Journal of Fluorescence, Vol. 15, No. 3, May 2005 (© 2005)
DOI: 10.1007/s10895-005-2634-z

Improved Routine Bio-Medical and Bio-Analytical Online Fluorescence Measurements Using Fluorescence Lifetime Resolution

Lutz Pfeifer,[1,5] Karsten Stein,[1] Ute Fink,[1] Alexander Welker,[2] Bianca Wetzl,[3] Petra Bastian,[4] and Otto S. Wolfbeis[3]

Received August 10, 2004; accepted January 11, 2005

Fluorescence techniques are widely used as sensitive detection methods in bio-analytics. The use of the bio-physical parameter fluorescence lifetime additional to the spectral characteristics of fluorescence has the potential to improve fluorescence-related detection methods in terms of selectivity in signal recognition, robustness against disturbing influences, and the accessibility of novel bio-chemical process parameters. This article describes the technical set up of a time-resolving instrument with either a fixed time-gated detection principle for improved evaluation of tissue metabolism by an online monitoring of the tissue autofluorescence or a direct fluorescence lifetime detection principle for lifetime-based fluorescent assays.

KEY WORDS: Time-resolved fluorescence; fluorescence lifetime; fluorescence assay; autofluorescence; metabolism.

INTRODUCTION

The measurement of fluorescence signals is of increasing importance in medical and bio-chemical analytics. An important topic of scientific work is the development of fluorescence-based methods for the *in-vivo* characterisation of human and animal tissues [1,2]. Aims of these efforts among others are the localisation and characterisation of cancer tissue, the characterisation and monitoring of the tissue metabolism and the investigation of ischemic tissue states [3–5]. In bio-analytics fluorescence measurements are routine in various applications in molecular biology, drug screening and medical diagnostics [6,7].

At present technologies used in routine analytics are steady state measurements of fluorescence intensities, of fluorescence spectra and fluorescence polarisation. The measurement and analysis of fluorescence lifetimes and of the time-resolved fluorescence polarisation have been limited so far to special research laboratories due to expensive laser and detection technology and the required detailed physical experience.

Fluorescence lifetimes of most fluorophores extend over about some picoseconds up to approximately 10 ns. The characteristic decay times of some fluorescing compounds may reach the microsecond scale (Pyren, metal complexes) or even the millisecond scale (lanthanide complexes).

For detection of fluorescence decay times light sources with nano- or sub-nanosecond pulse durations of modulated light intensities with frequencies up to 1000 MHz are required. The time-resolved fluorescence signals are recorded by detectors with bandwidths on gigahertz scale and the corresponding fast detection electronics. Usually this is done with the methods of

[1] IOM Innovative Optische Messtechnik GmbH, Berlin, Germany.
[2] Deutsches Herzzentrum Berlin, Stiftung des bürgerlichen Rechts, Berlin, Germany.
[3] Institute for Analytical Biochemistry and Sensors, University Regensburg, Germany.
[4] Chromeon GmbH, Regensburg, Germany.
[5] To whom correspondence should be addressed. E-mail: info@iom-berlin.de

time-correlated single-photon counting or MHz phase modulation measurement.

The importance of the detection and analysis of the fluorescence lifetime is the direct correlation of this physical value based on the chemical state of the fluorophore molecules under investigation [8]. Fluorescence lifetimes are dependent on the chemical structure of the molecules and their interaction with solvent molecules or bio-molecules such as proteins and peptides, nucleic acids or molecules of the cellular membrane. In contrast to simple spectrally resolved measurements the detection of fluorescence lifetimes may yield direct information about binding events, the localisation of a certain fluorophore in a cell or the presence and concentration of quencher molecules.

This article describes a technical approach for the development of lifetime resolving fluorescence detection systems for routine bio-analytical applications. It is based on robust and reliable Nitrogen lasers now available which produce intense laser pulses at 337 nm with durations between 0.5 ns and 3.5 ns. On this basis, compact and powerful fluorescence instruments with a great variety of excitation wavelengths can be designed. Using such instruments fluorescence lifetimes even in the sub-nanosecond range may be recovered. Applications in different fields of bio- and medical analytics are presented to demonstrate objectives and advantages of lifetime-resolved fluorescence detection.

MATERIALS AND METHODS

The basic set-up of the time-resolving detection system is shown schematically in Fig. 1.

For excitation of the fluorescence signals, a Nitrogen laser (type MNL 200, LTB GmbH, Germany) with 100 μJ pulse energy and 0.7 ns pulse duration is used. The excitation wavelength is 337 nm. The laser beam is focused by a cylindrical lens into a dye laser. The dye laser consists of

