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TIME-GATED AUTOFLUORESCENCE MICROSCOPY OF MOTILE GREEN MICROALGA IN AN OPTICAL TRAP

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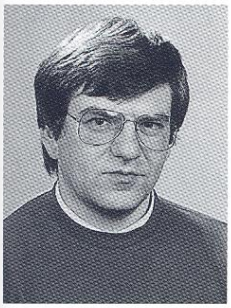

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Abstract - Ultrafast time-gated fluorescence imaging of optically trapped single motile cells is presented. The biflagellar green microalga *Haematococcus pluvialis* was confined with picoNewton trapping forces in the focal volume of a high numerical aperture objective by near infrared multitraps. Trapping radiation of 100 mW power at the sample was provided by a Nd:YLF laser (1047 nm) operating in the cw mode. Simultaneously, cellular autofluorescence was excited with a 633 nm picosecond 80 MHz laser diode. An ultrafast gated intensified slow scan CCD camera system with a tunable gate width (200 ps-1 ms) and tunable time-delay (0-20 ns) between excitation and detection was used as fluorescence detector. We demonstrate fluorescence imaging of high temporal (sub-ns) and high spatial (sub-µm) resolution and fluorescence lifetime determination of intracellular autofluorescence based on chlorophyll excitation. Exposure to the herbicide DCMU resulted in an increase of fluorescence intensity and lifetime by 250% and 150%, respectively.

Key words: Laser tweezers, optical trap, green alga, time-gated microscopy, fluorescence lifetime, chlorophyll

INTRODUCTION

Sensitive studies with high spatial resolution of motile cells and cell organelles are limited or not possible due to sample movement by picoNewton motility forces. A variety of cells, such as bacteria or spermatozoa, may achieve velocities higher than 50 $\mu m\ s^{-1}$. The problem of sample motion can be overcome by the application of laser tweezers with trapping forces larger than the motility forces. Using optical tweezers sensitive fluorescence measurements on motile cells, such as autofluorescence imaging with high spatial and temporal resolution, may become possible. High resolution imaging in the sub-nanosecond and sub-micron range can be performed by time-gated microscopy. Time-gated microscopy is a useful contrast enhancing imaging technique based on ultrafast temporal resolution. Time-gates of detection as well as time delays between excitation and detection in the ps (10^{-12}-10^{-9} s) and ns (10^{-9}-10^{-6} s) region allow fluorescence lifetime determination and fluorophore separation. In combination with light microscopy fluorescence lifetime imaging with submicron spatial resolution (τ-mapping) is possible. In addition, the use of a time delay allows the suppression of reflected, scattered, and transmitted fluorescence excitation photons. The fluorescence lifetime is an intrinsic property of the fluorophore and its microenvironment and independent on concentration. Time-gated microscopy can be performed either in the time domain (e.g. Bugiel *et al.*, 1989; König and Wabnitz, 1990; Schneckenburger *et al.* 1998) or in the frequency domain (e.g. Lakowicz, 1983; So *et al.*, 1998). In combination with pulsed excitation sources, high sensitive CCD (charged coupled device) cameras with time-gated intensifier units can be used. In this paper we demonstrate the combination of laser trapping and time-gated microscopy. In particular, we present time-gated autofluorescence images of the single motile alga *Haematococcus pluvialis* in an 1047 nm trap. The green microalga can achieve velocities up to 80-100 μms^{-1} in the breast stroke mode (Braune *et al.*, 1994).

