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Lasers in the Life Sciences 3(1), 1989, pp. 47–53
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Investigation of Cells by Fluorescence Laser Scanning Microscopy with Subnanosecond Time Resolution

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(Received November 16, 1988; January 23, 1989)

The spatial distribution of the fluorescence decay time τ ("τ-image") together with that of the mean fluorescence intensity I_F is obtained in fluorescing cell objects using an experimental arrangement consisting of a mode-locked argon ion laser, a microscope equipped with a scanning stage, and a time-correlated single photon counting system. As an example, Ehrlich ascites carcinoma cells incubated with Photofrin II were investigated. One- and two-dimensional scans of the fluorescence intensity as well as the fluorescence decay time are presented which show significant variations of the decay process as a function of the position within the cell.

KEYWORDS: *laser scanning microscopy, fluorescence decay time, hematoporphyrin derivative, photodynamic tumor therapy*

INTRODUCTION

Fluorescence microscopy is widely used in biology and medicine for the study of autofluorescence or fluorescence of attached probe molecules in cells and tissues in order to get information on morphological and functional properties of the object.

Another source of information on the molecular level can be opened if relaxation processes (*e.g.*, the fluorescence decay) are investigated with high time resolution [1]. Parameters of the fluorescence decay are sensitive to the configuration of molecular species, their mobility, and various interactions with the environment.

A combination of high spatial and temporal resolution can be achieved if the excitation is performed by a focused spot of a pulsed laser source and the fluorescence is measured by a time-resolving detection system. Because of the high required sensitivity, time-correlated single photon counting (TCSPC) [2] is advantageously applied. This method usually allows a time resolution of a few 100 ps and offers rather short measuring times if a mode-locked continuous-wave laser is used as high repetition rate excitation source.

Docchio *et al.* [3] as well as Schneckenburger [4], Rodgers *et al.* [5, 6], and Minami *et al.* [7] equipped subnanosecond fluorometers with a microscope and studied the fluorescence decay in a fixed small object region selected by the focused laser spot.

The approach of the present paper is to use primarily the concept of laser scanning microscopy: The object is illuminated point by point by a focused laser beam and the corresponding response is detected at each point. Not only reflected, transmitted, or fluorescence intensities, but also other physical parameters derived from the detected signal can be used for the construction of an image after data processing. Obviously, an image can be formed from parameters of the fluorescence decay (*e.g.*, "τ-image"). So the aim of this work was to display the decay time τ together with the mean fluorescence intensity I_F as a function of the position of the object with respect to the laser spot. This should allow to compare the information contained in I_F and τ and to get a survey of the spatial extension of variations of these two quantities.

EXPERIMENTAL ARRANGEMENT AND METHOD

A scheme of the setup is shown in Figure 1. A mode-locked argon ion laser (ILA 120 from Kombinat VEB Carl Zeiss Jena equipped with an acoustooptic modelocker) is used as excitation source which provides a continuous train of picosecond pulses (pulse duration: $t_P \approx 100$ ps; pulse separation: $T = 8.1$ ns; $\lambda = 514.5$ nm).

After attenuation the laser beam is focused onto the object by the objective of an epimicroscope JENALUMAR. A single additional negative lens at the entrance of the beam into the microscope is used to focus the laser in the object plane and to obtain a rather flat intensity distribution in the objective pupil which yields high spatial resolution. The resolving power as derived from the scan across a straight edge was found to be 0.6–0.7 μm for a high aperture objective (Apochromat 50×/0.95 ∞/0.17-A).

The fluorescence originating from the object is collected in backward direction by the objective and passes through a dichroic plate and color filters for eliminating scattered light. In the image plane a pinhole which is larger than the image of the laser spot removes background and scattered light.

The setup for time-resolving detection of fluorescence by the method of TCSPC consists of the electronic system SPC 100 (ZOS Berlin) and the photomultiplier FEU-77 (U.S.S.R.).