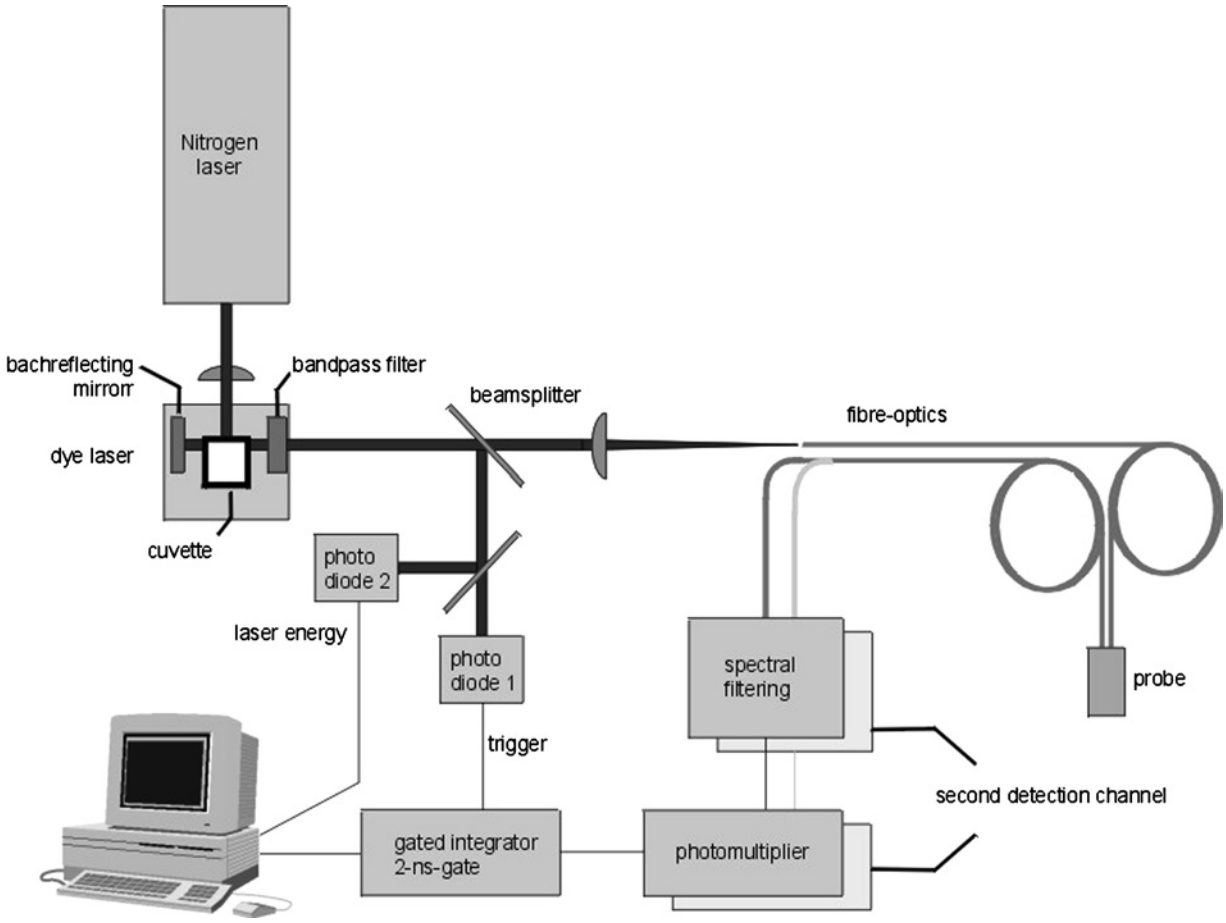

Fig. 1. Set-up of the time-resolving fluorescence detection system.

a cuvette filled with a laser dye solution, a back-reflecting mirror and a bandpass filter for a spectral clean-up of the dye laser beam. Cuvette and bandpass filter are mounted in a high precision black case. This case enables as well the easy exchange of the cuvette in the dye laser as the protection of the laser dye from light that could cause photo destruction of the dye molecules. Additionally, the dye laser contains a beam splitter facilitating the measurement of the laser pulse energy and the generation of a trigger signal with two photo diodes. After the beam splitter the laser beam is focused into an optical fibre by means of achromatic lens.

This simple set up represents a reliable laser source for the flexible production of sub-nanosecond laser pulses with wavelengths between 337 and 1000 nm using a variety of different laser dyes (Radiant Dyes, Wermelskirchen, Germany). Due to the nearly completely closed construction of the laser cuvette housing, the working cycle with almost constant pulse energy reaches at least 5 million pulses demonstrated by an experiment with Coumarin 152A at 488 nm where the single pulse energy was monitored over 5 million pulses.

The excitation light is transferred into the sample by an optical fibre via a fibre-optic probe. Typical core diameters of the used fibres range from 100 μm to 600 μm depending on the outline of the probe. Within the probe usually one fibre is used for excitation and a second fibre for collection of the sample fluorescence. For bioanalytical monitoring experiments the fibre-optic probe is outlined with a probe tip made of a stainless steel cannula. In this cannula the fibres are glued in and polished to optical quality. For measurements, where a direct contact between probe and sample is not possible, a detection head is applied which focuses the excitation light into the sample by means of lenses. Using a dichroic mirror, the fluorescence signal is coupled into the second fibre. The value of the laser pulse energy lead into the sample usually ranges between 0.5 and 2 μJ.

The second fibre guides the collected fluorescence light back to the detector. For detector, a photomultiplier (H 5783, Hamamatsu, Japan) is utilised. Before the light reaches the photocathode of the PMT, it is spectrally filtered by a bandpass filter to prevent back-scattered excitation light of saturating the detector. This module, consisting of the PMT, the bandpass filter, and the collimating lens can be doubled in the technical set up. Thus, two fluorescence signals at two different wavelengths can be detected simultaneously.

The signals resulting from the PMT are characterised by a pulse shape. Due to the short duration of the laser pulses (0.7 ns) and the fast detectors rise time (0.65 ns), every detector pulse carries the characteristic temporal fluorescence behaviour of the fluorescence process provided that the fluorescence lifetime is longer than approximately 1 ns (see Fig. 2b).

The time-resolved signal recovery is performed using a gated integrator with a 2-ns-gate. The gate position is controlled and adjusted from a micro controller. The precision of the adjustment is 0.1 ns. The total gate delay is limited to 25.5 ns. This means that fluorescence decays can be sampled up to 25,5 ns with a maximal resolution of 0.1 ns (see Fig. 2b).

The sampled signal intensities are digitalised and displayed in terms of relative fluorescence units (rfu) on a PC. A maximal value of 100 rfu corresponds to the saturation level of the integrator. In order to improve signal-to-noise ratio, averaging over a variable amount of laser pulses can be performed.

Operation Modes

There are two different modes of operation possible in depending on the application.