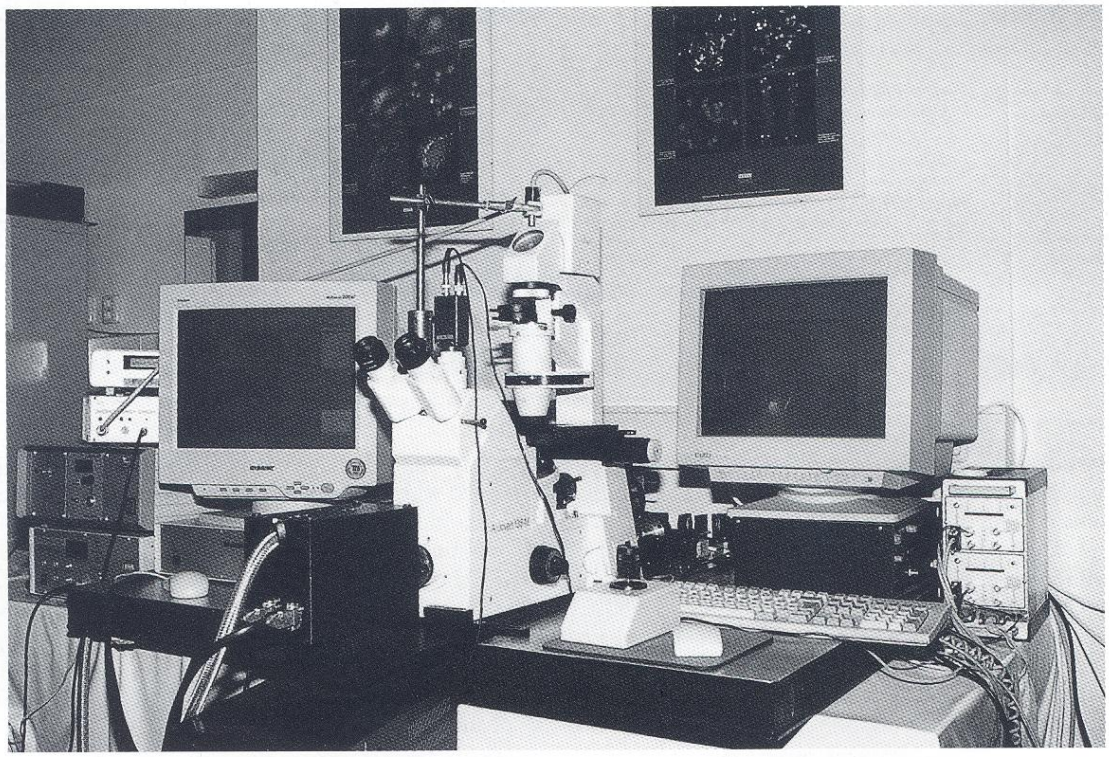

Fig. 1 *Photograph of the experimental set-up.*

MATERIALS AND METHODS

Laser Tweezers

The trapping beam was provided by a 1047 nm, 1W Nd:YLF laser (Coherent ADLAS, Lübeck, Germany) operating in the cw mode with 100 mW power at the sample. The beam passed a galvanometric mirror scanning unit with diffraction limited optics (SL Microtest, Jena, Germany). Mirror control was done by a special software with integrated video overlay in such a way that movements of the computer mouse on the overlay resulted in variation of the trapping beam position. In addition, the trap position in dependence on trapping time could be determined by a computer program. To achieve quasi simultaneously multiple sample trapping the system used fast hopping of the single laser beam between up to 10 positions. The hopping time to change position was less than 1 ms and the dwell time on one position was variable from 2 to 200 ms. Hopping time and dwell time were shorter than sample reaction time. The tweezers system was attached to an inverted microscope (Axiovert, Zeiss) with two camera ports. In order to focus the beam to its diffraction limit the beam was expanded to overfill the entrance pupil of a 63x oil immersion objective (NA+1.25. Optical cell manipulation with tweezers was controlled with a halogen lamp (white light) irradiation and taped with a standard video camera.

Fluorescence Excitation and Detection

Autofluorescence of alga was excited with a 80 MHz, 55 ps laser diode emitting at 635 nm (PicoQuant, Berlin, Germany). The diode was mounted above the halogen light illumination path in such a way that white light as well as excitation radiation passed through the condenser. The red beam was focused onto the sample by variation of the condenser position.

