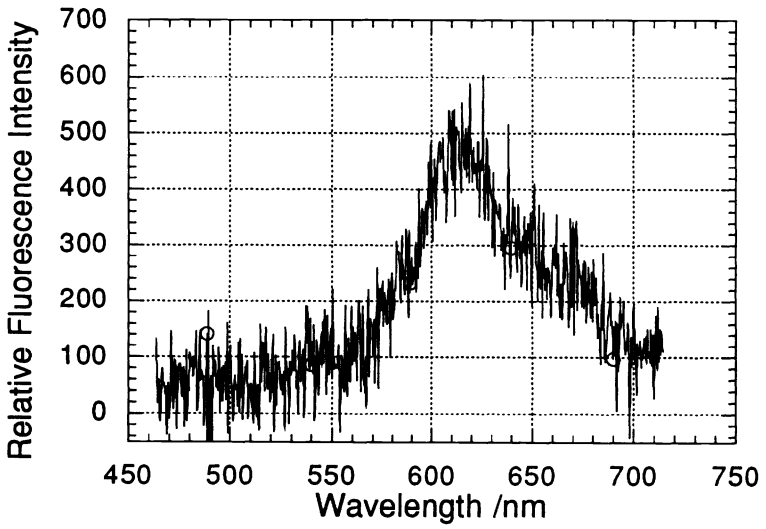
4. RESULTS

CW-induced Nonlinear Effects

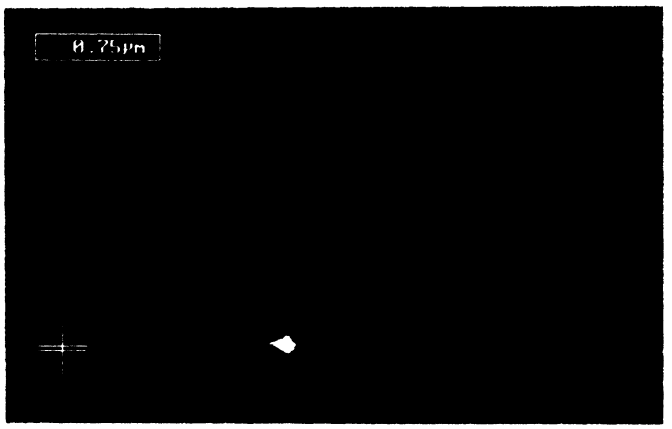
Highly focused cw NIR beams are able to induce two-photon excited visible fluorescence. Fig. 1 demonstrates clearly the squared dependence of NIR-induced Rhodamine fluorescence intensity on microbeam power indicative for two-photon excitation. The visible fluorescence of various fluorophores such as Rhodamine 123, Acridine Orange, and Propidium Iodide (PI) was detected either spatially resolved (fluorescence imaging) or spectrally-resolved (fluorescence spectrum), Fig. 2. Another evidence for cw-induced two-photon excitation was given by photochemical reaction. In this case, we used UV sensitive photochromic detection layers. These transparent layers contain photosensitive spiropizane compounds which undergo reversible heterolytic bond breakage during UV exposure resulting in fluorescent photoproducts with absorption bands in the blue, and lifetimes of several minutes[13]. Using the motorized scanning stage (3 scans in x- and y-direction), we were able to create a transient fluorescent cross. A lateral and axial linewidth of 0.75 μm and >1 μm, respectively, was determined by confocal fluorescence imaging (λ_{exc} = 488 nm, λ_f > 510 nm), Fig. 3.
Microbeams at 760 nm and 800 nm were used to trap motile human spermatozoa. The power was chosen to be 105 mW at the sample. In a first experiment, we labeled sperm cells with a Live/Dead kit containing the live-cell stain SYBR^{TM}14 with a 515 nm fluorescence maximum and the dead-cell stain PI emitting in the red. Without any further light exposure as the trapping beam itself, cells exhibited luminescence in a submicron-sized region in the sperm head (Fig. 3). No such emission was found in non-trapped cells. The emission was more intense in 760 nm traps than in 800 nm traps. It was even possible to recognize the NIR-excited fluorescence spot with the naked eye (eyepieces). In the case of 760 nm traps, the emission spot changed color from green to red in correlation with loss of vitality after about 1 min trapping. 800 nm traps did not induce color changes up to 10 min trapping.
A very weak fluorescence signal was found in optically-trapped unlabeled sperm cells, Fig. 4. However, in the case of 760 nm traps we found a significant autofluorescence increase of nearly two orders with trapping time. At radiant exposures of about 10 GJ/cm^2, the blue/green autofluorescent became clearly visible (eyepieces). No autofluorescence modifications were found in the case of 800 nm-trapped cells.



