

Time-resolved microspectrofluorometry and fluorescence lifetime imaging of photosensitizers using picosecond pulsed diode lasers in laser scanning microscopes

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Abstract. This work describes the time-resolved fluorescence characteristics of two different photosensitizers in single cells, in detail mTHPC and 5-ALA induced PPIX, which are currently clinically used in photodynamic therapy. The fluorescence lifetime of the drugs was determined in the cells from time-gated spectra as well as single photon counting, using a picosecond pulsed diode laser for fluorescence excitation. The diode laser, which emits pulses at 398 nm with 70 ps full width at half maximum duration, was coupled to a confocal laser scanning microscope. For time-resolved spectroscopy a setup consisting of a Czerny Turner spectrometer and a MCP-gated and -intensified CCD camera was used. Time-gated spectra within the cells were acquired by placing the laser beam in “spot scan” mode. In addition, a time-correlated single photon counting module was used to determine the fluorescence lifetime from single spots and to record lifetime images. The fluorescence lifetime of mTHPC decreased from 7.5 to 5.5 ns during incubation from 1 to 6 h. This decrease was probably attributed to enhanced formation of aggregates during incubation. Fluorescence lifetime imaging showed that longer lifetimes were correlated with accumulation in the cytoplasm in the neighborhood of the cell nucleus, whereas shorter lifetimes were found in the outer cytoplasm. For cells that were incubated with 5-ALA, a fluorescence lifetime of 7.4 ns was found for PPIX; a shorter lifetime at 3.6 ns was probably attributed to photoproducts and aggregates of PPIX. In contrast from fluorescence intensity images alone, different fluorescence species could not be distinguished. However, in the lifetime image a structured fluorescence distribution in the cytoplasm was correlated with the longer lifetime and probably coincides with mitochondria. In conclusion, picosecond diode lasers coupled to a laser scanning microscope equipped with appropriate detection units allows time-resolved spectroscopy and lifetime imaging with high spatial resolution and provides numerous possibilities in cellular and pharmaceutical research. © 2003 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1528595]

Keywords: time-resolved spectroscopy; laser scanning microscopy; pulsed diode lasers; lifetime imaging; PDT; mTHPC; 5-ALA.

Paper 01075 received Nov. 8, 2001; revised manuscript received Apr. 16, 2002, and Aug. 14, 2002; accepted for publication Aug. 30, 2002.

1 Introduction

The use of time-resolved methods in biomedical research has been a great challenge in the recent years. This includes time-gated microspectrofluorometry as well as time-resolved fluorescence imaging. The potential of time-gated microspectrofluorometry and some applications, for example the detection of different conformations of the coenzyme nicotinamide adenine dinucleotide (NADH) as well as flavin molecules, were summarized by H. Schneckenburger et al.¹ Especially, time-resolved energy transfer spectroscopy appeared to be an ap-

propriate tool for measuring mitochondrial metabolism in living cells.² Also different species of tumor-localizing porphyrins, which are of great importance in photodynamic therapy (PDT),³ could be distinguished by time-resolved methods, i.e., monomeric and aggregated porphyrin molecules as well as ionic species located at different cellular sites.^{4,5} Time-resolved fluorescence imaging in combination with a laser scanning microscope was performed by Bugiel et al.⁶ and Buurman et al.⁷ For excitation, argon-ion lasers were used in combination with electro-optic or acousto-optic modulators. As an alternative technique, time-gated video mi-

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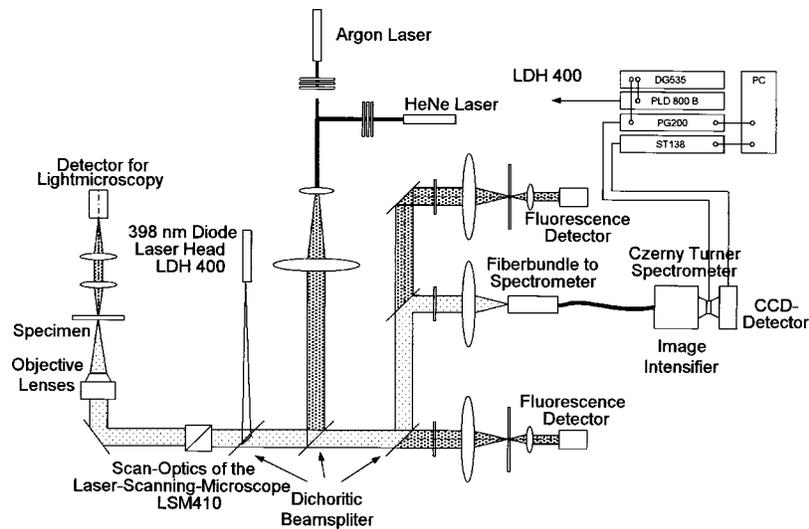