1. In the first mode, online measurements with fixed and adjusted integration gates are performed. This method should be used if one or more fluorophores with known fluorescence lifetime characteristics have to be monitored. If the lifetimes of two fluorophores differ significantly the "time-gated" signal recognition can be used to improve the differentiation between both signals despite spectral overlapping. This can be performed by positioning the gate for the fluorophore with the short lifetime in the signal maximum and delaying the gate for the fluorophore with the long lifetime by some nanoseconds (Fig. 2a). Though the signal separation for both fluorophores is not complete a significant improvement of the separation is achieved and it can be further improved by an additional spectral filtering of the superimposed signal. In this way a long-term monitoring of superimposed fluorescence signals with improved separation is possible.
2. In the second operation mode it is possible to observe the change of a fluorophore lifetime during a process with a certain precision to quantify process parameters. In such cases the repeated measurement and analysis of complete decay curves is required. This is the case in fluorescent assays where the fluorescence lifetime of the involved marker molecule is changing on a binding event. The evaluation of the fluorescence lifetime using fluorescence decay curves (Fig. 2b) is performed by a numerical deconvolution procedure (IOM

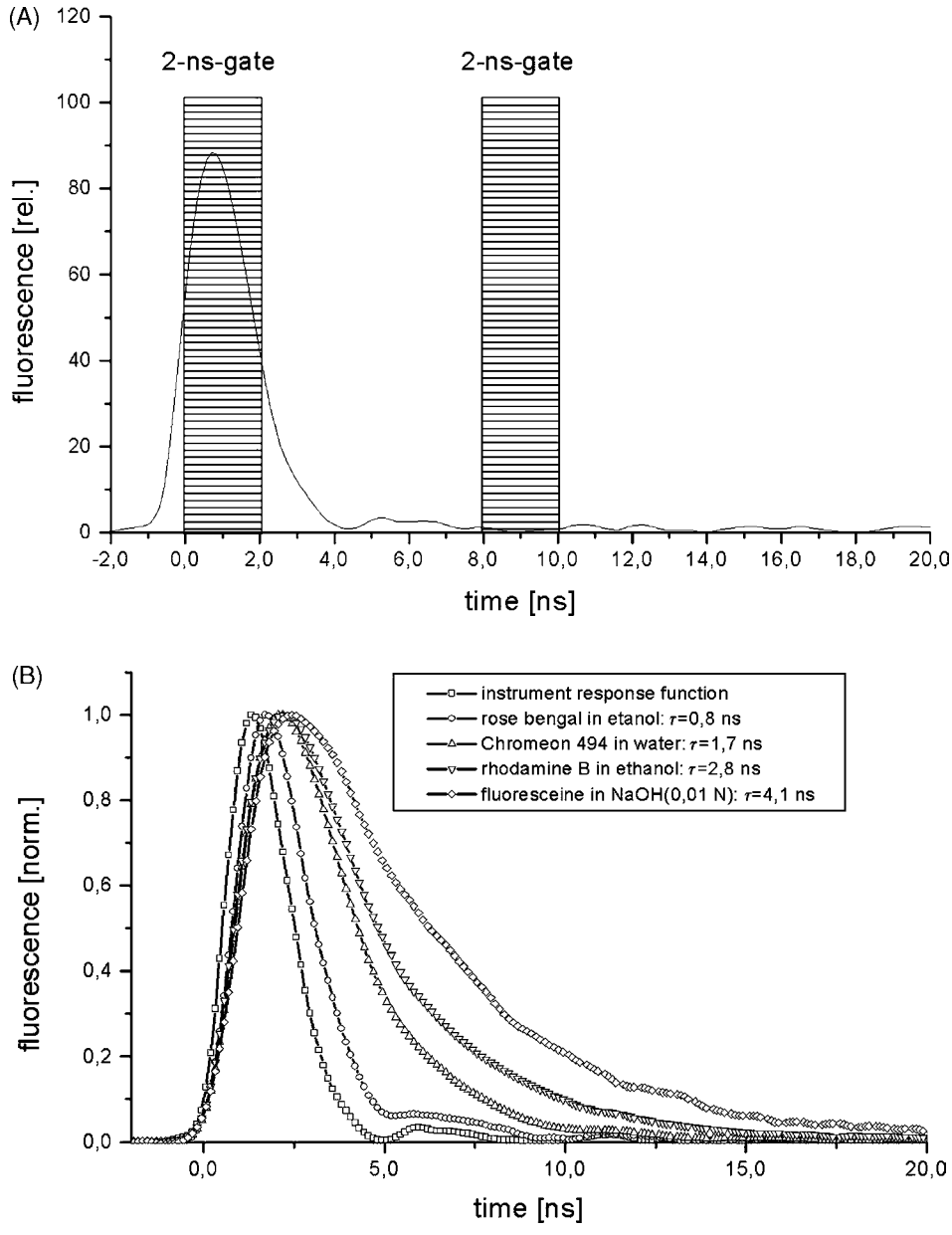

Fig. 2. (a) Response of the PMT after detection of reduced back-scattered laser light and comparison with 2-ns integration gates. (b) Fluorescence decay of different dyes detected with the time-resolving set up.

GmbH, Germany), which is based on the least square fitting algorithm by Marquardt and Levenberg. The software displays only the analysed lifetimes of the assay as a result of the process under investigation.

Figure 3 shows the measured fluorescence decay curve of fluorescein in NaOH as an example. The numerical fit curve is in good accordance to the observed fluorescence curve.

RESULTS AND DISCUSSION

Time-Resolved Signal Gating

The gating method based on fixed integration gates has to be used when fluorohores with significantly different lifetimes have to be monitored and when the signal ratio is of main interest instead of the signal intensities.