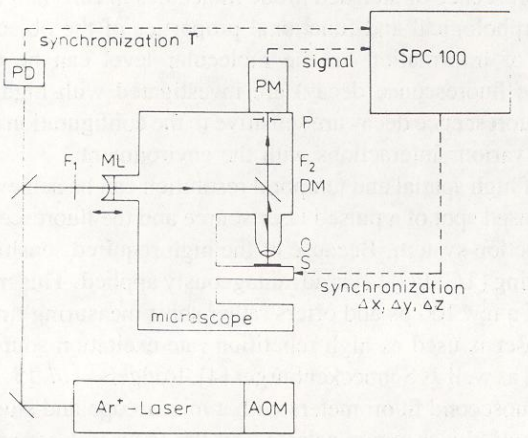

FIGURE 1 Experimental arrangement. AOM = acoustooptic modelocker; PD = photodiode; F_1 = attenuator; ML = matching lens; DM = dichroic mirror; O = objective; S = scanning stage; F_2 = block filters; P = pinhole; PM = photomultiplier; SPC 100 = electronic system for TCSPC

For a more detailed description see [8]. Single photon events are accumulated in a multichannel memory (256 channels per curve, maximum 65535 counts per channel, channel width usually 48.3 ps or 20.1 ps) yielding a histogram which represents the fluorescence decay. The time resolution of the whole system as given by the halfwidth of the response to scattered excitation light amounts to about 300 ps. Taking advantage of the high repetition rate of the excitation of ≈120 MHz, a rather high count rate up to 2×10^5/s can be processed which renders possible short measuring times. On the other hand, this corresponds to the detection of one photon per more than 600 excitation pulses. This elucidates the high sensitivity of the method.

For object scanning an x–y stage with a minimum step width of 0.5 μm is supplied to the microscope. The synchronization of step motion and measurement is realized by programs for the microprocessor of the system SPC 100.

This experimental arrangement allows two operation modes:

1) Recording of decay curves at fixed object position in a usual TCSPC measurement. For measuring times in the order of a few minutes decay curves with an excellent signal-to-noise ratio and a large dynamic range can be accumulated which are suited for a very accurate and detailed analysis of the time course of the relaxation process.

2) Much faster recording of one- and two-dimensional plots of the mean fluorescence intensity I_F and the fluorescence decay time τ while scanning the object. This is achieved by an interval method (cf. [9]) which gives a rather accurate estimate of τ (under the assumption of a single exponential decay law) within a measuring time per object step of about 1 s.

Three time intervals are chosen in the following way (cf. Fig. 2): Interval I begins after the

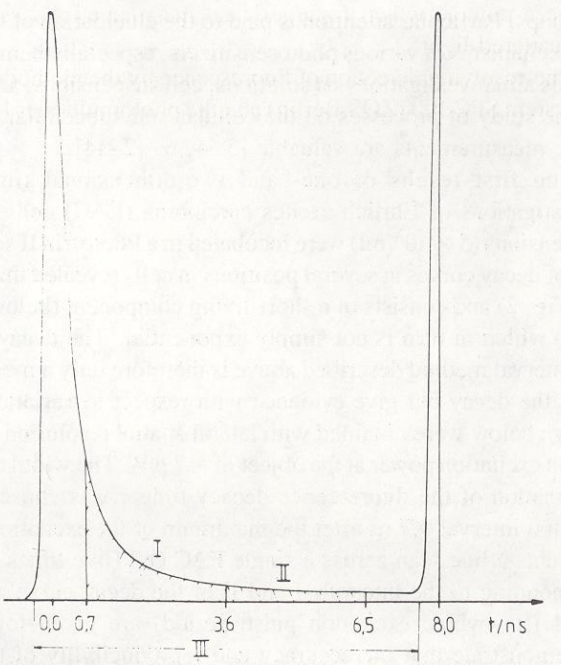

FIGURE 2 Fluorescence decay curve for an Ehrlich Ascites carcinoma cell with Photofrin II. The intervals for the following spatial scans are indicated.

end of the excitation pulse in order to suppress scattered excitation light and to be sure of free decay. Interval II of equal width ΔT begins just after the end of interval I. Interval III covers the whole excitation pulse period.

With these assumptions the parameter τ of the decay law

$$i_F(t) = i_F(0) \times \exp(-t/\tau) \tag{1}$$

and the stationary fluorescence intensity I_F (mean value over the whole excitation period) can be obtained by

$$\tau = \frac{\Delta T}{\ln(N_1/N_2)} \tag{2}$$

$$I_F \propto N_3 \tag{3}$$

from the sums of counts within the three intervals. After each object step only these sums have to be evaluated and stored instead of the whole decay curve.