Fig. 1 Experimental setup for laser scanning microscopy and time-resolved microspectrofluorometry.

scopy with highly intensifying camera systems has been used for imaging of tumor localizing dyes.⁸ Moreover, dyes whose fluorescence lifetimes change upon binding of signaling ions or molecules were used in confocal fluorescence lifetime imaging.⁹

So far, most approaches in time-resolved microspectrofluorometry and fluorescence microscopy depend on expensive and complex lasers, such as mode-locked solid state, dye, or gas lasers or other pulsed laser systems. The advantage of using a mode-locked Ti:sapphire system, especially in the case of multiphoton NIR microscopy, is discussed in Ref. 10. Spatially resolved mapping of fluorescence decay curves of fluorophore-labeled living cells were obtained by placing the laser beam in “spot scan mode” in a pixel of interest and using time-correlated single photon counting (TCSPC) to acquire the decay characteristics.¹⁰ This method allowed the opening of a fourth dimension of fluorescence imaging by adding a picosecond time resolution to the 3-D images of a confocal laser scanning microscope (4-D imaging).^{11,12}

Only recently, new developments in diode laser technology suggest the usage of such lasers for a wide range of applications, i.e., laser scanning microscopy and time-resolved fluorescence investigations.¹³ Diode lasers could improve the handling of time-resolved experiments significantly, because they are developed to be turnkey systems that provide their nominal optical energy a few minutes after the system has been started. Furthermore, diode lasers are cheap compared to traditionally used mode-locked lasers. Recently, a short-pulsed diode laser emitting at 640 nm was used in combination with a confocal microscope to study the burst of fluorescence photons from mononucleotide molecules labeled with Cy5.¹⁴ This technique allowed counting and identification of labeled analyte molecules in a given sample within microcapillaries with time-resolved detection.

Within this work we describe the potential use of a short-pulsed diode laser emitting at 398 nm¹⁵ coupled to a confocal laser scanning microscope for detecting time-resolved fluorescence spectra and fluorescence lifetime images of two photosensitizers that are actually under clinical trials. In detail, the

nanosecond fluorescence decay characteristics of meta-tetra(hydroxyphenyl)chlorin (mTHPC) as well as 5-aminolevulinic acid (5-ALA) induced protoporphyrin IX (PPIX) were studied with subcellular resolution.

2 Materials and Methods

2.1 Experimental Setup

2.1.1 Time-resolved spectroscopy

In order to perform time-resolved subcellular fluorescence spectroscopy, a short pulsed diode laser emitting at 398 nm (LDH 400, PicoQuant GmbH, Berlin, Germany) was coupled to a laser scanning microscope (LSM410, Carl Zeiss, Germany), in addition to the standard laser sources of the LSM410, an argon-ion laser emitting light at 488 nm and 514 nm and a HeNe laser emitting at 633 nm. For this, a coupling unit for external laser sources (LSM410 NLO, Carl Zeiss, Germany) was placed directly in front of the scan mirrors. Figure 1 shows a schematic diagram of the experimental setup. A custom-made dichroitic beamsplitter (420 DCLP, AHF Analysentechnik, Tübingen, Germany) was used to guide the diode laser beam to the scan optics and to transmit the fluorescence signal to the detection setups. The laser beam was focused on the specimen via a 40x objective lens (Plan-Neofluar, 40x, NA=0.75, Carl Zeiss, Germany). The astigmatic and divergent beam of the diode laser was only slightly collimated so that the back aperture of the objective was still overfilled. For time-resolved spectroscopy a Czerny-Turner spectrometer SP-150 (Acton Research Corporation, Acton, MA, USA) was coupled to one of the detection channels of the LSM410 using a bundle of optical fibers. After dispersion the fluorescence light was intensified by a microchannel-plate (MCP) (Princeton Instruments Inc., Monmouth Junction, NJ, USA), which could be used in continuous and gated mode. A cooled CCD detector (Princeton Instruments Inc., USA) finally acquired the time-resolved fluorescence signals from the MCP, which were transferred to a PC for further data processing. The fluorescence photons emitted from the fluorophore

