

Time-resolved microfluorescence in biomedical diagnosis

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Abstract

A measuring system combining subnanosecond laser-induced fluorescence with microscopic signal detection was installed and used for diverse projects in the biomedical and environmental field. These projects are ranging from tumor diagnosis and enzymatic analysis to measurements of the activity of methanogenic bacteria which effect biogas production and waste water cleaning. The advantages of this method and its practical applicability are discussed.

Introduction

Time-resolved microfluorescence has proved to be a highly sensitive method, not only for the detection of fast kinetics, but also for analysing microscopic biological samples such as single cells¹, bacteria², nucleic acids^{3,4}, chromosomes⁵ or amino acids⁶. By using this method a high spatial resolution is combined with the measurement of fluorescence decay curves down to the subnanosecond range. Therefore, even low fluorescence signals of complex samples with spectrally overlapping bands can be detected on the basis of their characteristic decay times. In addition, stray light and background luminescence arising from the samples or the optical detection system are discriminated easily. An experimental setup for using this measuring technique was recently installed in our institute and applied to diverse projects in biomedical and environmental diagnosis, as described in the following.

Experimental apparatus

The samples were placed in a fluorescence microscope (Fig. 1) or a chamber for micro-cuvettes, and irradiated alternatively by the picosecond pulses from a modelocked UV Ar⁺ laser (364 nm) or a cavity dumped dye laser operated at 420 nm.

Pulses with a repetition rate of 250 kHz or 80 kHz, respectively, were selected from the original 82 MHz pulse train of the Ar⁺ laser in order to limit the average power density to 0.05 ... 0.1 mW/mm² and to avoid heating or damaging of the samples. The fluorescence decay curves were obtained after appropriate spectral filtering using a single photon counting system with a time-to-pulse height converter and statistical accumulation of the measured events. The time-resolution of the entire detecting system was about 0.3 ns.

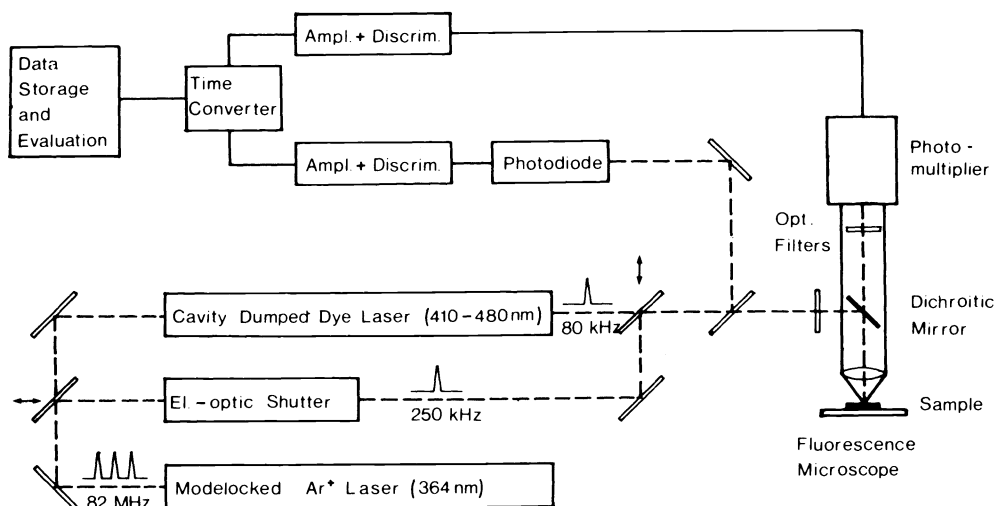


Figure 1. Experimental setup for time-resolved fluorescence microscopy.

Tumor diagnosis

For diagnosis and phototherapy of tumors fluorescent dyes, e.g. hematoporphyrin derivative (HpD), which are retained selectively in tumor cells, are used increasingly. The photosensitizing properties of HpD are well known, but many questions concerning the intracellular distribution and the phototherapeutic efficiency of distinct HpD components are still unresolved. Time-resolved fluorescence microscopy seems to be useful in treating these questions. First of all, this method allows different fluorescent compounds to be separated. As demonstrated in Fig. 2 two components with decay times of (9 ± 2) ns and (1.6 ± 0.5) ns were measured for Brown-Pearce tumor cells incubated with HpD.