1. Intensity of Rhodamine 123 fluorescence vs. power of the cw laser microbeam (760 nm). The squared dependence indicates a two-photon excitation process



2. Two-photon excited Propidium Iodide fluorescence spectrum generated with a 1064 nm cw mirobeam (150 mW) of a Nd:YAG laser.



3. Confocal laser scanning image of fluorescent photoproducts which were generated with 760 nm radiation of a cw Ti:Sapphire laser (equipped with an etalon to provide single-frequency radiation) via a two-photon excitation process in the focal plane

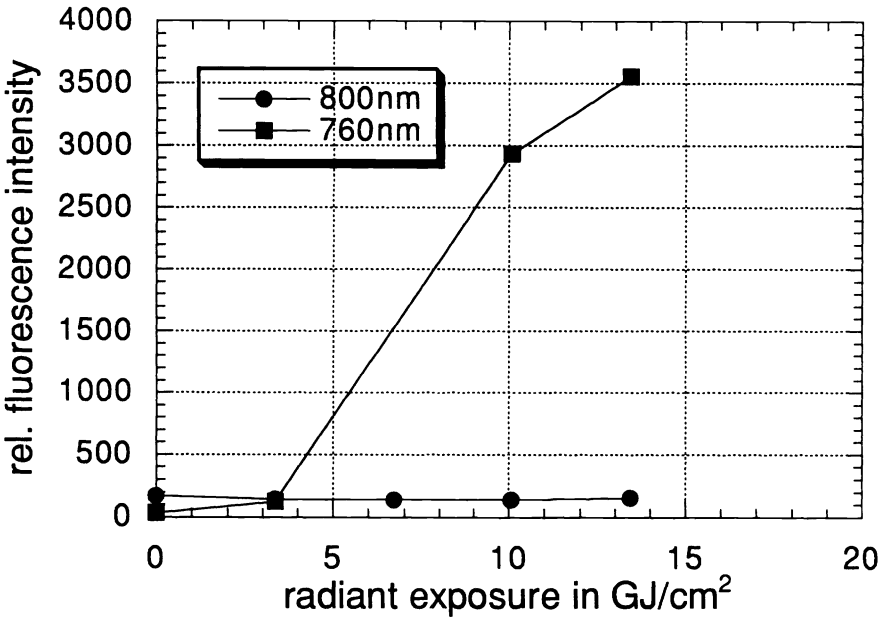
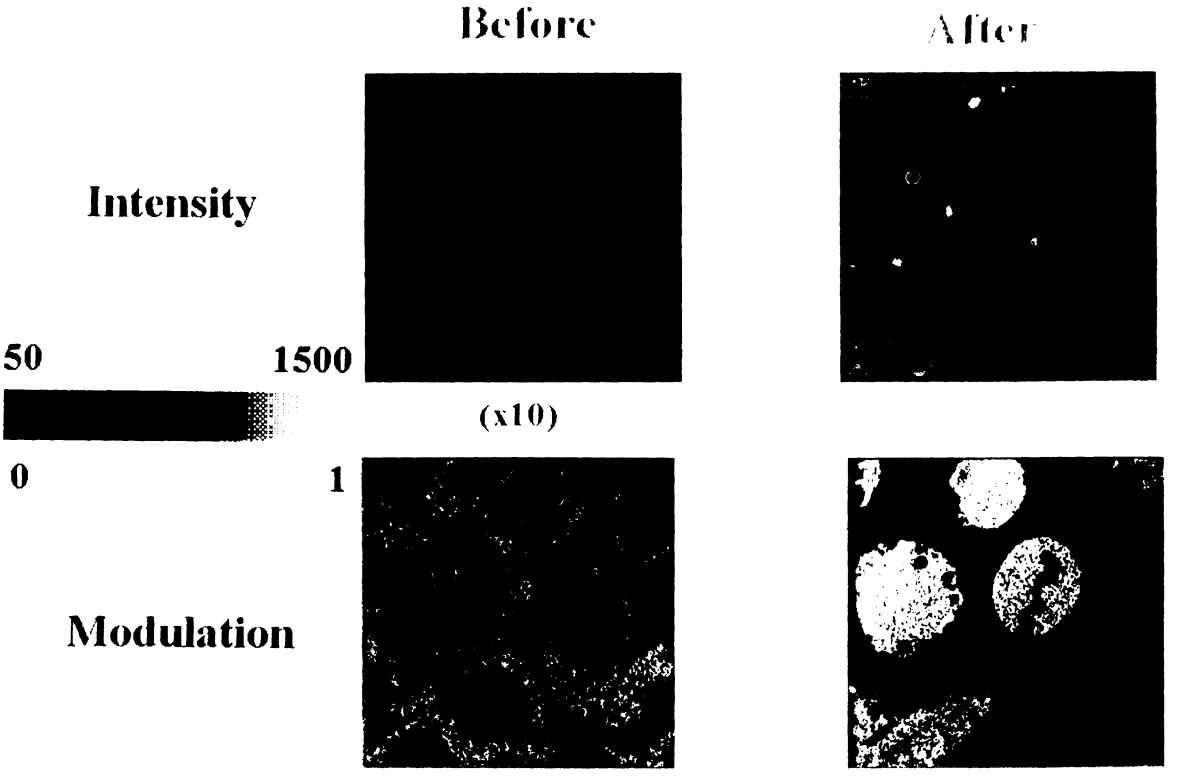