were recorded within a detection gate of 5 ns following the excitation pulse after a certain delay. In gate sweeping mode this delay was increased in steps of 2 ns. So a series of spectra was acquired, from which the spectral characteristics and the fluorescence lifetimes of the investigated specimen could be calculated. The MCP was sensitive from UV to NIR but no detailed information about the spectral sensitivity was available. Because only relative changes of the spectra were evaluated no attempt was made to correct the observed intensities. However, as usually done, calibration of the wavelength was carried out using the lines of a multicolor HeNe laser. The calibration was verified by means of the emission spectrum of the fluorophore Cy5 (Amersham Pharmacia Biotech, Little Chalfout, UK).

In the time-resolved mode a specific wavelength range was selected to calculate the fluorescence decay curve. For each time-resolved spectrum, we placed the excitation beam in "spot-scan mode" in a pixel of interest within a cell and collected the fluorescence of 10^5 successive excitation pulses at a pulse repetition rate of 10 kHz. With this repetition rate the power of the laser beam at the excitation site was measured to be $0.29 \mu\text{W}$. With an average energy of 29 pJ per excitation pulse and an illumination spot diameter of $4.4 \pm 0.5 \mu\text{m}$ this corresponds to an energy density of $1.97 \pm 0.23 \text{ J/cm}^2$ for every recorded spectrum.

2.1.2 Time-correlated single photon counting

As an alternative to time-resolved spectroscopy, fluorescence lifetime measurements were carried out using the TCSPC module SPC-330 (Becker & Hickl GmbH, Berlin, Germany), which was attached to the LSM410. The fluorescence, excited by the LDH-C 400, was wavelengths selected by two beam-splitters [420 DCLP and FT560 (Carl Zeiss, Germany)] and the fluorescence emission filter LP 590 (Carl Zeiss, Germany). A photomultiplier (R5783, Hamamatsu Photonics Deutschland GmbH, Germany) was mounted to the second descanned detection channel of the LSM410. With this equipment fluorescence decay characteristics with a time resolution ≤ 100 ps and a spatial resolution in the subcellular region could be detected. The pulse repetition rate of the diode laser was now set to the maximum of 40 MHz, resulting in a short total acquisition time of less than 1 s for every fluorescence decay curve, depending on the maximum number of counts.

2.1.3 Fluorescence lifetime calculations

In both applications, time-resolved spectroscopy and TCSPC, the fluorescence lifetime was determined by a nonlinear curve fitting and deconvolution process based on the Levenberg-Marquardt algorithm. This algorithm uses a least squares method to fit a model function, mostly a mono- or double-exponential decay to the acquired data. Fit parameters were the decay times and the proportional coefficients.

2.1.4 Fluorescence lifetime imaging

For high-resolution fluorescence lifetime imaging based on time-correlated single photon counting, a novel TCSPC board, the SPC-730 (Becker & Hickl GmbH, Berlin, Germany), was attached to the LSM410. This system allows synchronization of the fluorescence detection with the position of the excitation spot within the specimen.¹¹ Every pixel of the fluores-

cence lifetime image was achieved by a software binning of 4×4 pixels of the LSM image. From this set of data, fluorescence lifetime images could be calculated using the SPCImage Version 1.6 software (Becker & Hickl GmbH). In combination with the SPC-730, the photomultiplier PMH-100 from Becker & Hickl was used at the second descanned detection channel of the LSM410, which allows a time resolution ≤ 200 ps. Wavelength selection was achieved for fluorescence lifetime imaging as described in Sec. 2.1.2.

2.2 Photosensitizers

Lipophilic mTHPC (CAR/97/00069) was provided by Scotia QuantaNova (Guildford, UK) and used without further purification. Stock solutions containing 1 mM of mTHPC were prepared in ethanol and stored at 4°C . Cell cultures were incubated with $5 \mu\text{M}$ mTHPC. 5-ALA, which is a precursor of the photosensitizer PPIX, was obtained from Fluka (Neu-Ulm, Germany). A 100 mM stock solution of 5-ALA was prepared in aqua bidest, and the pH was subsequently neutralized with NaOH. Cell cultures were incubated with 1 mM 5-ALA. Only freshly prepared solutions were used for incubation.