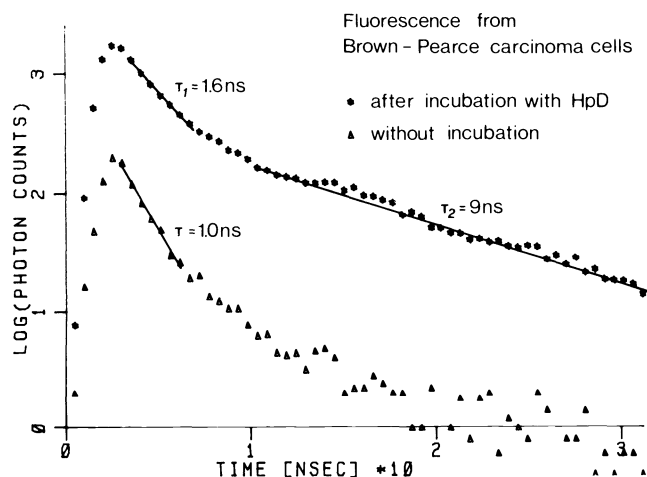


Figure 2. Fluorescence decay curves of Brown-Pearce carcinoma cells (excitation wavelength 420 nm, detection range 610-690 nm). The semilogarithmic plot reveals two exponential components for the cells incubated with HpD.

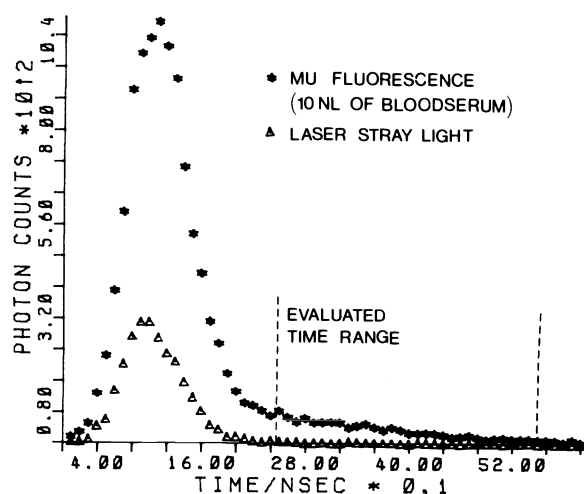
According to results obtained from HpD solutions⁸ these components might be attributed to monomers and aggregates with different phototherapeutic efficiencies, respectively. Also the autofluorescence of the cells can be distinguished from HpD fluorescence according to its decay time of about 1 ns, as obtained from the non incubated reference cells.

Preliminary measurements of the distribution of HpD within single urinary bladder cells (spatial resolution 10 μ m) showed higher fluorescence intensity around the cell nucleus as compared to different parts of the cytoplasm. This seems to prove a higher amount of HpD within the nucleus, the nuclear membrane or adjacent particles such as mitochondria.

Fluorogenic enzyme reactions

Fluorogenic enzyme reactions are used in the determination of small numbers of enzyme molecules or enzyme linked antibodies (enzyme immunoassay). Although the detection sensitivity of these reactions is usually rather high (one enzyme molecule typically produces $10^4 \dots 10^5$ fluorescent molecules per minute), it remains limited due to stray light and background fluorescence from the solvent or products of non-specific reactions.

An increase in detection sensitivity can be obtained using time-resolved measurements. Fig. 3 shows the fluorescence decay curve of 4-methylumbelliferone (MU) produced by enzyme coupled IgM antibodies against cytomegalovirus from only 10 nl of blood serum.



One short-lived component (decay time < 1 ns) is strongly superimposed by scattered laser light, but a second fluorescent component decaying with (2.0 ± 0.5) ns does not show this superposition. The integral fluorescence intensity of this component was determined within the time range indicated in Fig. 3 as a function of the quantity of blood serum. A linear relationship was obtained down to quantities of only 10 nl corresponding to about 10^{-15} moles of enzymatically produced MU. This detection limit is by 2 orders of magnitude lower than the limits so far obtained in conventional clinical tests.

Figure 3. Time-resolved fluorescence of enzymatically produced 4-methylumbelliferone (MU) in comparison with scattered laser light (excitation wavelength 364 nm, detection range 430-470 nm)

Methanogenic bacteria

Bacterial cells are often identified on the basis of specific fluorescent coenzymes. This also holds for methanogenic bacteria which are important for both biogas production and waste water treatment. Direct quantitative fluorometric determination of coenzyme F_{420} present in all methanogenic species, however, has so far been prevented by interference with other fluorescent compounds. Using time-resolved detection different decay times of (0.7 ± 0.3) ns and (2.5 ± 0.5) ns for F_{420} and 7-methylpterin, another fluorescent compound, respectively were obtained from samples of *Methanobacterium thermoautotrophicum*, and allowed these components to be separated.