This is the case when the intrinsic fluorescence of cells and tissues has to be detected. By measuring this

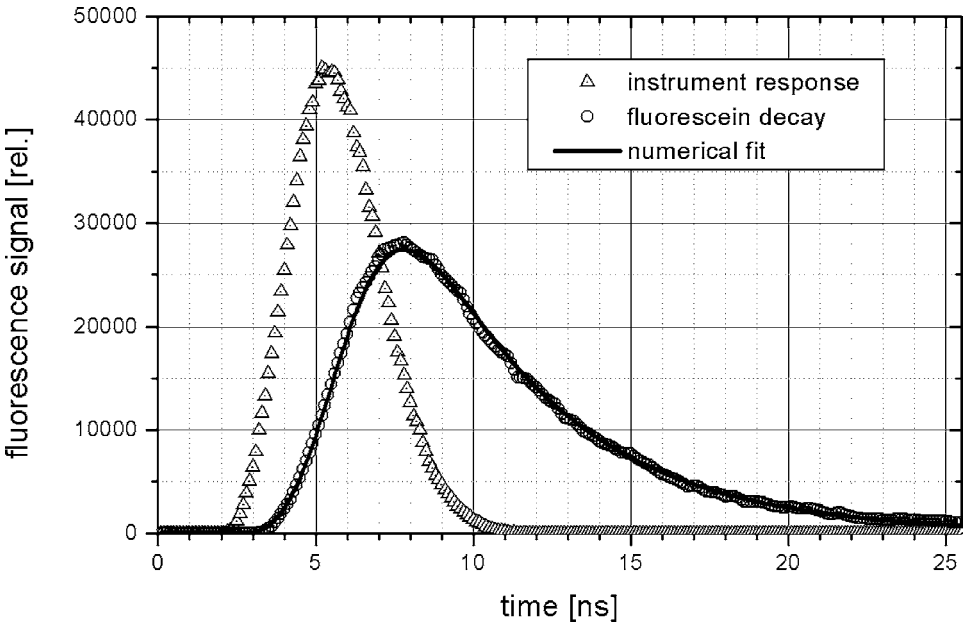

Fig. 3. Fluorescence decay curve of fluoresceine in NaOH together with the instrument response function and the fit curve.

so-called autofluorescence, an online monitoring of the metabolic state of cells and tissues becomes possible: the pyridine nucleotide Nicotinamide-Adenine-Dinucleotide (NADH, reduced form) is one of the predominant fluorophores of the cells under UV-excitation whereas different flavine species (oxidised form) have similar fluorescence properties. These molecules play an important role in the metabolic chain and their relative concentrations reflect the type and intensity of cellular metabolism [9].

If a selective and simultaneous measurement of the NADH and flavine molecules is achieved an online monitoring of the metabolic state of complete explanted organs with intended use in organ transplantation could be realised. The knowledge of the organ state is basic information that could be very helpful for the success of organ transplantations. Furthermore, the characterisation of the tissue metabolism could be exploited for information concerning abnormal tissue transformations, e.g. in tumors [10–14].

Selective Simultaneous Detection of NADH and Flavine

The reduced NADH is characterised by absorption maximum at 340 nm, a maximum of the fluorescence spectrum at 465 nm and a mean fluorescence lifetime of 0.4 ns [15]. Flavines have absorption maxima at 375 and 450 nm, a fluorescence maximum at 535 nm, and fluorescence lifetimes between 2.3 and 5.0 ns, depending on the type of flavine molecule [16]. Both molecules can be excited with UV light from a Nitrogen laser at 337 an.

Figure 4a displays the fluorescence spectra of NADH and FAD (Flavine-Adenine-Dinucleotide). This diagram illustrates that a selective detection only of NADH could be achieved by simple spectral filtering of the superimposed fluorescence intensity of both molecules. Detecting FAD at 530 nm would result in a significant signal contribution of NADH to the measured signal. As shown in Fig. 4b, the fluorescence decay of FAD occurs significantly slower than those of NADH.

On the basis of this spectral and temporal behaviour of the fluorescence signals of both molecules the gate position for the selective detection of both fluorescence signals was fixed. The 2-ns-gate for the detection of NADH is positioned into the temporal maximum of the fluorescence whereas the gate for the flavine detection is delayed by 9 ns. At this time the short NADH fluorescence has completely disappeared. The signal reduction of the flavine fluorescence is compensated by an increase in the PMT amplification voltage.

By means of aqueous solutions of NADH and FAD the performance of the time-gated set up was demonstrated (Fig. 5). The fibre-optic probe used consisted of one fibre with 100 μm core diameter for fluorescence excitation and two fibres with 200 μm core diameter for fluorescence detection. The external diameter of the probe tip was 1.0 mm.