2.3 Cell Culture

Cultures of rat epithelial cells (RR 1022, American Type Culture Collection No. CCL47) were grown in M199 medium (GibcoBRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin) at 37°C and 5% CO_2 . The cells were seeded on microscope slides in a density of 50 cells/ mm^2 and were allowed to grow for 36 h. The medium was replaced and cells were incubated in the exponential growth phase for 5 h with 5-ALA or alternatively between 1 and 6 h with mTHPC, respectively. Microscopic observation was performed immediately after removing the incubation medium and rinsing twice with isotonic NaCl solution at 37°C .

3 Results

With the described detection setups the subcellular fluorescence characteristics of mTHPC and 5-ALA induced PPIX were studied. In order to first prove the capability of the diode laser LDH-C 400 for fluorescence investigations in combination with laser scanning microscopy, we compared the fluorescence images excited by the diode laser with images excited by the standard argon-ion laser. Though the beam parameters and the beam profile of the unshaped LDH-C 400 are pretty poor it was possible to acquire high-resolution phase contrast and fluorescence images. Figure 2 (see Color Plate) demonstrates RR 1022 cells incubated 4 h with 1 mM 5-ALA. In Figure 2(a) the argon-ion laser was used for excitation and, in Figure 2(b) the LDH-C 400. The excitation power per pixel was in both cases in the same order of magnitude ($180 \mu\text{W}$ LDH-C 400 at 40 MHz; $230 \mu\text{W}$ Ar⁺ laser). The fluorescence of the cells was observed in the red detection channel of the LSM; additionally the phase contrast was observed in the green channel. Absorption of ALA-induced PPIX at 514 nm is approximately 1/10 of the absorption at 398 nm. Therefore, fluorescence of PPIX after excitation with 514 nm was not visible under the experimental conditions. However, the spatial resolution of the two images was nearly

the same. Therefore, the LDH-C 400 allows excellent image quality, both for fluorescence and phase contrast microscopy. In addition, Figure 2 provides other important information. It could be expected that excitation at 398 nm induces damaging of the cells. However, under the used experimental conditions significant morphological changes of the cells were not observed. Formation of blebs and bubbles could be detected only after applying multiple scans, which was also observed in the case of Ar^+ laser excitation.

The most important advantage of the LDH-C 400 is the ability to measure subcellular time-resolved spectra and fluorescence lifetimes. Following wavelength calibration, we first measured the steady-state emission spectrum of the known fluorophore Cy5, in order to demonstrate the accuracy of our system. The fluorescence of Cy5 was excited with the LDH-C 400. The emission spectrum of Cy5 ($2 \mu\text{M}$ in PBS) exhibits a maximum fluorescence at 670 nm (see Figure 3), which is exactly the same as reported in the *Handbook of Molecular Probes*. Measuring fluorescence spectra is therefore possible with our technical equipment.

Figure 4 demonstrates a series of time-gated fluorescence spectra of mTHPC incubated in a concentration of $5 \mu\text{M}$ for 4 h in RR 1022 cells. The spectra were obtained from one cell by placing the laser beam intracellularly in a "spot of interest." Signals were recorded within a detection gate of 5 ns. The delay after the excitation pulse was increased in steps of 2 ns. From these signals the decay curves were monoexponentially fitted at the emission wavelength around 652 nm and the fluorescence lifetime was calculated to be 6.1 ± 0.1 ns. In comparison, a lifetime of 6.54 ± 0.58 ns was calculated when using TCSPC instead of time-resolved spectroscopy. Within the standard deviation, the results were similar.

The cellular distribution and accumulation of photosensitizers can be subject to change during incubation. Therefore, the fluorescence lifetime of mTHPC was determined dependent on the incubation time, which varied from 1 to 6 h. With increasing incubation time the fluorescence lifetime decreased from 7.5 ± 0.5 to 5.5 ± 0.1 ns, which is demonstrated in Figure 5.

For lifetime imaging, cells were incubated with $5 \mu\text{M}$ mTHPC for 6 h. The lifetime image was built up from the average decay (monoexponential fitting), which was calculated in every detected pixel of the scanned area (128×128 pixels). The lifetime was represented in pseudocolor (data not shown). However, excitation at 398 nm is not optimal for mTHPC; the absorption maximum is at 416 nm. The quality of the pseudocolored image was therefore poor. Nevertheless it seemed that short lifetimes below 5 ns were found in the cytoplasm in the neighborhood of the cell nucleus, whereas longer lifetimes could be detected in the outer cytoplasm. In the "pure" fluorescence intensity image these differences could not be seen.