Fig. 4 Two-photon excited autofluorescence in a single sperm cell vs. radiant exposure (105 mW)

Nonlinear Effects induced with Femtosecond Pulses

Two-photon excited autofluorescence images of single CHO cells were obtained with a 730 nm scanning microbeam. Figure 5 shows a typical fluorescence intensity image. Autofluorescence was found to arise from mitochondria, mainly located around the non fluorescent nucleus. We scanned the cell for a total time of 30 min (average power: 2 mW) without significant changes in the intensity (bleaching less than 20%). To record a time-resolved image we increased the power up to 4 mW. Fig. 6 (lower image) demonstrates the spatial distribution of the mean lifetime of CHO cells as i demodulation data. The pixel lifetime histogram shows a 2.2 ns mean lifetime which is typical for NAD(P)H.
Laser beams of 4 mW were used for probing UVA-induced (one-photon absorption) autofluorescence modifications. CHO cells were exposed to low-power 365 nm radiation from a mercury lamp. A radiant exposure of 300 J/cm^2 led to significant modifications in autofluorescence (Fig. 6),
Interestingly, the nucleoli which showed no fluorescence emission prior to exposure became the brightest intracellular fluorescent sites after UVA exposure. The mean cellular fluorescence intensity increased about 60-fold compared to non-exposed cells. The intensity changes were accompanied with modifications in lifetime distribution. The center of the lifetime distribution shifted from 2.2 ns to 1.8 ns.
In order to prove if the NIR scanning beam itself is capable to induce similar ("UVA-like") autofluorescence modifications, we increased the mean power up to 6 mW (0.8 kW peak power). Fig. 7 demonstrates the typical luminescence behavior of CHO cells. The first scan of three cells revealed the similar intensity pattern as in Fig. 5. Interestingly, the third scan of exactly the same cellular areas 32 s later revealed modifications in cellular luminescence pattern. In particular, a small intracellular region near the nucleus of two cells showed increased intensity. Further scanning resulted in further signal increase up to 200 fold and in enlargement of the previously induced luminescent area. The highly luminescent areas were also visible with the eye (using the eyepieces, using only 650 nm short pass filters) as "white" flashes during interaction with the scanning beam. All cells (n=20) showed similar luminescence increase. Morphologically, most of the cells were completely destroyed after 10 scans. These cells showed severe membrane damage (membrane blebbing), loss of cell material, or even disruption into small pieces.

5. Autofluorescence imaging of a single CHO cell excited with 730 nm pulses. Autofluorescence is mainly from mitochondria. The nucleus is non-fluorescent.



6. Intensity imaging and imaging of demodulation data of CHO autofluorescence prior to (left images) and after low-power UVA stress (right images).

5. DISCUSSION

We performed two-photon exited autofluorescence imaging of single cells. In particular, we demonstrated that NIR trapping beams, used as novel micromanipulation tools, are capable to excite endogenous fluorophores. Major location of intracellular NAD(P)H is the midpiece of the sperm cell[14]. Because the trapping beam interacts with the sperm head, the trapping beam should be unable to induce intense autofluorescence. However, the intrinsic motility forces of the cell which are not constant over time result in time-dependent changes of intracellular microbeam position. This may include transient interaction with the sperm midpiece. More significant is the fact (known from one-photon UVA experiments[14]) that photodamage to spermatozoa results in autofluorescence relocalization. In particular, the nucleus becomes the brightest fluorescent site. By this time, intense autofluorescence can easily be excited with the cw, NIR trapping beam resulting even in visible blue/green emission (eyepiece).