An ultrafast gated intensified slow scan CCD camera system PicoStar HR-14 (LaVision, Göttingen, Germany) with a tunable gate width (minimum: 200 ps), tunable time-delay between trigger pulse and detection, a maximum gate repetition rate of 110 MHz, spectral response 200-900 nm, and sensitivity in the single photon range was used as detector. The camera was mounted to the second camera port of the microscope and triggered externally using the sync-output of the ps laser diode. Fluorescence was detected by the use of a 800 nm short pass filter to avoid detection of trapping radiation. In some cases, an additional 650-725 nm bandpass filter was used. Image processing was performed with WinSC 5.0 software (LaVision, Göttingen).

Microbeads

Three types of red-absorbing micrometer-sized beads from Molecular Probes were used: a) 4.1 ± 0.3 µm 660/680

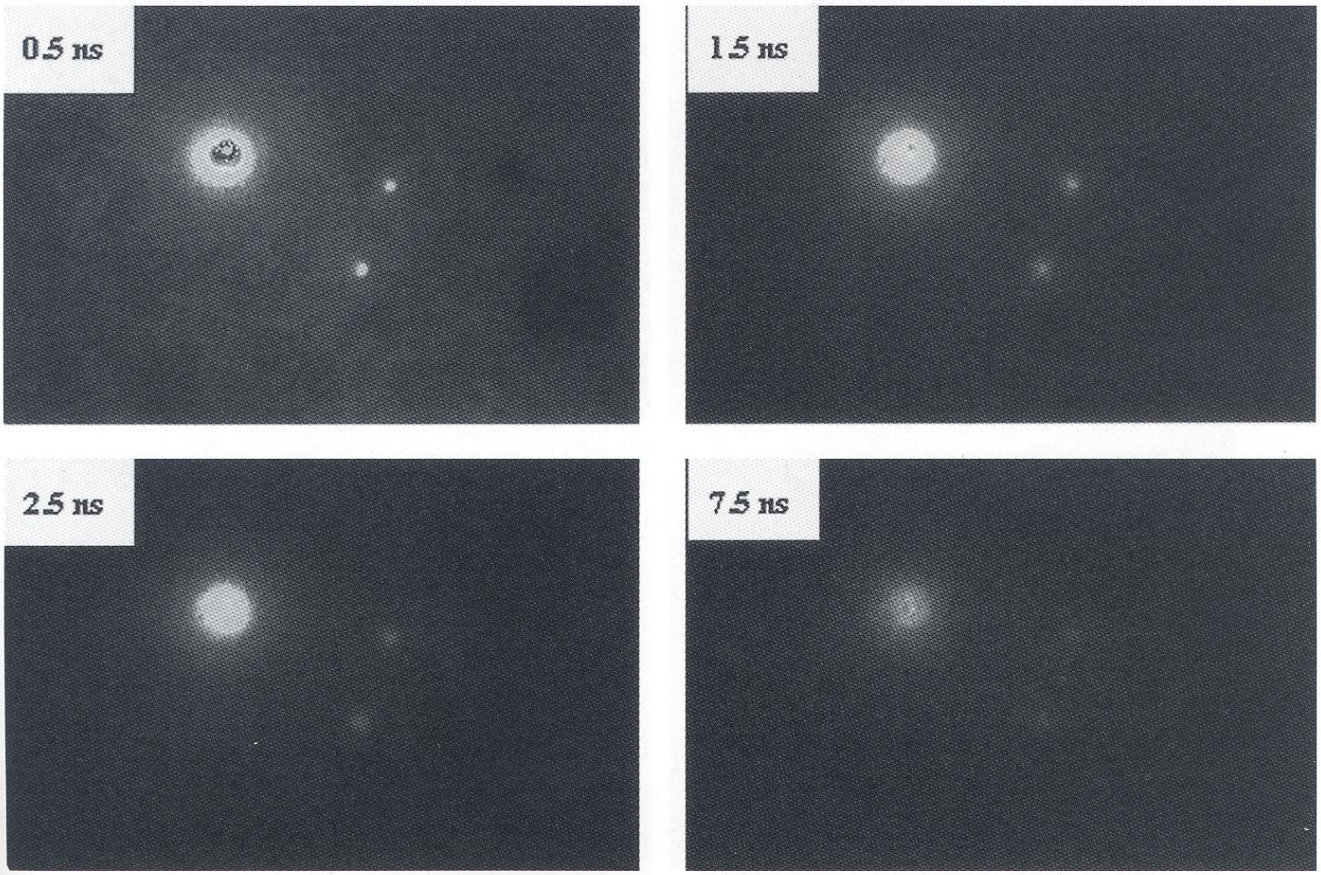

Fig. 2 *Time gated fluorescence images at different time-delays of fluorescent microbeads confined in an optical multitrap.*

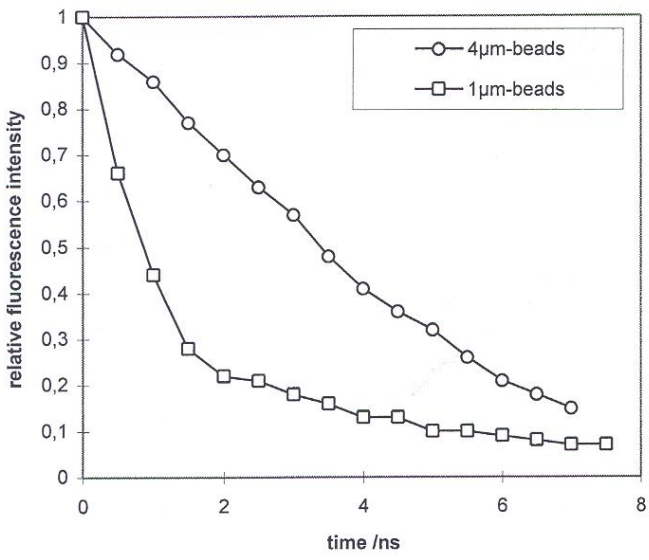

Fig. 3 *Fluorescence decay curve of 4 µm and 1 µm microspheres.* The mean fluorescence intensity vs. time delay is depicted. Mean lifetimes of 4.5 ns and 1.2 ns were determined.

(absorption maximum/emission maximum) microbeads, b) 4 µm 625/645 microbeads, and c) 1.03 ± 0.02 µm 633/720 polystyrene microbeads with a typical polystyrene density of 1.055 g/cm^3.

Cells

The motile unicellular volvocalean microalga *Haematococcus pluvialis* was used. The green alga is of biotechnological interest due to the synthesis of antioxidative pigments (e.g. astaxanthin). The algae were grown autotrophically in glass flasks as described in detail by Grünewald *et al.* (1997). For experiments alga suspension was filled into sterile cell chambers consisting of over-sized coverslips as windows, a silicon spacer with opening and a metal frame. Trapping and fluorescence excitation was perfomed through a coverslip window. In part, the biflagellate was exposed to the herbicide and photosynthesis II blocker DCMU (3-(3,4-dichlorophenyl)-1.1-dimethylurea, Sigma).