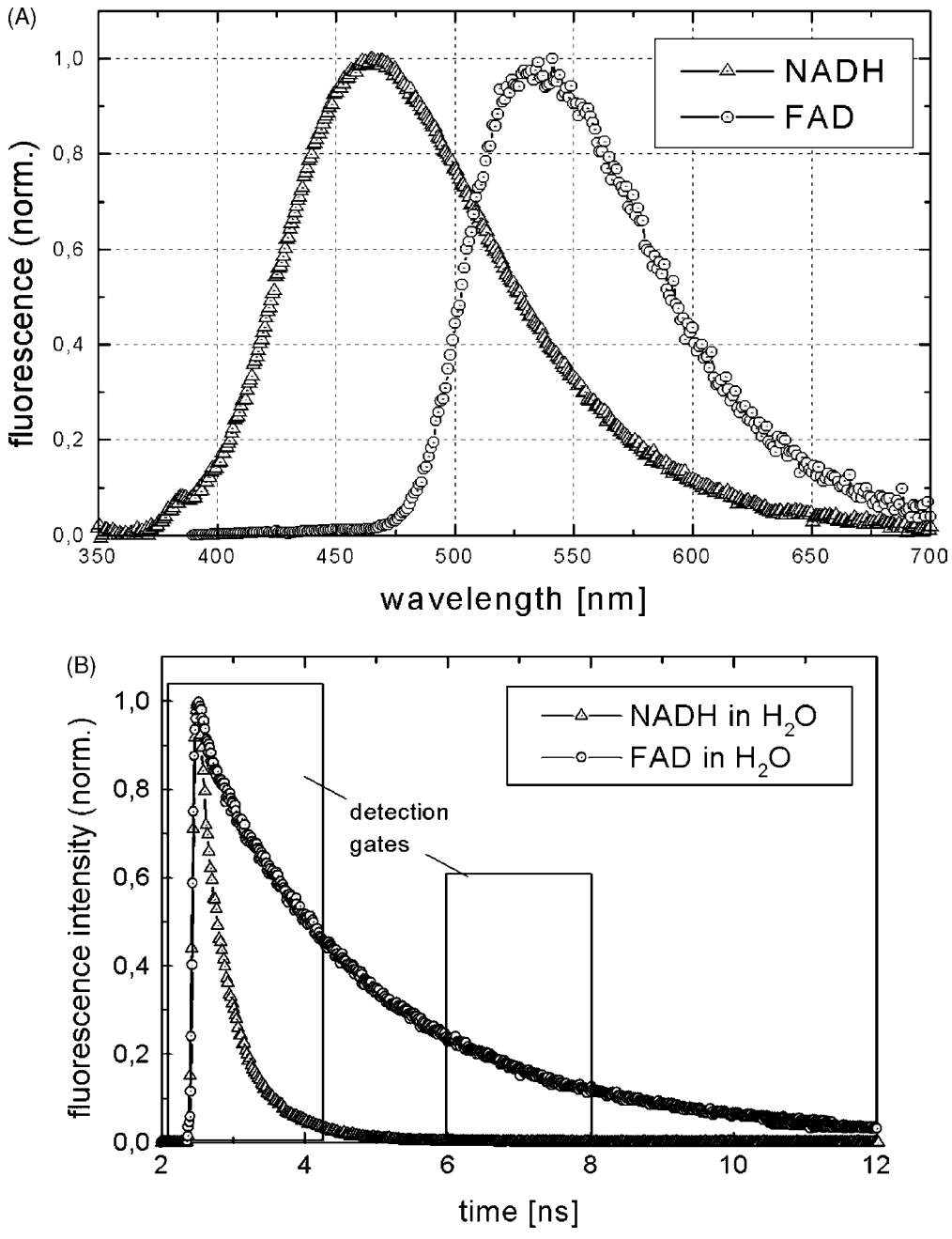

Fig. 4. (a) Normalised fluorescence spectra of NADH and FAD in water. (b) Fluorescence decay curves of NADH and FAD (measured with a time-correlated single photon counting set up).

Sequentially, the fluorescence signals of the probe in air, water, NADH solution ($c_1 = 1 \times 10^{-5}$ mol/l), FAD solution ($c_2 = 1,67 \times 10^{-5}$ mol/l), and the mixture of NADH and FAD, have been acquired. The technical set up was equipped with two detection channels. For the spectral filtering of the NADH channel, a 10-nm-bandpass filter centred at 456 nm was used. In the flavine channel, a 10-nm-bandpass filter was centred at 532 nm. The temporal position of the integration gate in the NADH channel was adjusted to the maximum of the signal. In the flavine channel, the gate position was delayed by 9 ns. Excitation of the fluorescence occurred with 337 nm.

While keeping the probe tip into the air, both channels showed no signal. In water the signal in the NADH

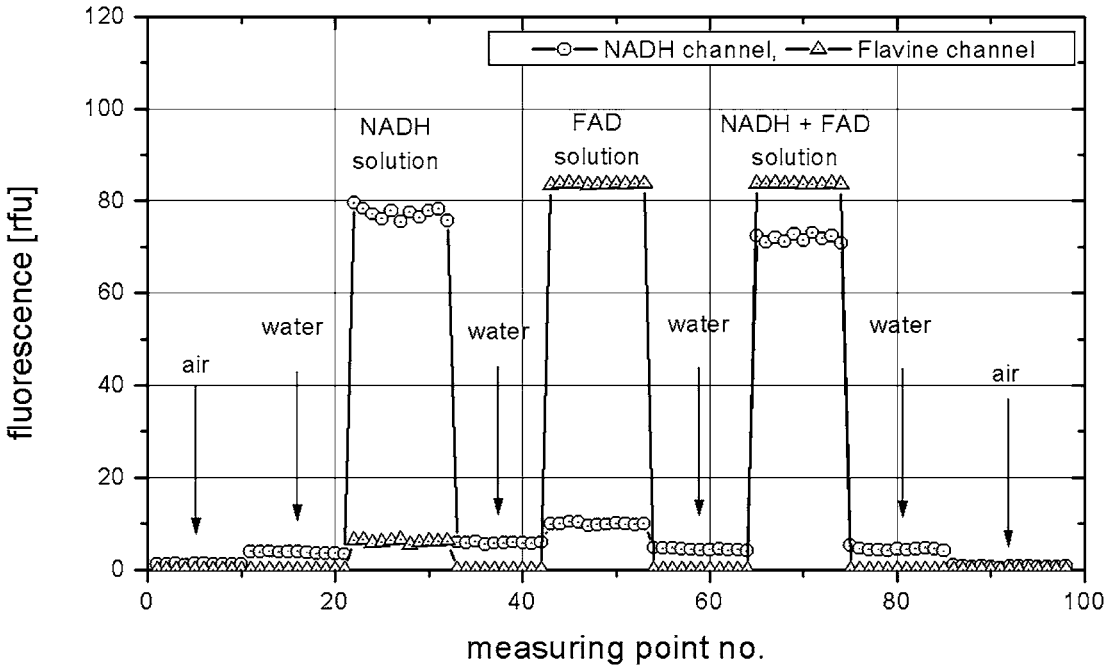

Fig. 5. Measurement of aqueous solutions of NADH and FAD by gated signal detection.