The information content of the measurements can still be increased by additional spectral analysis of the fluorescence whereby a reduction of about 10 nm can be already sufficient, or by spatial depth discrimination with the help of a pinhole in the image plane with a size near the extension of the imaged laser focus which allows optical sectioning with a depth resolution comparable to the lateral resolution [10, 11].

APPLICATION IN THE INVESTIGATION OF INTRACELLULAR HPD FLUORESCENCE

Fluorescence diagnostics and photodynamic therapy of tumors is a field of intense work of many research groups. Particular attention is paid to the elucidation of the tumor localizing and destructing mechanisms of various photosensitizers, especially hematoporphyrin derivative (HpD). To this aim investigations on solutions, cell suspensions, and tumor models are performed. For the study of processes on the cellular and subcellular level time-resolved microfluorometric measurements are valuable [3, 4, 6, 12–14].

In the following first results of one- and two-dimensional time-resolved microfluorometric investigations of Ehrlich ascites carcinoma (EAC) cells with HpD are presented. EAC suspensions (5×10^6/ml) were incubated in a Photofrin II solution for one hour.

The recording of decay curves at several positions in cells revealed that the decay is rather complicated (cf. Fig. 2) and consists of a short-living component (below 1 ns) and a slower decay (several ns) which in turn is not simply exponential. The decay time τ determined according to the interval method described above is therefore only a mean value that cannot fully characterize the decay but give evidence with respect to variations.

The scans shown below were obtained with lateral spatial resolution (spot size) of about 0.7 μm and a mean excitation power at the object of ≈7 μW. The width of the intervals I and II for the determination of the fluorescence decacy time τ was chosen to be 2.9 ns, the beginning of the first interval 0.7 ns after the maximum of the excitation pulse (cf. Fig. 2).

Figure 3 represents a line scan across a single EAC cell (five times the same cell). The intensities corresponding to the intervals I and II of the decay curve as well as the mean intensity (interval III—whole excitation pulse period) are given together with τ. The repeated scans demonstrate that the accuracy and reproducibility of the measurement is satisfying. Within the cell there is a region of lower intensity which can be probably

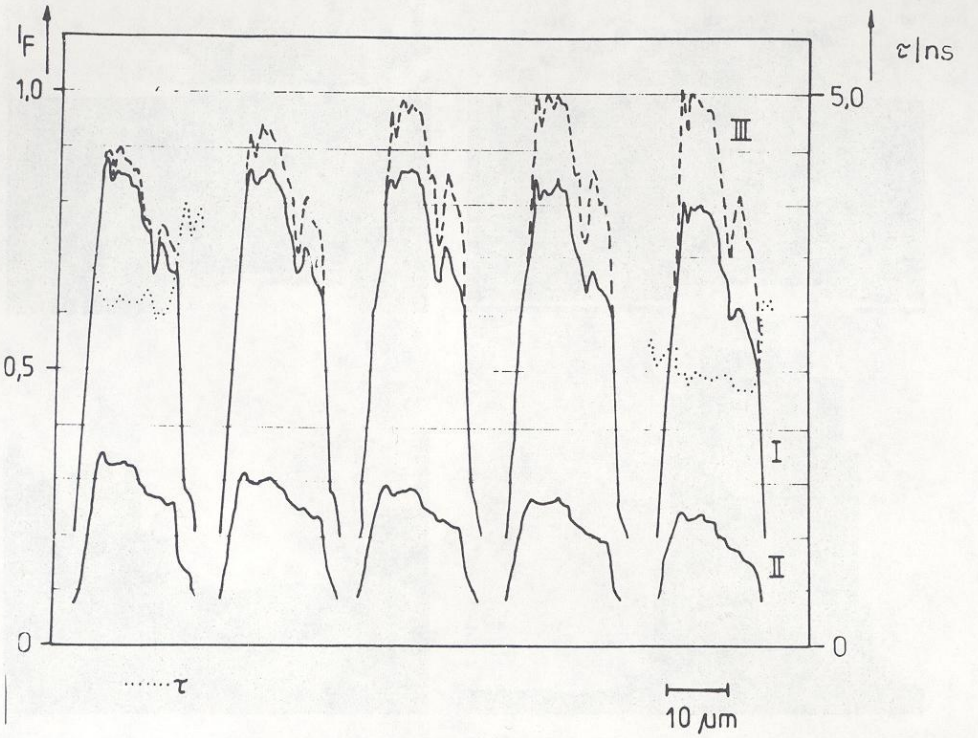

FIGURE 3 Line scan of an EAC cell (5 times the same cell), step width 0.5 µm. The intensities in the intervals I, II, and III are depicted together with τ.