RESULTS

In order to test the novel system time-gated fluo-

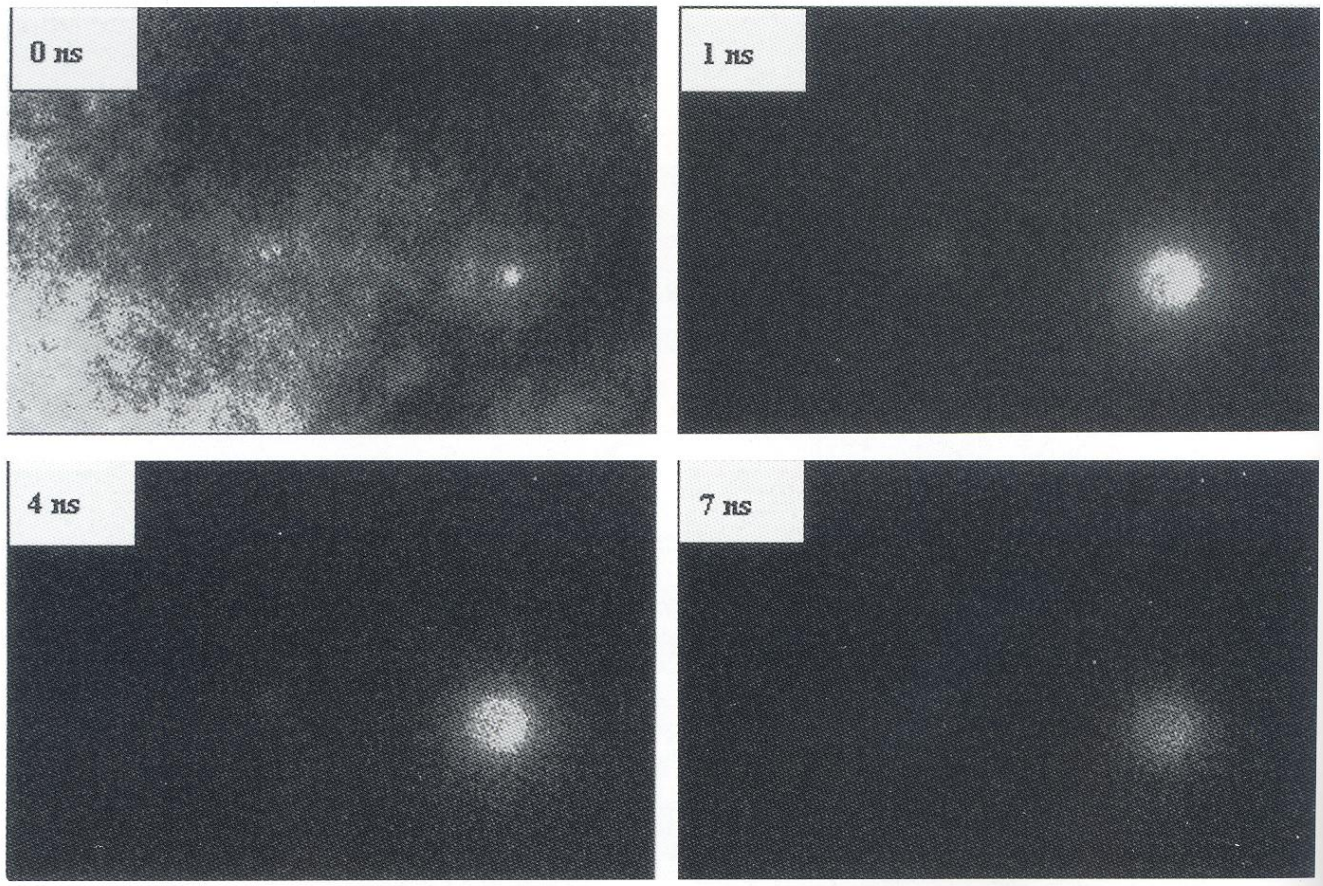

Fig. 4 *Time-gated fluorescence images of a 4 µm trapped microsphere.* No time-delay leads to the detection of excitation and fluorescence photons whereas a delay results in high-contrast fluorescence images.