Laser scanning microscopy by means of pulsed laser sources was employed to detect intracellular distribution of fluorescent coenzymes (fluorescence intensity imaging) as well as to determine spatially-resolved fluorescence lifetimes (τ-mapping). CHO cells exhibited blue/green autofluorescence with a mean lifetime of 2.2 ns attributed to NAD(P)H in mitochondria. Exposure to 365 nm radiation of a high-pressure mercury lamp (1 mW, 300 J/cm^2) resulted in oxidative stress correlated with increased autofluorescence intensity and onset of nuclear fluorescence. Interestingly, the fluorescence lifetime decreased. It is known, that binding of free NAD(P)H to proteins result in increase of fluorescence quantum yield and lifetime increase[10]. Possible explanations are therefore increased NAD(P)H concentration by enhanced biosynthesis or by transformation of the cell in a more reduced state, or binding of free NAD(P)H to proteins with a lower binding constant as in the case of binding NAD(P)H to mitochondrial proteins.

The cellular response to pulsed NIR microbeams depended significantly on peak power. Peak powers above a threshold value of about 0.5 kW (average power: 6 mW) at 730 nm resulted in onset of luminescence with a two order higher intensity than the intracellular NAD(P)H fluorescence. The luminescence, accompanied with morphological cell destruction, was localized in the mitochondria region. The photon flux of highly focused 0.5 kW pulses can be estimated to be about 10^{31} photons cm^{-2}s^{-1} (during pulse duration). This enormous photon flux could be sufficient to create intracellular plasma formation. The formation of highly-luminescent spots occurred always around the nucleus, in the region of high mitochondria concentration. Interestingly, the luminescence enhancement does not appear always with the first scan which implies a memory effect. We propose that the scanning beam induces a change of the optical properties (absorption coefficient, scattering coefficient, dielectrical constant) providing somehow the conditions for the generation of optical breakdown within the cell.

6. REFERENCES

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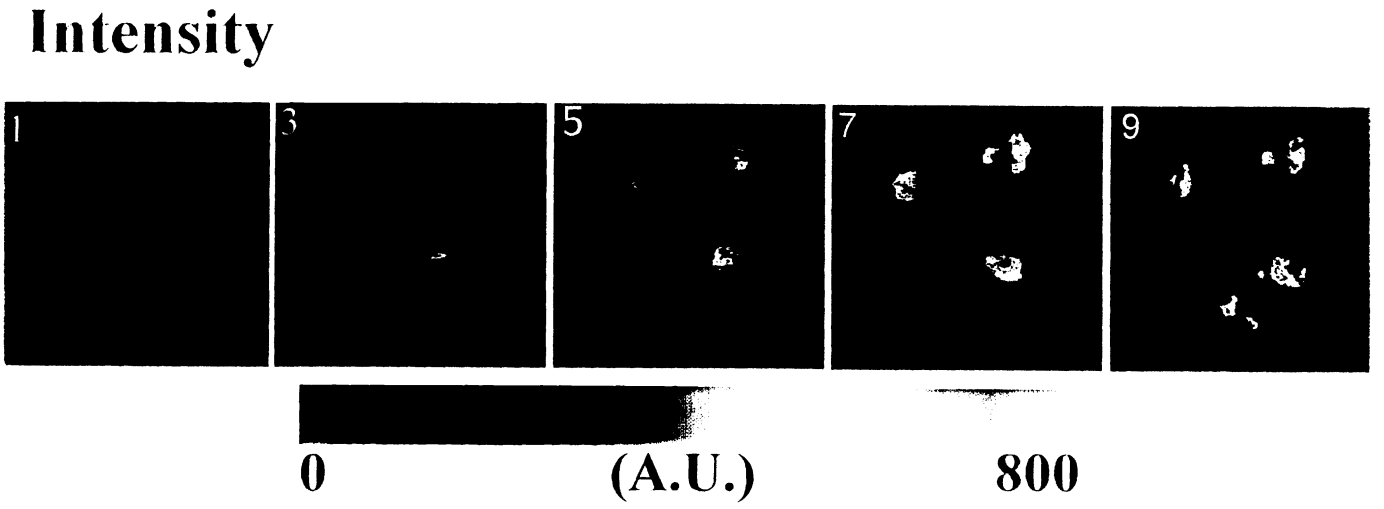

7. Photoinduced modifications of cellular luminescence as a function of the number of scans.