In another approach, RR1022 cells were incubated with 5-ALA and the decay characteristics of the resulting PPIX were measured. PPIX is well known to develop photoproducts during PDT which fluoresce approximately at 668 nm, whereas the main peak of PPIX is at 635 nm. The time-gated fluorescence spectra of cells, incubated 4 h with 1 mM 5-ALA, are demonstrated in Figure 6. A monoexponential fit was used to determine the fluorescence lifetimes. A fluorescence lifetime of 7.44 ± 0.56 ns was calculated at a fluores-

cence wavelength of 625 ± 15 nm, which was correlated with the main band of PPIX, whereas a lifetime of 3.6 ± 0.46 ns was calculated at a wavelength of 680 ± 20 nm, which was correlated mainly with the photoproduct of PPIX and aggregated species.

Figure 7 (see Color Plate) demonstrates the fluorescence lifetime image of 5-ALA PPIX. Cells were incubated 4 h with 1 mM 5-ALA. Since excitation at 398 nm corresponds perfectly to the maximum absorption band of PPIX, the quality of the image was much better than in the case of mTHPC. The lifetime represented in pseudocolor differs significantly within the cells. A longer lifetime approximately at 6 ns correlates with a specific localization of PPIX inside organelles in the neighborhood of the cell nucleus, which probably represent mitochondria. The diffuse fluorescence in the cytoplasm decayed faster below 2 ns.

In order to prove the specific localization of PPIX, which seemed to be caught up in organelles and not freely diffusing in the cytoplasm, lifetime imaging was performed before and after photobleaching. Due to that process, the overall fluorescence intensity decreased. Interestingly, the localized component that decayed slowly completely vanished, whereas the faster decaying fluorescence was retained (data not shown). The latter probably originates from aggregates or photoproducts of PPIX and from the autofluorescence of the cells. It is known that irradiation during PDT causes photobleaching of the active photosensitizer. While in the "pure" intensity image this specific photobleaching could not be observed, lifetime imaging allowed the intracellular follow-up of this process and thus discrimination of the localization of the photodynamically active component from other fluorescing molecules in the cells.

4 Discussion

This work describes the time-resolved fluorescence characteristics of cells, incubated with different photosensitizers that are of potential clinical interest, in particular, 5-ALA PPIX and mTHPC. For subcellular detection, laser scanning microscopy was performed using a picosecond pulsed diode laser at 398 nm, which has so far not been used in this combination. In spite of the poor beam parameters of this laser it was possible to acquire high-resolution fluorescence images of cells incubated with the photosensitizers, although we did not regard beamshaping. The direct comparison with standard argon-ion laser excitation showed no significant difference in the achievable spatial resolution; even single organelles could be discriminated. Compared with IR laser sources (i.e., pulsed Ti:sapphire lasers for multiphoton excitation) one would expect damaging effects on the cells. However, with the LDH-C 400 we observed damaging (blebs, bubbles, etc.) only after applying multiple scans, which was also observed with other laser sources (for example, Ar^+ laser).

The second-generation photosensitizer mTHPC, which induces cell death when excited with light in the visible region, is currently under clinical trial.¹⁶ From time-resolved fluorescence measurements in ethanol the lifetime of the lowest vibrational state of S_1 was assigned to 10 ns.¹⁷ Using the LDH-C 400 for excitation, we observed a decrease in the fluorescence lifetime from 7.5 to 5.5 ns in the cytoplasm of cells during increasing incubation time. The different results