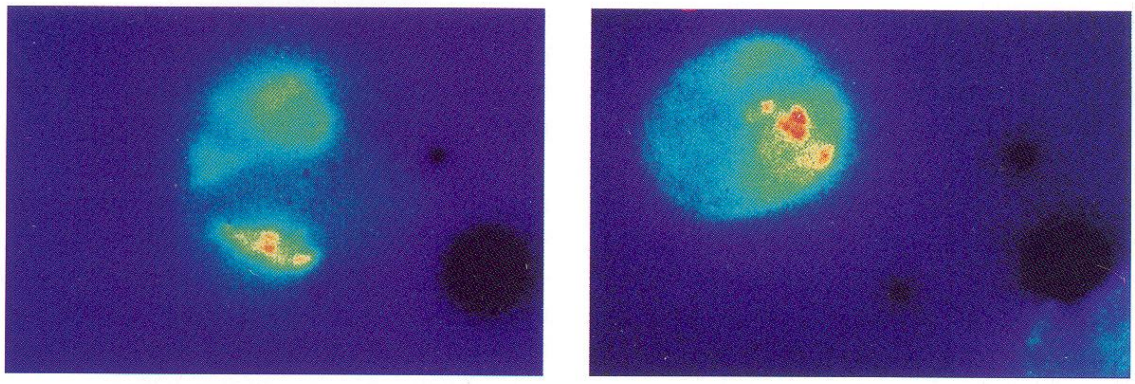

Fig. 5 *Time-gated* (500 ps gate) *autofluorescence images of herbicide-exposed microalga.* Application of the herbicide resulted in increased fluorescence intensity.