In addition, active and inactive bacterial cells could be differentiated from measurements of fluorescence bleaching. When the irradiating power density was raised to about 5 mW/mm^2 or more, a significant photobleaching effect was observed. This fading was limited to the short-lived fluorescence due to coenzyme F_{420} when the bacteria were in an active state (Fig. 4), but affected all fluorescent components (F_{420} , 7-methylpterin and long-lived background fluorescence) when the bacteria were inactive (Fig. 5). Accordingly, by using this method it seems possible to control the activity of methanogenic bacteria during biogas production.

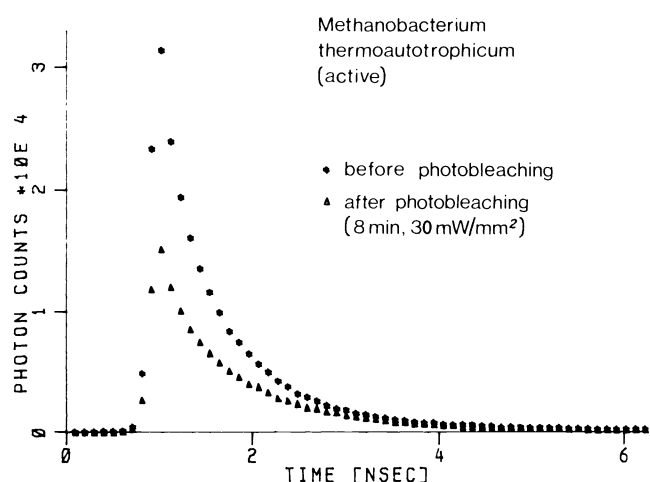


Figure 4. Time-resolved fluorescence of *Methanobacterium thermoautotrophicum* in active state (excitation wavelength 420 nm, detection range 460-570 nm). Reduction of the short-lived fluorescent component due to photobleaching.

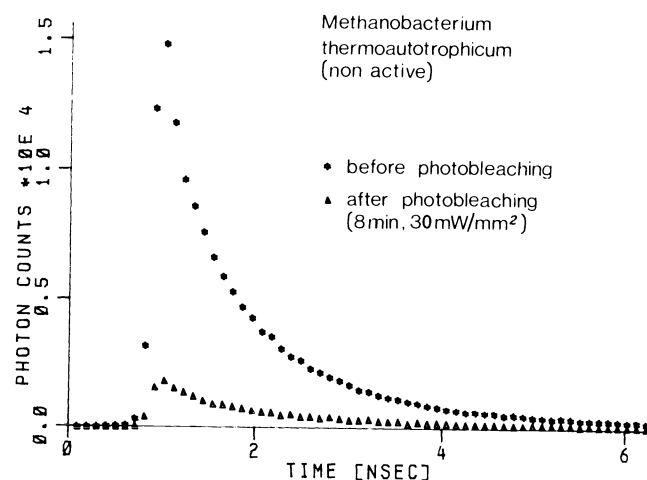


Figure 5. Time-resolved fluorescence of *Methanobacterium thermoautotrophicum* in inactive state (excitation wavelength 420 nm, detection range 460-570 nm). Reduction of all fluorescent components due to photobleaching.

Discussion and Outlook

As demonstrated for 3 projects, time-resolved microfluorescence is a powerful method for analysing complex samples in biomedical diagnosis or environmental analysis. However, according to its complexity the measuring system described in this paper seems to be reserved for basic or applied research. For routine applications, both the excitation source and the detection system must be modified, e.g. by using flashlamps or pulsed lasers and less sensitive detection electronics. In this case the temporal resolution is reduced from the subnanosecond to the nanosecond or even microsecond range, thus limiting the applicability of the method to processes with sufficiently long time constants. A diagnostic instrument for measuring long-lived fluorescent probes coupled to antibodies or antigens¹⁰ has recently been developed. Further apparatus, e.g. for measuring long-lived radiophotoluminescence of glass dosimeters¹¹ are in the stage of refinement. But also for surveying the activity of methanogenic bacteria in biogas reactors or sewage treatment plants, time-resolved fluorescence seems to be an appropriate method, if instead of nanosecond decay times, the time course of photobleaching² is determined within the range of several seconds.

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