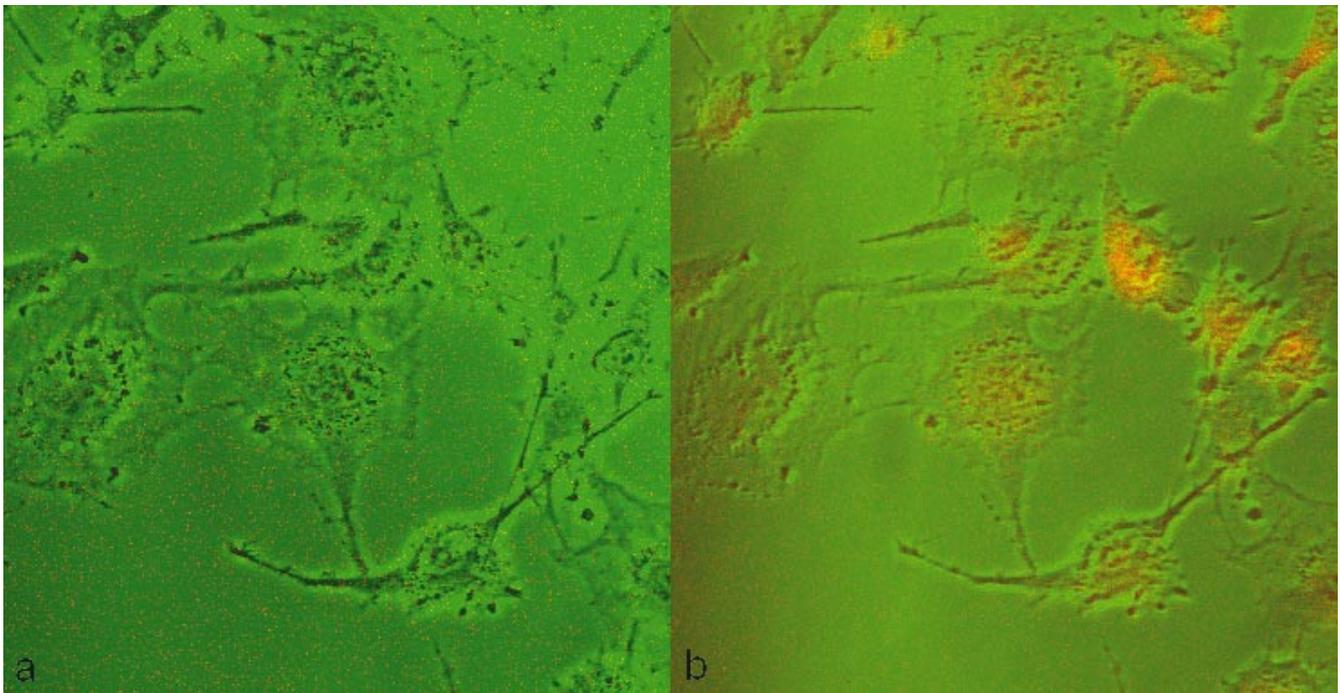


Fig. 2 Fluorescence and phase contrast images of RR1022 cells incubated 4 h with 1 mM 5-ALA using the LSM410 (objective 40x, NA 0.75). (a) Cells were excited using the argon-ion laser (514 nm) and (b) cells were excited using the short pulsed diode laser (398 nm).

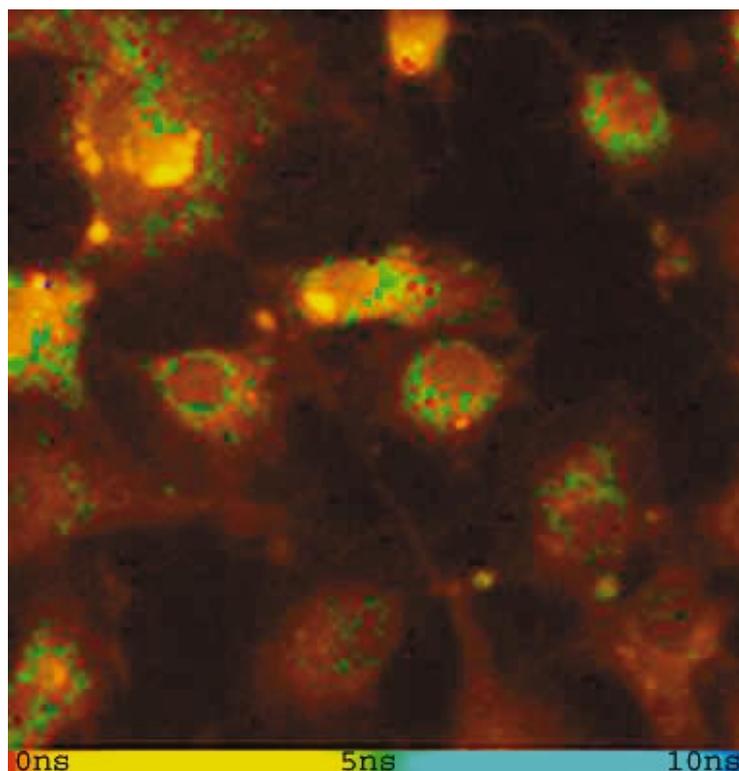


Fig. 7 Lifetime image of RR 1022 cells, incubated 4 h with 1 mM 5-ALA. Lifetimes are represented in pseudocolor.