identified with the nucleus. The differences between successive scans are due to photoinduced processes. Whereas the intensity in the intervals I and II decreases, I_F(III) is increasing which can be explained by an increase in the amplitude of a short-living component not covered by the intervals I and II. The decay time τ also offers reproducible structures not simply correlated with those in the intensity but with a tendency to increase at the cell boundary. Similar relations can be seen in a two-dimensional scan of another cell (Fig. 4).

CONCLUSIONS

These preliminary results demonstrate first of all that the method and its experimental realization described above are suited for obtaining distinct one- and two-dimensional pictures of I_F and τ for relevant cell objects. Conclusions concerning the spatial distribution of photosensitizers, components of them or other molecular species giving further insight into mechanisms of their uptake and photodynamic action should be possible.

However these measurements also revealed that individual cells of the same suspension behaved differently with respect to the value and spatial distribution of I_F and τ. Therefore reliable conclusions can only be drawn after the investigation of a greater number of cells of an as much as homogeneous suspension.

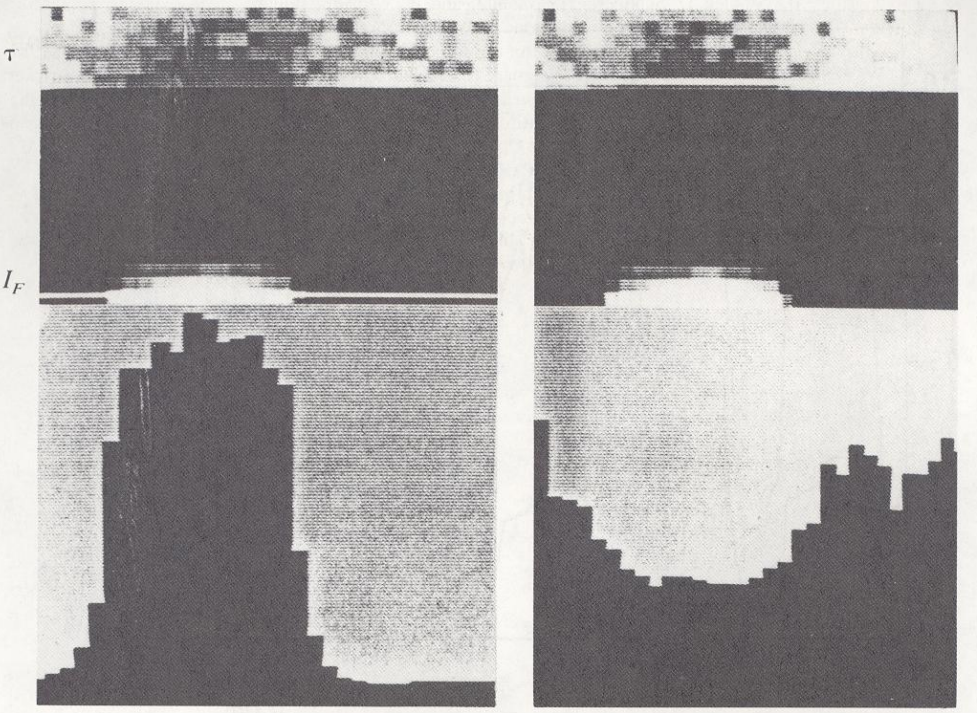

FIGURE 4 Two-dimensional scan of a part of an EAC cell, step width 1 μm. Upper part: $\tau(x, y)$ and $I_F(x, y)$, lower part: histograms along the lines indicated in the upper part.

Acknowledgments

The authors wish to thank Dr. H. Schubert for providing the EAC cell suspensions and Prof. B. Wilhelmi for helpful discussions.

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