channel was slightly increased, and in the flavine channel it remained at zero. While keeping the probe tip in the NADH solution, the signal in the NADH channel was increased, as expected, to nearly 80 rfu whereas the flavine signal remained approximately at zero. This illustrates that the underlying NADH signal in the flavine channel (see Fig. 4a) is extensively discriminated by the delayed positioning of the integration gate. Thereafter the probe tip was cleaned in water. The probe tip in the flavine solution revealed a significant increase in the flavine channel only. The NADH signal slightly increased to 10 rfu. In the fluorophore mixture, both signals were detected.

Time-resolved Online Monitoring of NADH and Flavine

The application of this method for online measurement of tissue autofluorescence provides several advantages compared to the common steady state fluorescence measurements.

In contrast to the detection of the fluorescence intensity of NADH, the selective and simultaneous measurement of different co-enzymes of the metabolic chain enables a better quantification of the metabolic state of the sample. The calculation of the ratio of two or more signals allows the compensation of artefacts that would strongly influence the intensity measurement in living tissues. Amongst others, such influences could be based on varying optical properties of the tissue, e.g. due to differences in light absorption due to varying blood flow in micro capillaries, or changed light scattering properties due to oedemas and necrotic tissue areas [17]. All differences in tissue spectral properties would greatly influence the detected signal intensities. Ratios of different signals are influenced to a much lower degree. The signal ratio is mainly dependent on changes of the intracellular molecular concentrations.

Further advantage of the time-resolved signal gating with low-repetition rates of excitation is the insensitivity of the method against light from the surrounding, e.g. from lamps or daylight. This is due to the small probability of the photons from these light sources to fall into the 2-ns-gate of the detection process. As a result, there is no need to darken the surrounding area of the region of interest during the measurement.

During a study at the German heart centre Berlin (DHZB) on explanted and *in vitro* perfused pig hearts, it has been shown that the selective and parallel detection of two intrinsic fluorophores and the calculation of the ratio of both signals represents a more objective method for monitoring the tissue metabolism than the former methods based on intensity measurements of single fluorophores [18–20]. In comparison to other methods, the time-resolved approach is influenced by changing light absorption and light scattering conditions to a significantly

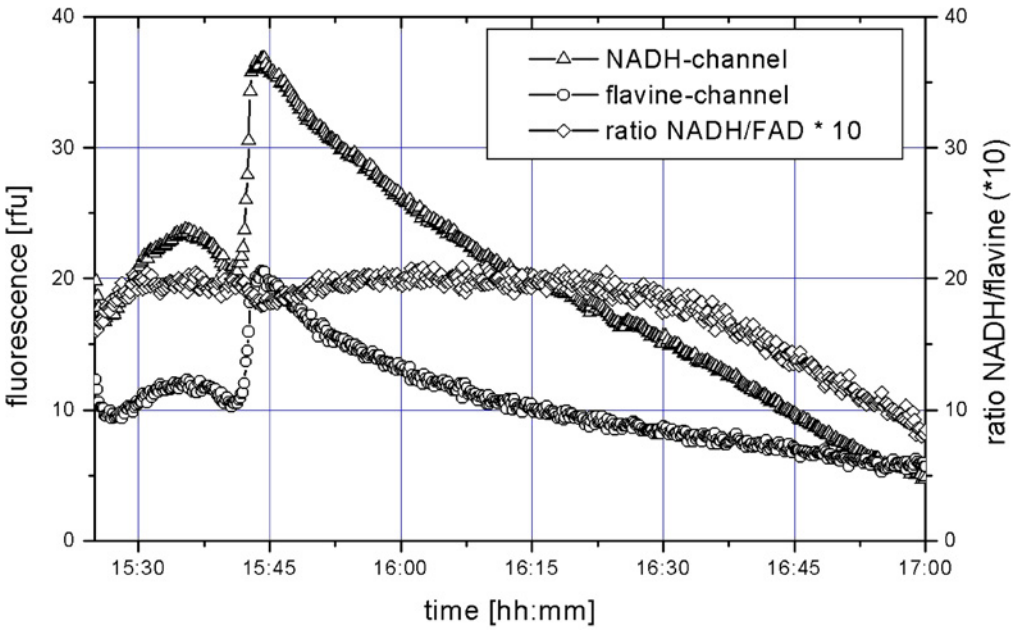

Fig. 6. Online monitoring of the NADH and flavine surface fluorescence of the beating pig heart.

smaller extent in the living tissue during periods of several hours.

Figure 6 exemplarily illustrates as an example the temporal course of the intrinsic NADH and flavine fluorescence during the steady state phase of a long term *in vitro* perfusion session of an explanted pig heart (for details see [18]). For the measurement, the same setting of the excitation and detection wavelengths and the integrator gates was used as previously for the solution experiment. The fibre-optic probe was positioned on the surface of the heart muscle in the area of the left vestibulum and remained there for the duration of the experiment. During the measurement, significant signal variations had been stated as well in the NADH as in the flavine signal. In total, the tendency directed towards decreasing fluorescence intensities. Despite the signal changes, which may be caused by varying perfusion conditions or a varying oxygen supply, the ratio of both signals remained nearly constant for about 60 min. After this interval the ratio of the signals began to fall additionally to the intensities. This may indicate the ongoing damage and the slow process of destruction of the tissue resulting in the formation of oedema and necrosis. This explanation of the process is supported by the parallel determination of biochemical blood parameters like pH, blood oxygen, blood CO_2 and the Troponin concentration. The latter is a biochemical marker of cell destruction in heart muscle tissue.