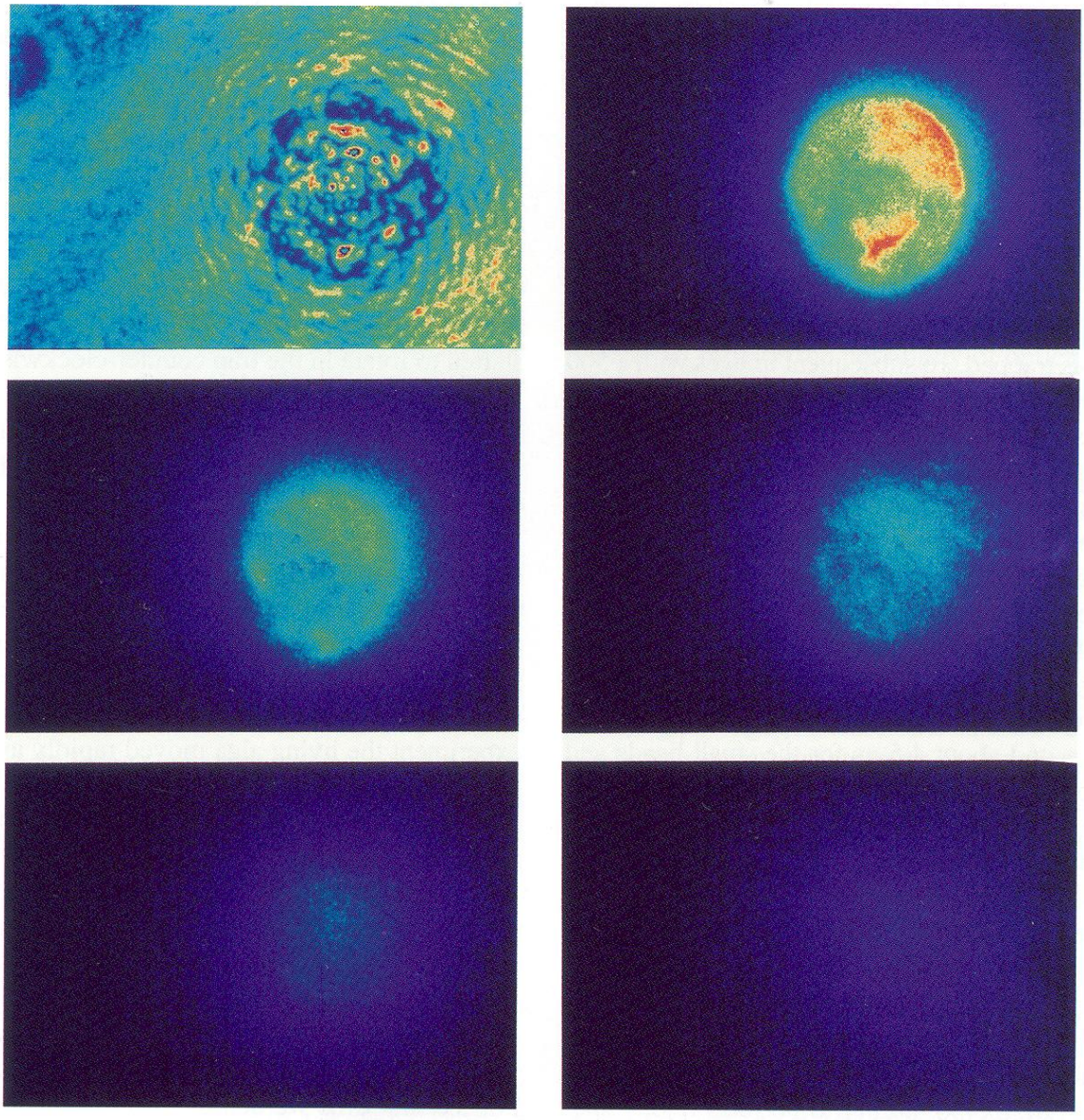

Fig. 6 *Series of time-gated autofluorescence images at different time delays of a living optically-trapped green alga.*

rescence microscope with NIR multitrap we started to image time-resolved emission of optically-trapped microbeads. Microbeads at different size and fluorescence lifetime were confined in the up to 10 foci of the multitrap. Using the fast trap scanner the beads could be confined "quasi-simultaneously" and could be moved individually in the lateral, vertical or rotation mode through the medium. The flexible multitrap based on only one trapping beam allowed "dancing" of the samples.

Simultaneously to the optical micromanipulation, the microbeads were excited with ps pulses at 633 nm to fluoresce in the far red. Fluorescence images were obtained with time gates of 300 ps and 500 ps. In order to get information on fluorescence decay times, series of time-gated images at different time delays between excitation and detection were taken.

The fluorescence images in fig. 2 show a multitrap which confined 1 µm microbeads at three trap positions. In the left position, an additional large 4 µm bead was trapped. 16 subsequent images with a 500 ps time gate and a 500 ps time delay were taken. The first image, 500 ps after the excitation pulse, indicates a broad, high contrast fluorescence image of the beads. With increasing time delay the fluorescence intensity drops with respect to the fluorescence lifetime. The small beads possess a shorter lifetime. Calculating the mean fluorescence intensity I of the beads by region of interest (ROI) processing, a typical mean lifetime τ [assumption of a monoexponential decay with $I(t) = I(0) \cdot \exp(-t/\tau)$] of 1.2 ns and about 4.5 ns for the small beads and the large microspheres was determined. A typical curve of fluorescence intensity versus time-delay is seen in fig. 3. Time-gated microscopy as a high contrast technique allows the discrimination of the fluorescence signal from backscattered (reflected) excitation radiation without additional long-pass or broadband filters (Fig. 4). Discrimination is obtained by choosing an appropriate time delay between excitation and detection. The microbead studies demonstrate the possibility to perform highly spatially-resolved time-gated fluorescence imaging of optically-traped microsized samples.

Trapping of the motile green alga was more complicated. Due to alga morphology, the trapping positions differed significantly from most animal cells where the nucleus appeared to be the main trap location. In the alga mainly regions near the outer membrane, likely the chloroplast with a high refractive index, were confined in the focus volume of the objective. Interestingly, the flagellate tried to escape from the trap. Using a single-trap arrangement, in 80% of the experiments we did not succeed in confining alga for more than 20-60 s. However, we managed long-term trapping in most of cells by use of the multitrap. In particular, we positioned three or four traps near the outer membrane. The attempt of the phototactic alga to escape the laser tweezers was not limited to time periods of white light exposure where the blue spectral part could trigger rhodopsin photoreceptors. We detected successful escapes also in the case of red/NIR exposure (trap + fluorescence excitation) without additional light sources. In part, confined algae started to rotate in the trap counter-clockwise.