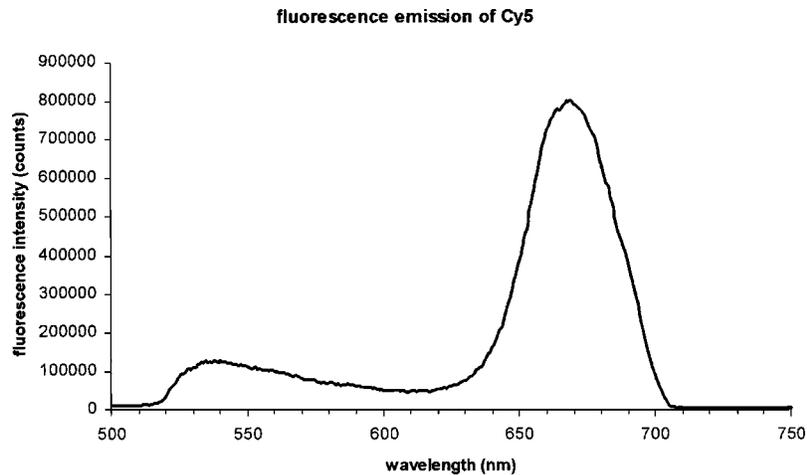


Fig. 3 Emission spectrum of Cy5 after excitation with the short pulsed diode laser (398 nm).

compared to Ref. 17 can be explained by the altered chemical environment. The decrease in the fluorescence lifetime during incubation probably reflects subsequent aggregation of mTHPC and binding to other sites within the cells. As spectral modifications were not detected, structural alterations of the molecule, which are for example observed during development of photoproducts, can be excluded.

To prove the results from time-resolved spectroscopy, the fluorescence lifetime of mTHPC was alternatively determined by TCSPC. Within the standard deviation, the results were the same. Thus, time-resolved spectroscopy, using the LDH-C 400 for excitation, is a valuable tool to determine the lifetime of photosensitizers within cells. Otherwise, TCSPC techniques provide the possibility for high-resolution lifetime imaging during laser scanning microscopy. With the SPC-730, which was attached to the LSM 410, we were able to observe the subcellular distribution of the fluorescence lifetime. To our knowledge, this is the first time that a fluorescence lifetime image was successfully detected using picosecond pulsed diode laser excitation in combination with a laser scanning microscope. In the case of mTHPC, short lifetimes up to 5 ns were found in the cytoplasm in the neighborhood of the cell nucleus, whereas longer lifetimes were detected in the outer

cytoplasm, which may be correlated with decreased accumulation and therefore decreased formation of short-lived aggregates, which normally decay fast.¹⁸

In addition to mTHPC, time-resolved measurements were performed in cells, which were incubated with 5-ALA. 5-ALA is approved for PDT in the treatment of actinic keratoses.¹⁹ 5-ALA produces mainly monomeric PPIX, which was attributed to a long-lived component with fluorescence lifetimes between 18 to 20 ns, measured in the chorioallantoic membrane (CAM) of fertilized eggs.¹⁸ Formation of the photoproduct photoporphyrin (PPIX) was correlated with a decreased fluorescence lifetime at 6 ns.¹⁸ Within this work, subcellular resolved detection led to a fluorescence lifetime of about 7.4 ns for PPIX and 3.6 ns for the photoproduct. The reason for the different results could be the use of different cellular systems. In the case of the CAM, 5-ALA was applied topically on a complex vascular system, whereas in this work the drug was accumulated in cell cultures. Also incubation time plays an important role with respect to lifetime characteristics. In the case of calf aorta endothelial cells, PPIX measured selectively in the plasma membrane exhibited a long-

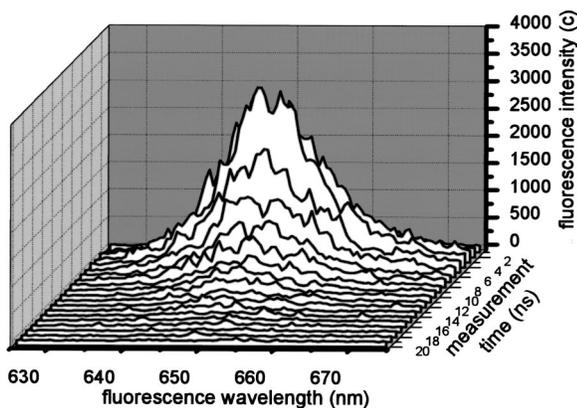


Fig. 4 Time-gated fluorescence spectra of RR1022 cells incubated 4 h with 5 μ M mTHPC detected in the cytoplasm of a single cell.