The end of the steady state phase of the *ex vivo* beating heart is indicated by a significantly reduced capacity of pumped blood, a decreasing beat frequency and starting arrhythmias of the heart beats.

This small study of 10 investigated hearts in total demonstrated the reproducibility of the method of time-gated signal detection of multiple intrinsic fluorophores of heart muscle tissue for the evaluation of the tissue metabolic state.

Online Analysis of Fluorescence Lifetimes

The online measurement of fluorescence decay curves and instantaneous analysis of fluorescence lifetimes is required when the bio-physical parameter of fluorescence lifetime indicates a distinct state of the sample under investigation. As a rule, this value indicates changes in the molecular structure and molecular interactions of the fluorophore molecule involved in processes to be analysed.

As a bio-physical constant, the fluorescence lifetime is independent on the measuring set up and other parameters influencing simple fluorescence intensity measurements, e.g. varying signal amplitudes due to varying sample volumes, light scattering, inner filter effect, etc. [6,7].

Pharmaceutical drug screening is another most important field of bio-analytics where detailed information about molecular conditions are required. Big compound libraries have to be screened for molecular binding reactions. The direct change in fluorescence lifetime of special

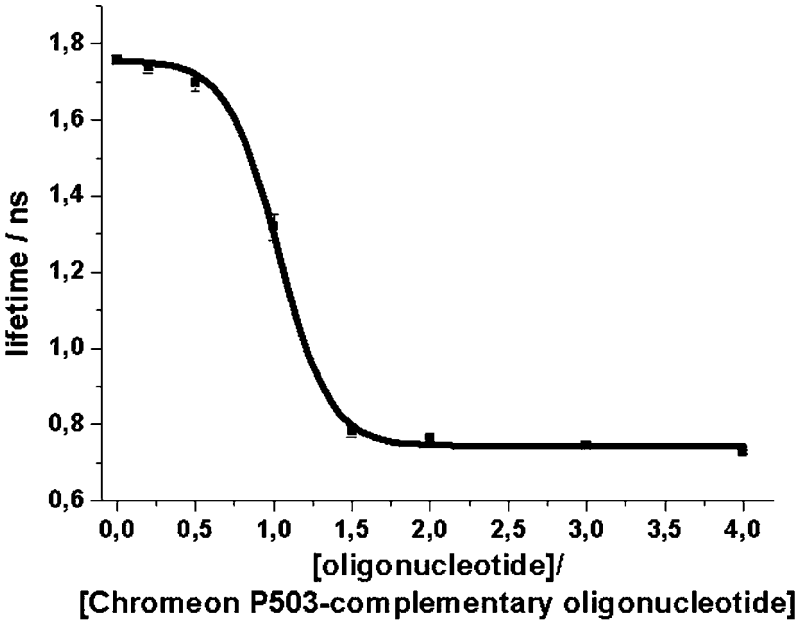

Fig. 7. Hybridisation of oligonucleotides as an example for a fluorescence lifetime assay.

fluorophores by binding events of biomolecules allows the design of simple and fast assays. Furthermore, these assays provide good sensitivity and robustness in comparison to assays based on intensity measurement. Thus, they will become of significant importance for an efficient screening for candidates in drug development [6].

An example for such a binding reaction represents the hybridisation of two complementary oligonucleotides. An amino-linked 15-mer oligonucleotide is labelled with Chromeon P503 (Chromeon GmbH, Regensburg, Germany). If the binding of the marked oligostrand with the unmarked counter strand occurs a significant and concentration dependent alteration in the fluorescence lifetime is achieved [21].

In this experiment, excitation occurred at 505 nm. Fluorescence was observed at 630 nm with a filter bandwidth of 50 nm. The samples have been prepared in a black 96-well plate (F96, Nunc, Danmark). Since a non-contact measurement was required the optical detection head with a dichotic mirror was placed above the plate and the cavities of the plate were measured sequentially.

A concentration series was performed with constant concentration of the marked oligonucleotide. The sample volume in each microplate cavity was set to 150 μl. After incubation of 30 min, the measurements were performed.

The numerical curve analysis used a single-exponential decay law for fitting of the fluorescence decay curves. This assumption yields the value of a mean fluorescence decay time even in cases where a mixture of hybridised and free oligonucleotides occurs in the solution and thus a multi-exponential law would be more appropriate.

It was found that the mean fluorescence lifetime in the hybridised state is near 0.8 ns and in the free state around 1.7 ns. This large shift in the fluorescence lifetime on binding makes the method suitable for the development of a fluorescence lifetime assay (Fig. 7).

SUMMARY

It has been demonstrated that technical concepts for the development of analytical instruments based on short-pulsed and low frequency Nitrogen lasers could be realised, which use the time-resolved approach of measuring fluorescence signals for an improvement of the significance and reliability of the applications behind the fluorescence detection.

Two modes of operation have been shown to be valuable for routine applications: the time-resolved signal gating and the online fluorescence lifetime measurement. Both methods use characteristic lifetime changes for the evaluation of bio-analytical processes.

REFERENCES

1. C. Ince, J. M. C. C. Coremans, and H. A. Bruining (1992). In vivo NADH fluorescence. *Adv. Exp. Med. Biol.* **317**, pp. 277–296.