Typical 633 nm excited fluorescence images of optically confined algae are seen in fig. 5. The red autofluorescence rises from chlorophyll a which is located in the chloroplasts. Fluorescence images taken without broad band filters at different time delays are depicted in fig. 6. The images demonstrate a fast fluorescence decay. We determined a mean lifetime of about 1.4 ns (Fig. 7). When the trapping beam was switched off at the end of the experiment the living alga moved rapidly away.

Additional application of the herbicide to trapped algae resulted in 2.5 fold enhanced intensity and in 1.5 fold fluorescence lifetime increase. The cell stopped immediatly attempts to escape from the trap.

DISCUSSION

For the first time we demonstrate time-gated fluorescence microscopy on motile cells using laser

tweezers. Two red/NIR lasers, one for fluorescence excitation, another for trapping, were coupled to an inverted fluorescence microscope equipped with an ultrafast CCD detector. High-sensitive time-gated autofluorescence measurements of optically-trapped motile green microalga with ps time resolution and submicron spatial resolution have been performed. Trapping of motile algae with a rather complicated morphology (several regions of high refractive index such as nucleus and chloroplast) and phototactic behaviour was possible mainly due to the application of a multi-trap system. The attempt of the phototactic green microalga *Haematococcus pluvialis* to escape the NIR trap without UV/blue light exposure may have possible explanations in the action of mechanoreceptors to the presence of pN trapping forces or in non-resonant two-photon excitation of blue/green light photoreceptors (e.g. rhodopsin) due to the MW/cm^2 intensity of the NIR trapping beam. That means, the simultaneous absorption of two 1047 nm trapping photons may result in the transition into an electronic state which requires normally excitation at about 1047 nm: 2 = 523 nm, the wavelength which corresponds nearly to the one-photon absorption maximum of rhodopsin. Within the focus volume rhodopsin molecules could therefore absorb NIR trapping photons.

In contrast to short-wavelength NIR traps used in spermatozoa studies (König, 1998), we found no destructive influence of the cw 1047 nm trapping beam at 100 mW. Also the low-level pulsed fluorescence excitation source did not harm the cell. Turning off the lasers the alga was found to be still motile.

Time-gated imaging as an efficient contrast enhancing technique at different time delays between excitation and detection allowed the suppression of scattered and reflected excitation radiation, the determination of fluorescence lifetimes, and the differentiation between fluorophores independently on fluorescence intensity fluctuations due to concentration variation, sample movement and bleaching. As shown trapping of motile cells in combination with time-resolved fluorescence measurements provide the possibility of optical micromanipulation as well as sensitive cell diagnostics.

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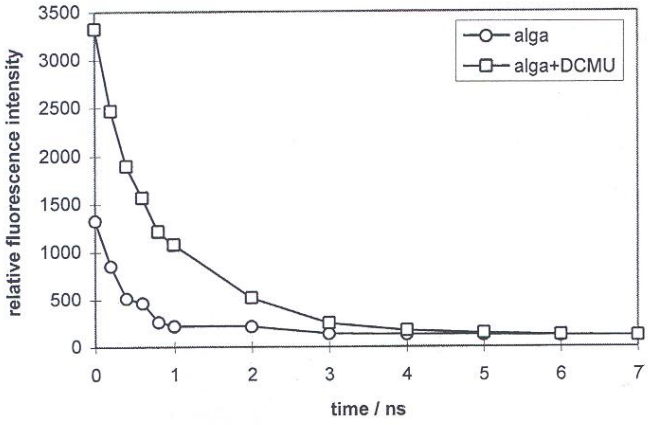

Fig. 7 *Fluorescence decay curve of intracellular chlorophyll fluorescence before and after exposure to the herbicide DCMU* (data from 200 ps time gates). DCMU-induced fluorescence intensity and lifetime increase by a factor of 2.5 and 1.5, respectively, were detected.

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