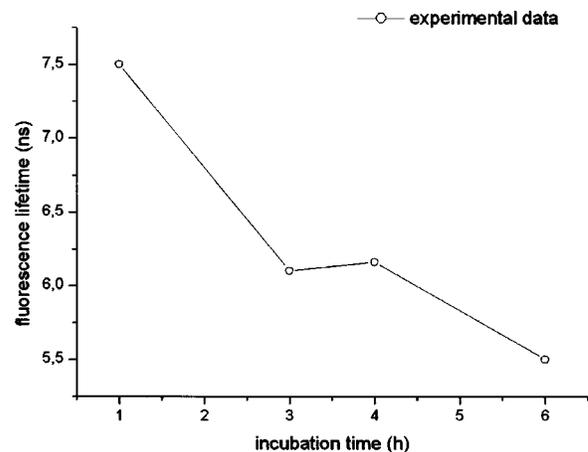


Fig. 5 Dependence of the fluorescence lifetime on incubation time. RR 1022 cells were incubated with 5 μ M mTHPC.

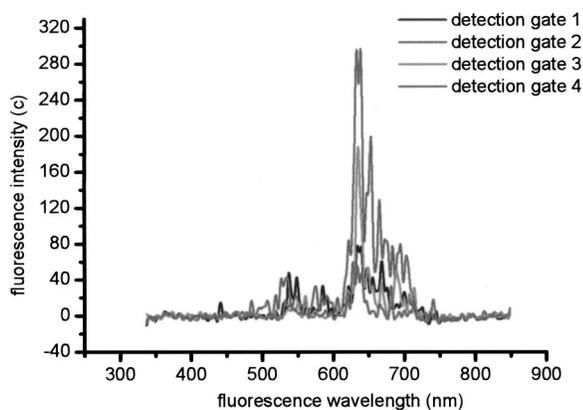


Fig. 6 Time-gated fluorescence spectra of cells, incubated 4 h with 1 mM 5-ALA. The spectra were detected in the cytoplasm of a single cell.

lived component at 14.5 ns after 1 h incubation, which decreased to 12.1 ns after 24 h incubation.²⁰ The efficiency of light-induced cell killing during PDT was correlated with the relative amount of the long-lived component.²⁰ From our fluorescence lifetime images a species with longer lifetime was correlated with a “structured” fluorescence distribution in the cytoplasm around the cell nucleus, which probably coincides with mitochondria. A species with shorter lifetime showed diffuse fluorescence distribution in the cytoplasm. During photobleaching the component with longer lifetime completely vanished. Photobleaching is normally observed for the “active” photosensitizer. We can therefore deduce that the structured fluorescence correlates to the photodynamically active PPIX. Observation of cellular events during PDT is therefore achievable by the described method.

From the discussion above, changes in the fluorescence lifetime reflect the dynamic behavior of photosensitizers, which probably influences the cellular response. Only recently the correlation between cell death mechanisms and fluorescence kinetics of photosensitizers could be demonstrated, using confocal laser scanning microscopy.²¹ In combination with time-resolved methods this offers the identification of different fluorescence species, which are involved in cell death. In conclusion, picosecond diode lasers coupled to a laser scanning microscope, which is equipped with appropriate detection units, is a valuable tool to follow up the cellular kinetics of drugs and provides numerous possibilities in pharmaceutical research.

Acknowledgments

We would like to thank Dipl. Biol. Nermin Akgün and Dilek Dayanakli for helping us with cell cultures. This work was financially supported by Zukunftsinitiative “Junge Generation” der Landesregierung Baden-Württemberg, Förderprogramm Biotechnologie, Grant 720.430-21/1, and in part by the BMBF grant IZKF 01 KS 9605/2.

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