2. I. J. Bigio and J. R. Mourant (1997). Ultraviolett and visible spectroscopies for tissue diagnostics: Fluorescence spectroscopy and elastic-scattering spectroscopy. *Phys. Med. Biol.* **42**, 803–814
3. R. R. Alfano, G. C. Tang, A. Pradhan, W. Lam, D. S. J. Choy, and E. Opher (1987). Fluorescence spectra from cancerous and normal human breast and lung tissus. *IEEE J. Quant. Electr.* **QE-23**(10), 1806–1811.
4. K. Svanberg, S. Andersson-Engels, L. Baert, E. Bak-Jensen, R. Berg, A. Brun, S. Colleen, I. Idvall, M.-A. D'Hallewin, C. Ingvar, A. Johansson, S.-E. Karlsson, R. Lundgren, L. G. Salford, U. Stenram, L.-G. Strömblad, S. Svanberg, and I. Wang (1994). Tissue charakterization in some clinical specialities utilizing laser-induced fluorescence. in R. R. Alfano and A. Katzir (Eds.), *Proceedings of the Advances in Laser and Light Spectroscopy to Diagnose Cancer and Other Diseases, SPIE Vol 2135* pp. 2–15
5. K. T. Schomacker, J. K. Frisoli, C. C. Compton, T. J. Flotte, J. M. Richter, N. S. Nishioka, and T. F. Deutsch (1992). Ultraviolett laser-induced fluorescence of colonic tissue: Basic biology and diagnostic potential. *Laser Surg. Med.* **12**, 63–78.
6. A. Fowler, D. Swift, E. Longman, A. Acornley, P. Hemsley, D. Murray, J. Unitt, I. Dale, E. Sullivan, and M. Coldwell (2002). An evaluation of fluorescence polarization and lifetime discriminated polarization for high throughput screening of serine/threonine kinases. *Anal. Biochem.* **308**, 223–231.
7. C. Eggeling L. Brand, D. Ullmann, and S. Jäger (2003). Highly sensitive fluorescence detection technology currently available for HTS. *DDT* **8**(14), 632–641.
8. J. R. Lakowicz (1999). *Principles of Fluorescence Spectroscopy*, 2nd edn. Plenum Press, New York.
9. B. Chance, P. Cohen, F. Jöbsis, and B. Schoener (1962). Intracellular oxidation–reduction states *in vivo Science* **137**(3529), 499–507.
10. H. A. Bruining, G. J. M. Pierik, C. Ince, and F. Ashruf (1992). Optical spectroscopic imaging for non-invasive evaluation of tissue oxygenation *Chirurgie* **118**, 317–323.
11. J. Eng, R. M. Lynch, and R. S Balaban (1989). Nicotinamide adenine dinucleotide fluorescence spectroscopy and imaging of isolated cardiac myocytes. *Biophys. J.* **55**, 621–630.
12. M. W. Riepe, K. Schmalzigaug, F. Fink, K. Oexle, and A. C. Ludolph (1996). NADH in the pyramidal cell layer of hippocampal regions CA1 and CA3 upon selective inhibition and uncoupling of oxidative phosphorylation. *Brain Res.* **710**, 21–27.
13. M. Büchner, R. Huber, C. Sturchler-Pierrat, M. Staufenbiel, and M. W. Riepe (2002). Impaired hypoxic tolerance and altered protein binding of nadh in presymptomatic App23 transgenic mice. *Neuroscience* **14**(2), 285–289.
14. S. Schuchmann, R. Kovacs, O. Kann, U. Heinemann, and K. Buchheim (2001). Monitoring NAD(P)H autofluorescence to assess mitochondrial metabolic function in rat hippocampal-entorinal cortex slices. *Brain Res. Protocols* **7**, 267–276.
15. J. R. Lakowicz, H. Szmacinski, K. Nowaczyk, and M. L. Johnson (1992). Fluorescence lifetime imaging of free and protein-bound NADH *Proc. Natl. Acad. Sci. USA* **89**, 1271–1275.
16. W. S. Kunz, A. V. Kuznetsov, K. Winkler, F. N. Gellerich, S. Neuhof, and H. W. Neumann (1994). Measurement of fluorescence changes of NAD(P)H and of fluorescent flavoproteins in saponin-skinned human skeletal muscle fibers. *Anal. Biochem.* **216**, 322–327.
17. L. Pfeifer, A. Welker, I. Grünwald, K. Willoh, K. Stein, and R. Hetzer (2004). Fluorometric characterisation of Metabolism in Isolated pig hearts, using Time-Resolved Spectroscopy, (*Submitted for publication*)
18. E. Chinchoy, C. L. Soule, A. J. Houlton, W. J. Gallagher, M. A. Hjelle, T. G. Laske, J. Morissette, and P. A. Iaizzo (2000). Isolated four-chamber working swine heart model. *Ann. Thorac. Surg.* **70**(5), 1607–1614.
19. H. Von Baeyer, K. Stahl, M. Häusler, M. Meissler, V. Unger, J. Frank, Ch. Grosse-Siestrup, G. Kaczmarczyk, K. Affeld, H.-J. Flaig, and B. Steinbach (1997). Eine neue methode zur ex-vivo-vollblut-perfusion isolierter warmblutorgane, dargestellt an der niere von schweinen. *Biomedizinische Technik* **42**, 61–68.
20. D. A. Scott, L. W. Grotyohann, J. Y. Cheung, and Jr. R. C. Scaduto (1994). Ratiometric methodology for NAD(P)H measurement in the perfused rat heart using surface fluorescence. *Am. J. Physiol.* 267 *(Heart Circ. Physiol.* 36), 636–644.
21. B. Wetzl, M. Gruber, P. Bastian, L. Pfeifer, K. Stein, and O. S. Wolfbeis (2004). Homogenous bioassays based on measurements of fluorescence lifetime. *Miptec conference Basel, Poster presentation.*